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CHARACTERIZATION OF THE ROLE OF MACROPHAGES IN THE PROGRESSION OF NON-ALCOHOLIC STEATOHEPATITIS

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common form of liver injury worldwide in relation to the diffusion of overweight and obesity. In about 15-20% of patients with NAFLD the disease evolves to non-alcoholic steatohepatitis (NASH), characterized by hepatocellular damage and lobular inflammation that often evolves to hepatic fibrosis and cirrhosis. In my doctoral project, I have investigated some aspect of the inflammation associated to NASH in order to get more insides on the mechanisms responsible for the progression to fibrosis. As experimental model, we used C57BL/6 and Balb/c mice in which NASH was induced by feeding a methionine and choline deficient (MCD) diet up to eight weeks. In these animals, we have observed that the evolution of NASH is associated with a progressive increase in hepatic macrophages that changes their function and morphology. In fact, in the early phase of the disease the onset of inflammation is characterized by a prominent M1 activation that account for inter-strain difference in the susceptibility to NASH between C57BL/6 and Balb/c mice. With the progression of the disease toward fibrosis macrophages show a decline in M1 responses in relation with an increased production by the macrophages them-selves of the anti-inflammatory protein Annexin A1 (AnxA1). Using AnxA1-null mice and recombinant AnxA1 it has been possible to determine that AnxA1 acts on macrophages by down-modulating M1 polarization through stimulation of IL-10 production. Furthermore, AnxA1 has also a functional role in controlling fibrogenesis by the regulation of galectin-3 production. Although the advanced phases of experimental NASH are characterized by a decline in macrophage M1 responses, this does not parallel with a lowering of hepatic inflammation. Experiments performed with NF-kB1-null mice have shown that this phenomenon can be ascribed to an enhanced liver recruitment of osteopontin-producing NKT cells. Indeed, also in wild type animals the number of liver NKT cells changes during the evolution of NASH declining in the early phases of the disease and expanding again in the advanced phases in relation to an increased hepatic production of IL-15, a cytokine involved in controlling T-cell survival.

Collectively, these results indicate that NASH progression involves multiple interactions between macrophages and NKT cells and they represent a starting point to investigate whether genetic differences in NF-kB1 and Annexin A1 may account for the inter-individual variability in the evolution of NASH as well as to test AnnexinA1 analogues as possible novel treatments to control NASH evolution.

Introduction

NAFLD/NASH epidemiology

In the recent years, obesity and its metabolic and inflammatory consequences have become significant health problems all over the world. The prevalence of obesity and overweight in adults continues to rise and contributes to morbidity and mortality that is estimated to cost $147 billion dollars a year in the US and up to 0,6% of the gross domestic product of European countries (1). Indeed, obesity is considered one of the main causes of the so 'called metabolic syndrome', a cluster of related clinical features that include insulin resistance, dyslipidemia and hypertension which is the major risk factor in the development of type 2 diabetes and cardiovascular diseases (2). The effects of the metabolic syndrome involve also the liver where the disease is commonly associated with an accumulation of an excess of triglycerides within the hepatocytes, a condition known as non-alcoholic fatty liver disease (NAFLD). NAFLD is characterized by liver fat accumulation or hepatic steatosis in the absence of excessive alcohol consumption (20g per day for men and 10g per day for women) and of any other specific case of steatosis, such as hepatitis B and C, toxic and autoimmune diseases, hypobetalipoproteinemia and Wilson disease (3). According to the American Association for the Study of Liver Diseases, NAFLD is chemically defined as fat accumulation in the liver exceeding from 5%-10% the organ weight or by histological detection of more than 5% of hepatocytes containing visible intracellular triglycerides (4).

Due to the growing diffusion of obesity, NAFLD is by now the most frequent hepatic lesion in western countries where it has evolved as a serious public health problem. NAFLD prevalence in the general population ranges from 3 to 15%, but reaches up to 70% among overweight individuals. Even if hepatic steatosis is often benign, in about 15-20% of NAFLD patients the disease can evolve to non-alcoholic steatohepatitis (NASH). NASH is characterised by hepatocyte injury, inflammation and fibrosis (3, 5). From present statistics, it seems that NASH will become the leading cause of transplantation in the USA by the year 2020 (6). A critical aspect of the growing diffusion of NASH is related to its progression to liver cirrhosis and, in some cases, to hepatocellular carcinoma (HCC). For example, 15% of NASH patients develop clinically and histologically evident cirrhosis and the death rate ascribed to NAFLD/NASH-derived cirrhosis accounts 12-25%, while end-stage NASH is responsible for 4-10% of liver transplants. After the development of cirrhosis, from 4% to 27% of NASH cases progress to HCC. Therefore, it is clear that this disease may significantly contribute to the rapid increase of HCC incidence (3).

A further worrying aspect of NAFLD/NASH epidemiology is its increasing diffusion among children and adolescents in relation to the growing of childhood obesity and overweight. Paediatric

NAFLD prevalence is between 3% and 10% reaching the 80% in obese children and it varies as such because it is influenced by the characteristics of the population, especially lifestyle habits. Furthermore, NAFLD in children is also strongly associated with several features of the metabolic syndrome, especially insulin resistance and type 2 diabetes and it increases the risk of the developing cardiovascular disease in adulthood (7). All these evidences make NAFLD/NASH the most frequent paediatric chronic liver disease all over the world.

Clinical and histological features of NAFLD/NASH

In a large proportion of patients, NAFLD is asymptomatic and they only show hepatomegaly variability combined with chronically elevated liver function test results, such ALT, AST and alkaline phosphatase in the absence of other clinical evidence for viral infection, alcohol abuse and autoimmune or metabolic liver diseases. The preferred imaging test in the diagnosis of fatty liver is the hepatic ultrasonography that reveals a 'bright' liver of increased echogenicity. Ultrasonography is sensitive and specific for steatosis but cannot effectively detect the presence of fibrosis that is the main complication of the disease. Fatty liver can also be diagnosed by abdominal computed tomography scan where the liver is less dense than the spleen and by magnetic resonance imaging based on the signal differences between fat and water and shows a good correlation with microscopic fat content. An important limitation of all these three imaging modalities is the inability of these techniques to differentiate isolated hepatic steatosis from steatohepatitis (8).

Therefore, liver biopsy and the observation of the liver histology are required to confirm the diagnose of NASH. Liver biopsy is also helpful for determining the severity of NASH and may provide clues about the future course of the disease. The primary histological feature of both NAFLD and NASH is steatosis that consists in the presence of triglycerides that are contained in hepatocytes as single large droplet or as smaller, well-circumscribed droplets admixed with cytoplasmic contents. The second lesion is hepatocyte ballooning that is considered a hallmark parenchymal injury. Ballooned hepatocytes are enlarged with a characteristic 'flocculent' appearance to the cytoplasm. In addition, they can present cytoplasmic aggregates referred as Mallory-Denk bodies. The two hepatocyte keratins, K8 and K18, are disrupted and no longer present in the cytoplasm of ballooned hepatocytes and the serological testing for K18 fragments has shown a sensitivity and specificity for NASH of 77% and 92%, respectively. The last lesion is inflammation of the parenchyma with or without portal inflammation. Histologically detectable inflammation comprises the tissue infiltration by lymphocytes, plasma cells, monocytes and macrophages. Natural killer T cells are increased in NASH-associated cirrhosis, but this cell population has not been evaluated in the early stages of the disease. Eosinophils and neutrophils are

occasionally present. Other features that may be seen in the lobules of a patient with NASH include apoptotic hepatocytes, hepatocyte nuclei filled with glycogen, enlarged mitochondria and iron accumulation. A histopathological component of NASH is the presence of chronic inflammation mainly consisting of mononuclear-cell infiltrate in the matrix of portal tracts. A study based on biopsy samples from 728 adults and 205 children showed a positive correlation between portal inflammation and fibrosis (5, 9, 10).

Pathogenesis of steatosis in NAFLD/NASH

A critical aspect in studying the pathogenesis of NAFLD/NASH is determined by the incomplete understanding of the mechanisms responsible for the progression from simple steatosis to NASH. This aspect is very relevant because the parenchymal damage and inflammation typical of NASH are the factors that determine the evolution to fibrosis and cirrhosis. The clinical and social relevance of NAFLD and NASH and their continuous growth worldwide have stimulated a number of studies to clarify the mechanisms leading to the disease in attempt to develop effective treatments able to block the evolution of the disease (11).

The pathogenesis of NAFLD/NASH is complex and implicates the cross talk between different metabolically active sites. The initial 'two hits' model describe insulin resistance and accumulation of fat, mainly in the form of the triglycerides, as the cause for the development of hepatic steatosis that primes the liver to a 'second hit', involving oxidative stress which leads to the development of NASH and fibrosis. This model has been expanded in 'multiple parallel hits' hypothesis in which different processes, beside oxidative stress, may contribute to liver inflammation. In this setting, endoplasmic reticulum stress, cytokines production by the adipose tissue and gut and immunity are considered as co-factors in the development of steatohepatitis (12, 13).

Mechanisms leading to steatosis

Insulin resistance

A key factor in the onset of the metabolic syndrome is the development of insulin resistance (IR). Insulin is a hormone produced by beta cells of the pancreas and has a central role in the regulation of carbohydrate and fat metabolism in the body. In normal condition, the insulin binding stimulates the phosphorylation of its receptor that induces the recruitment and the activation by tyrosine phosphorylation of adaptor molecules IRS-1 and IRS-2, which, in turn, recruit signalling molecules that activate downstream effectors. Instead, serine phosphorylation of IRS proteins by serine kinases attenuates insulin signalling. Insulin resistance is referred as an impaired insulin-mediated glucose uptake in skeletal muscle, but also involves the liver (impaired insulin-mediated

suppression of glucose production) and the adipose tissue (impaired insulin-mediated suppression of lipolysis). To overcome IR and promote glucose storage, insulin-resistant individuals increase insulin secretion and reduce insulin clearance. Many factors, such as pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) release and cellular stresses, can promote the serine phosphorylation of IRS proteins by stimulating a variety of kinases, thus impairing insulin signalling (14). An important target of insulin is the adipose tissue where the hormone regulates glucose uptake and the activity of triglyceride lipase that controls the release of free fatty acids (FFAs) into the circulation. As a consequence of insulin resistance in the adipose tissue, the antilipolytic effect of the hormone is reduced, leading an increase in circulating FFAs. Therefore, subjects with NAFLD are insulin resistant at the level of: muscle because they have reduced glucose uptake; liver because they exhibit impaired suppression of hepatic glucose production; and adipose tissue because they show high lipolytic rates and increased circulating FFAs (12).

Effects of circulating free fatty acids: A large body of evidence indicates that the accumulation of triglycerides within the hepatocytes results from a derangements in fatty acids metabolism in both the liver and the adipose tissue. As a consequence of insulin resistance, the increase in the lipolysis in both visceral and subcutaneous fat leads to high levels of FFAs that, by flowing though the portal circulation, reach the liver, where promote triglycerides synthesis within the hepatocytes. Nonetheless, additional factors can contribute to steatosis, in particular an increased dietary fat intake, an enhanced *de novo* lipogenesis (DNL), a decreased FFAs oxidation and an impaired hepatic lipid transport through very-low density lipoproteins (VLDL) (2) (Fig.1).

Dietary habits that include excessive carbohydrate consumption, especially in the form of high fructose corn syrup, may be common causes of the DNL. This is supported by studies in mice that have confirmed the importance of dietary sugars in steatohepatitis and by a study in humans, which has linked fructose consumption with more advanced fibrosis (15).

The liver is able to *de novo* synthesize fatty acids (FAs) through a complex cytosolic polymerization in which malonyl-CoA undergoes several cycles of metabolic reactions to form one palmitate molecule. It has been shown that in humans, NAFLD is associated with increased hepatic expression of several genes involved in DNL. It is known that even if the contribution of DNL during fasting is small in normal subjects (5%), in patients with NAFLD, DNL is elevated, reaching a rate of 25%. Lipogenesis in the liver is regulated by three nuclear transcription factors: the sterol-regulatory element binding protein-1c (SREBP-1c), the carbohydrate responsive element binding protein (ChREBP) and the peroxisome proliferator-activated receptor-γ (PPAR-γ). Severe insulin resistance and increased circulating glucose stimulate hepatic SREBP-1c- and ChREBP-mediated

transcription of the enzymes involved in lipid synthesis, resulting in increased rates of de novo FAs biosynthesis. ChREBP also leads to the transcription of genes involved in glycolysis, converting the glucose excess into acetyl-CoA that is the precursor of FAs biosynthesis. The peroxisome-proliferation-activated-receptors (PPARs) are a subfamily of the nuclear-receptor family and regulate gene expression in response to ligand binding. After ligand binding, PPARs undergo conformational changes that allow the recruitment of coactivator proteins. There are three isoforms of PPARs (α, γ and β) with a central role in the sensing of nutrient levels and in the regulation of lipid and glucose metabolism: they are the receptors for various fatty acids. PPARα has a central role in hepatic lipid metabolism because many PPARα target genes are involved in mitochondrial and peroxisomal β-oxidation of fatty acids. PPARγ agonists are able to increase insulin-mediated adipose tissue uptake and storage of FFAs and inhibit hepatic fatty acids synthesis; moreover, they have anti-inflammatory action increasing adiponectin levels and reduce resistin, TNF-α, IL-6 and C-reactive protein levels (2, 12). Healthy individuals express PPAR- γ at very low levels in the liver, but in human NASH, it is transcriptionally upregulated, activates lipogenic enzymes and exacerbates steatosis (14).

It is well known that the oxidation of intrahepatocellular FAs occurs primarily within mitochondria and to a much lesser extent by peroxisomes and microsomes. Genetic or experimentally induced deficiencies of mitochondrial oxidative enzymes involved in fatty acids oxidation enhances triglyceride accumulation. Moreover, subjects with NAFLD have evidence of hepatic mitochondrial structural and functional abnormalities.

The liver exports water-insoluble triglycerides by packing them into very low-density lipoproteins (VLDL). These lipoproteins are secreted into the systemic circulation and are used to deliver triglycerides to the peripheral tissues. A large fraction of intra-hepatocellular FAs that are not oxidized are esterified to triglycerides, which are than incorporated into VLDL to be secreted. Therefore, the secretion of VLDL is a mechanism to reduce intrahepatic triglyceride content. It has been observed that even if VLDL-triglyceride secretion rate is greater in subjects with NAFLD than in those with normal intrahepatic triglyceride content, it is not able to adequately compensate for the increased rate of triglyceride production (2).

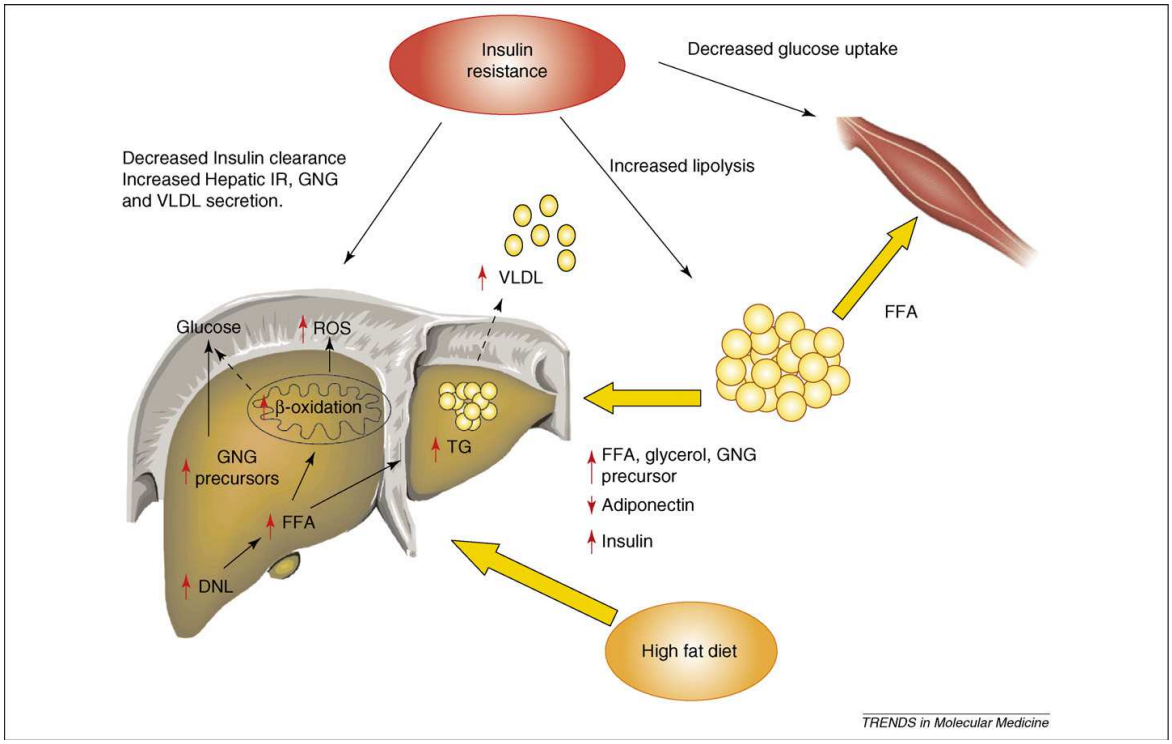

Fig. 1: Mechanisms of accumulation of fat in NASH (Marra *et al*., Trends Mol Med., 2008).

Alterations in adipokines secretion

Beside its metabolic functions, the adipose tissue is very active in secreting a variety of cytokines collectively known as adipokines that are implicated in controlling food intake, the metabolic cross talk between adipose tissue, muscle and liver and modulate endocrine and immune functions. NAFLD subjects exhibit decreased adiponectin levels and have instead high circulating levels of leptin and resistin. On the liver, the changes in adipokines influence lipid metabolism as leptin and resistin stimulate FFAs oxidation and favour hepatic fat, while adiponectin has an anti-inflammatory activity and improves insulin sensitivity (13, 16). Different studies have demonstrated that in obese animals, adipocitokines negatively correlate with hepatic triglycerides and the administration of recombinant adiponectin decreases hyperglicemia and the levels of plasma FFAs ameliorating steatosis and transaminase release. Adiponectin is also reduced in patients with hyperinsulinemia and insulin downregulates type 1 and 2 adiponectin receptor expression in the liver. Thus, lower amounts of circulating adiponectin and reduced expression of adiponectin receptors might contribute to hepatic steatosis in hyperinsulinemia (16). On the other hand, in patients with NASH, serum leptin levels are positively correlated with hepatic fat content, fibrosis and inflammation as well as with serum lipids, glucose, insulin, C-peptide and ALT (2). In obese subjects, the enlargement of adipose tissue, in particular of visceral fat, is associated with fat infiltration by monocytes that transform in activated macrophages releasing pro-inflammatory

cytokines and chemokines, such as TNF-alpha (tumor necrosis factor-alpha), IL-6 (Interleukin-6) and CCL2 (or MCP-1 – monocyte chemoattractant protein-1). Of these cytokines, TNF-α contributes to hepatic steatosis by inducing insulin resistance and increasing circulating FFAs, while both TNF-α and IL-6 impair mitochondrial FFAs oxidation (13).

Mechanisms leading to hepatocyte injury in NASH

Mitochondrial dysfunction and oxidative stress

It has been observed that the insulin resistance and the FFAs and cholesterol accumulation within the hepatocytes cause mitochondrial dysfunction characterized by increased mitochondrial dimensions, presence of crystalline inclusions and impaired electron transport chain enzyme activity. These alterations contribute to the impairment of hepatic FFA oxidation that allows the triglyceride accumulation in NAFLD (17). Furthermore, alterations in the mitochondrial electron transport chain are an important source of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals. In turn, ROS-dependent lipid peroxidation promotes a self-sustaining loop that leads to further mitochondrial damage and causes mitochondrial DNA (mtDNA) mutations (11). The decreased activities of mitochondrial respiratory complexes increase TNF-α expression, which cause additional lipid peroxidation of mitochondrial membranes, worsening mitochondrial functions. Moreover, the induction of cytochrome P450 isoforms involved in the FFAs metabolism, in particular CYP2E1 and CYP4A10 isoenzymes, also contributes to the oxidative damage (11). The cytochrome P450 system is a group of protein with diverse functions, including the metabolism of drugs, toxins, carcinogens, fatty acids and steroids. It is able to convert non-polar to polar compounds for conjugation by phase II enzyme or for direct excretion, requiring oxygen activation, which results in the generation of ROS. CYP2E1 metabolizes polyunsaturated fatty acids such as linoleic and arachidonic acids to generate ω-hydroxylated fatty acids. An increased CYP2E1 is evident in obesity, fatty liver and NASH in both humans and rodents. For example, CYP2E1 is induced in rats fed a high fat diet compared to control animals suggesting that the amount of fat in the liver is important in regulating CYP2E1. In the absence of substrates, CYP2E1 has a high oxidase activity leading to the transfer of electron to oxygen and the production of superoxide anion and hydrogen peroxide. Thus, the stimulation of CYP2E1 activity in NAFLD can play a critical role in ROS generation. ROS are toxic for cells since they react with macromolecules, denature proteins, inactivate enzymes and cause RNA and DNA damage (18). Furthermore, the interaction of ROS with phospholipids stimulates the oxidative breakdown of unsaturated fatty acids in a process known as lipid peroxidation (18) which damages cell membranes and generates a variety of toxic compounds including malonildialdehyde (MDA) and 4-

hydroxy-nonenal (4-HNE) (18). Within the body there are a number of enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (ferritin, glutathione, vitamin E, A, C, uric acid) mechanisms that maintain physiological levels of ROS and prevent cellular damage by ROS. Under physiological conditions, there is a balance between the rate of ROS generation and the rate of ROS removal; but an excess of ROS production causes oxidant stress, leading to apoptosis and necrosis (19). As a consequence of an increased ROS production, NAFLD/NASH patients show increased markers of oxidative damage that correlate with the severity of necro-inflammation and fibrosis (20, 21), suggesting that oxidative injury might be involved in triggering steatohepatitis. Indeed, MDA and HNE can covalently bind to hepatic proteins and are able to inhibit cytochrome c oxidase of mitochondrial complex IV or stimulate the synthesis of extracellular matrix proteins by hepatic stellate cells. Furthermore, lipid peroxidation products may initiate a cascade that activates the transcription factor NF-kB, which will lead to increased transcription of inflammatory cytokines, adhesion molecules, chemokines and death ligands by hepatocytes and non-parenchymal cells (22). Furthermore, lipid peroxidation products originating from phospholipid oxidation can act as damage-associated molecular patterns (DAMPs) and promote inflammation through the interaction with both soluble and cell-associated pattern recognition receptors (23, 24). Inflammatory cytokines contribute to mitochondrial dysfunction by interfering with the mitochondrial respiratory chain and by forming superoxide anion.

Lipotoxicity and endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an intracellular organelle involved in the synthesis, folding and trafficking of proteins. Under stress conditions, unfolded proteins accumulate in the ER and initiate a response trying to restore the organelle function. The unfolded protein, however, are able to induce the activation of stress kinases, such as c-Jun N-terminal kinases 1/2 (JNK1/2), which can contribute to the induction of ER stress-related apoptosis and to the activation of the pro-apoptotic caspases (22). In the recent years, increasing evidences indicate a role for the direct toxicity of circulating free fatty acids (FFAs) and their metabolites in causing endoplasmic reticulum stress and cell death, a phenomenon known as lipotoxicity (25-27). Indeed, hepatocyte incapability to esterify such an excess of FFAs triggers endoplasmic reticulum stress and JNK1/2 activation (26, 27). As previously mentioned, JNK-1 isoform is implicated in causing insulin resistance, thus contributing to hepatic steatosis. One aspect of JNK involvement in NASH that has received particular attention concerns its role in causing lipotoxicity. Studies by Gore's group have demonstrated that lipotoxicity can be 'reproduced' *in vitro* exposing liver cells to saturated FFAs, such as stearic acid (26, 27). In this experimental model, JNK promotes the induction of the p53 up-regulated

modulator of apoptosis (PUMA) and the subsequent activation of Bax and JNK-mediated phosphorylation inactivates the anti-apoptotic proteins Bcl-2 and Bcl-XL sensitizing the cells to the death-ligand TRAIL (26, 27). Accordingly, JNK activation is evident in liver biopsies from NASH patients and pharmacological or genetic JNK inhibition prevents lipotoxicity *in vitro* and ameliorates steatohepatitis in rodent models of NASH (26-28). The combined action of oxidative stress and lipotoxicity along with toxic activity of cytokines as TNF-α is by now considered the main cause of hepatocyte death through either apoptosis or necrosis. In particular, hepatocyte apoptosis is a prominent feature of NASH and it correlates with the disease severity (26, 27). Apoptotic hepatocytes are engulfed by Kupffer cells, which results in their activation and inflammation. The elevated circulating level of hepatocyte death markers can predict NASH in overweight, obese and morbidly obese patients (29).

Inflammatory mechanisms in the progression of NASH

Mechanisms leading to the onset of inflammation

Inflammation, along with hepatocyte damage, is the main feature of the progression from simple steatosis to steatohepatitis through molecular mechanisms closely linked each other. Several factors have been proposed to contribute to the onset of inflammatory responses. For instance, FFAs and oxidative stress stimulate NF-kB-mediated production of TNF-α and IL-6 by hepatocytes. In turn, these cytokines trigger Kupffer cells to secrete inflammatory mediators, which recruit to the liver other phagocytic cells (22). Pattern-recognition receptor, including Toll-like receptors (TLRs), contribute to the pro-inflammatory responses in fatty livers (30). TLRs responses can be activated by fatty acids and lipid peroxidation products and, in turn, the signal pathways associated to TLR stimulation activate NF-kB, and thereby further amplify and sustain inflammatory signal (22). Consistently, in rodent models of NASH, the interference with NF-kB activation protects from the development of steatohepatitis and reduces the expression of TNF-α and intracellular adhesion molecule-1 (30). Adipokines can be regarded as further regulators of inflammation in NAFLD/NASH, with leptin and adiponectin exerting, respectively, pro- and anti-inflammatory actions (14, 22). In line with these findings, NASH patients show an increased hepatic expression of cytokines genes that correlates with the severity of liver lesions (14) (Fig.2).

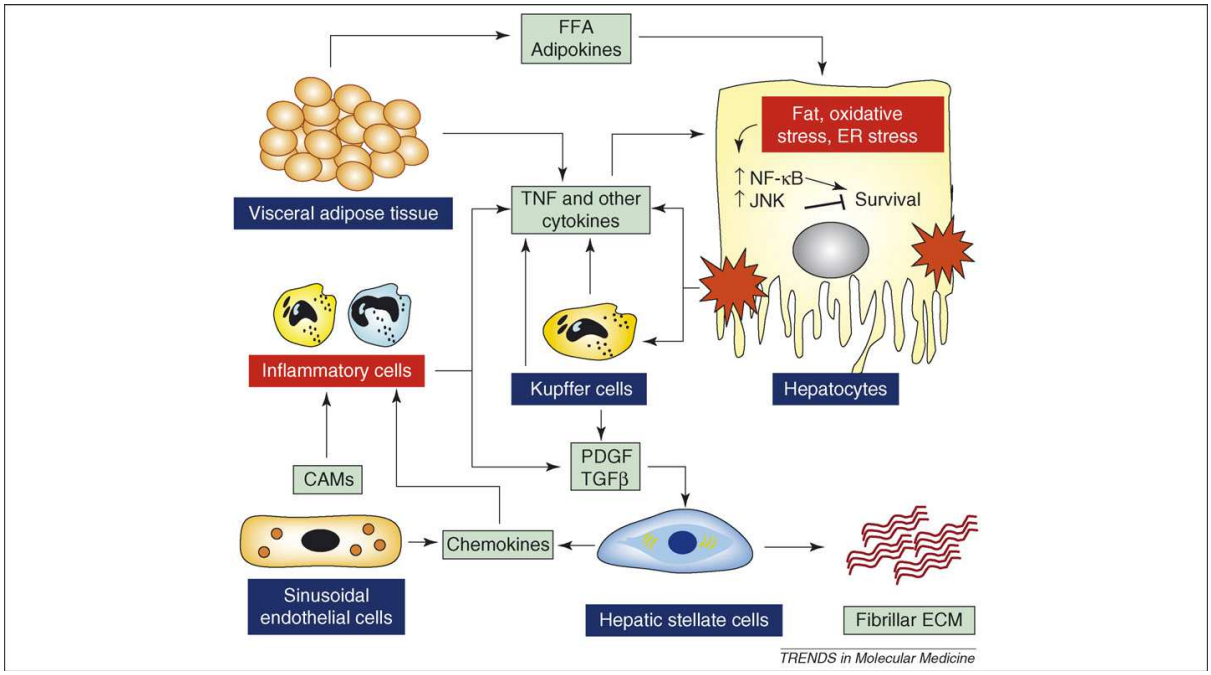

Fig. 2: Mechanism of inflammation in NASH (Marra *et al*., Trends Mol Med., 2008).

Interaction between inflammatory cells in the progression of NASH

Although many observations indicate that several pro-inflammatory mechanisms operate in NASH, the overall picture is still rather confused. In particular, the reason why only a fraction of the subjects with steatosis develops chronic hepatic inflammation remains unclear. Inflammatory reactions result from the interplay between innate and adaptive immunity. The first comprises physical and chemical barriers, humoral factors (complement and interferon-γ), phagocytic cells (neutrophils and macrophages) and lymphocytic cells (natural killer and natural killer T cells) that recognize invading pathogens as well as tissue injury providing a rapid response that recruits immune cells to sites of infection and activates the specific response of the adaptive immune system.

Adaptive immunity is activated when the innate or non-specific immune system cannot efficiently destroy the foreign organism. There are two types of specific immune response: humoral mediated by B cells that are able to produce antibodies recognizing antigens and cellular mediated by T lymphocytes. Antigenic specificity, immunologic memory and self-nonself-recognition characterize adaptive immunity (31). Available evidence suggests that adaptive immune responses are prevalent in NASH and mainly involves macrophages. In this scenery, the factors that control macrophage recruitment and activation are considered important in understanding the disease evolution. Nonetheless, a possible role of adaptive immunity is also emerging and might have an important role in driving macrophages responses. Indeed, in atherosclerosis, as well as in several auto-immune diseases, the interaction of lipid peroxidation products with cellular proteins leads to the formation

of immunogenic adducts that induce both humoral and cellular immune responses (32, 33). High titres of IgG against some of the antigens originating from oxidative stress, namely malondialdehyde- (MDA) derived adducts, are detectable in about 40% of adult NAFLD/NASH patients and in 60% of children with NASH (34, 35). In these latter, high antibody titres associate with more severe lobular inflammation and 13 fold increased risk of a NAFLD Activity Score ≥5 (35), while in adults anti-MDA IgG are an independent predictor factor of fibrosis (34).

Resident liver macrophages, also known as Kupffer cells, derive from circulating monocytes and represent about 20% of non-parenchymal cells in the liver. Once arrived to the liver, macrophages reside within the sinusoidal vascular space and differentiate to perform specialized functions, such as phagocytosis, antigen processing and presentation; they also produce cytokines, prostanoids, nitric oxide and reactive oxygen intermediates. Upon activation by bacterial antigens, such as lipopolysaccharide, Kupffer cells modulate the activation of various immune cells including dendritic cells, T lymphocytes and neutrophils. It is well known that the behaviour of macrophages is heterogeneous, depending on the different environmental setting (36). Their activation ranges between two separate polarization states: the “classically activated” pro-inflammatory M1 and the “alternatively activated” anti-inflammatory M2 states. These states mirror the Th1-Th2 polarization of T cells (37). Pro-inflammatory mediators (TLR ligands and IFN-γ) induce M1 polarized macrophages; they have IL-12^{high}, IL-23 ^{high}, IL-10^{low} phenotype, they secrete pro-inflammatory cytokines, such as TNF-α, IL-6, IL-12 and activate iNOS (inducible nitric oxide synthase) and they participate as inducers and effectors in polarized Th1 responses. M2 polarized macrophages are induced by IL-4, IL-13, immune complexes and glucocorticoid hormones and are characterized by the production of anti-inflammatory cytokines, as IL-10, and the enhancement of arginase-1, an enzyme with iNOS blocking properties. They have an IL-12 ^{low}, IL-23 ^{low}, IL-10 ^{high} phenotype and participate in polarized Th2 reactions as well as in promoting the killing and encapsulation of parasites. M2 polarized macrophages are also involved in tissue repair and remodelling and have immunoregulatory functions. It is noteworthy that M2 macrophages are present in established tumors where support cancer cell growth and migration (36) (Fig.3).

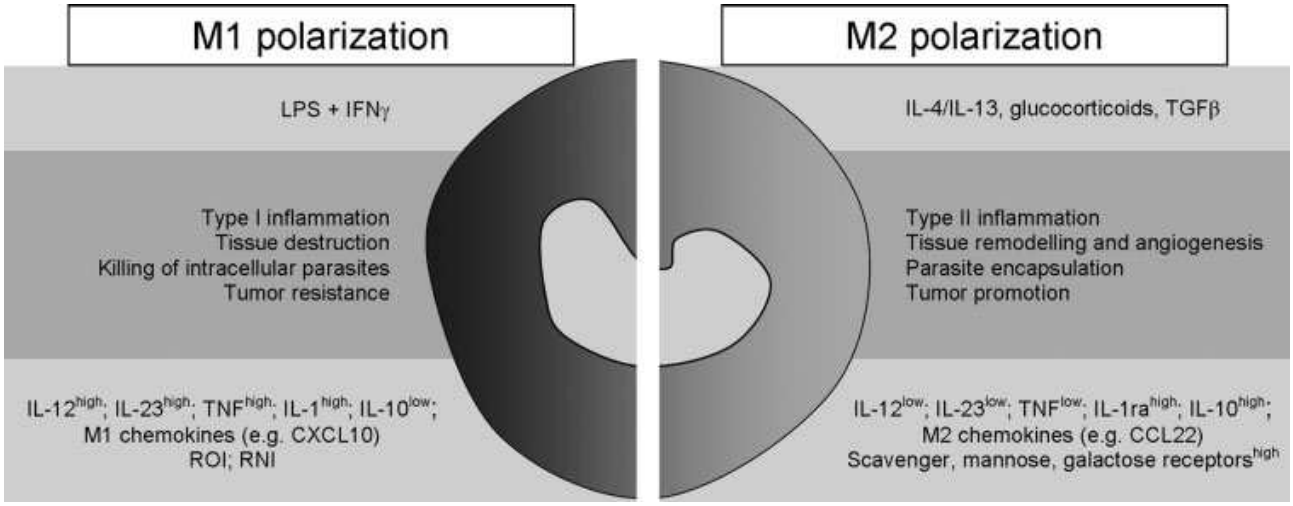

Fig. 3: Macrophage polarized activation (Mantovani *et al*., Eur. J. Immunol., 2007)

Adipose tissue macrophages (ATMs) are important players in obesity-associated pathology. During the lean state, ATMs display a M2 phenotype and maintain adipocyte function, insulin sensitivity and glucose tolerance. Upon mice feeding with a high-fat diet, the development of obesity is associated with ATMs phenotype switch from an anti-inflammatory M2 polarization state to a pro-inflammatory M1 polarization state (38). This switch from M2 to M1 polarization is also related to CCR2-dependent recruitment of other circulating monocytes and M1 activated ATMs are the main responsible factors for the pro-inflammatory state associated to obesity. Under physiological condition, Kupffer cells also display a prevalent M2 differentiation and some evidences suggest that a M2/M1 polarization shift might occur also in the liver during the evolution from NAFLD to NASH. Indeed, Tosello-Trampont and colleagues have recently reported that inducing NASH in mice by feeding for ten days methionine and choline deficient (MCD) diet, Kupffer cells get activated realising TNF-α and promote a subsequent infiltration of pro-inflammatory $CD11b^{int}Ly6C^{hi}$ monocytes (39). Moreover, the depletion of Kupffer cells by clodronate-containing liposomes decreases the incidence of NASH development by impairing steatosis, liver damage, monocyte infiltration and the production of inflammatory chemokines (39). In a similar manner, it has been shown that hyperlipidemic mice receiving high fat high cholesterol diet show bloated foamy Kupffer cells due to the scavenging of oxidized lipoproteins (40). Lipid accumulation within Kupffer cells is associated with a cascade of pro-inflammatory events leading to the initiation of the inflammation. In fact, the target deletion of scavenger receptors in the macrophages reduces hepatic inflammation, lipid oxidation and fibrosis, without affecting steatosis (41).

As previously mentioned, on top of resident macrophages, new macrophages can be recruited from circulating monocytes at the onset of NASH and infiltrate the liver. In mice as in humans,

circulating monocytes can be distinct in different subsets on the basis on antigens and receptors exposed on the cell surface. The widest group consists on Ly6C^{high} (Gr1^{high}) monocytes that are distinguished by CCR2^{high} CX3CR1^{low} expression and the presence of VLA-2 and CD62L adhesion molecules; they are called 'classical' or 'inflammatory' monocytes due to the extensive capacity of secreting pro-inflammatory mediators (TNF-α, iNOS, IL-12, IFN-γ) and migrating to inflamed tissues. The human counterpart of this population is CD14^{++}CD16^{-} (42, 43). The second subset of monocytes has the following phenotype: Ly6C^{low} (Gr1^{low}) CCR2^{low} CX3CR1^{high}. They are called 'non-classical' or 'resident' monocytes' and correspond to human CD14^{+}CD16^{+} monocytes. The high expression of CX_3CR1 might explain their long live span. Moreover, they express different adhesion molecules, for example LFA-1, in comparison to the 'classical' counterpart (42, 44) (Fig. 4). There are controversial reports about the formation of this cell type as some experimental evidences indicate that the Ly6C^{low} monocytes derive from Ly6C^{high} cells during maturation (45) and others sustain that Ly6C^{low} monocytes in parallel arise from distinct precursor in the bone marrow, independent from the Ly6C^{high} subset (46, 47). Tacke and colleagues have demonstrated that the infiltrating and inflammatory Ly6C^{high} monocytes present in liver diseases rapidly down-regulate Ly6C surface expression upon transmigration into the liver (43). Nonetheless, the actual functions of CX_3CR1^{high} monocytes during the evolution of hepatic injury is still poorly understood.

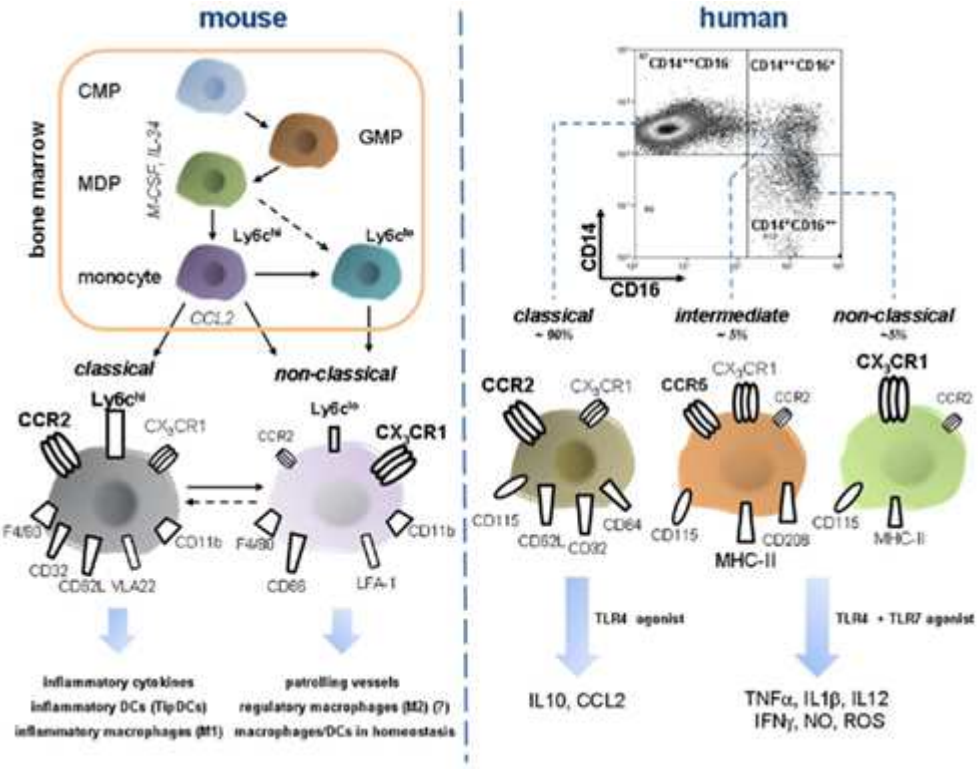

Fig. 4: Murine and human monocyte subsets (Zimmermann *et al.*, Front Physiol., 2012).

Several evidences show that in both mice and humans hepatic macrophage infiltration in liver with NASH is controlled by CCR2 and its main ligand CCL2. In the injured liver, CCL2 is produced by activated stellate cells, hepatocytes, macrophages and endothelial cells, representing a central role for liver inflammation. Patients with NASH have increased hepatic CCL2 expression as well as CCR2-dependent macrophage infiltration. Baeck and colleagues have demonstrated that the pharmacological inhibition of CCL2 specifically blocks the hepatic monocyte infiltration in models of chronic liver injury in mice, inhibits intrahepatic levels of pro-inflammatory cytokines and ameliorates steatosis progression without affecting hepatic fibrogenesis (48). Even if CX_3CR1 and its ligand fractalkine (CX_3CL1) are up-regulated in biopsies of patients with acute and chronic liver injury including NASH, CX_3CR1 deficient mice develop greater hepatic fibrosis than wild-type animals in two independent models of fibrosis. The possible explanation is that monocytes and macrophages lacking CX_3CR1 undergo increased cell death following liver injury, which perpetuates inflammation, promotes inflammatory monocyte infiltration into the liver, enhancing liver fibrosis (44).

Inflammatory infiltrates in the adipose tissue of obese subjects is characterized by the combined presence of activated macrophages and both B- and T-lymphocytes. In particular, cytotoxic CD8+ T cells are increased in fat pads of mice receiving a high fat diet and express an activated phenotype characterized by the release of pro-inflammatory mediators, which are implicated in the recruitment and activation of macrophages in the adipose tissue. The role of CD8+ cells in initiating and propagating adipose tissue inflammation has been confirmed by the fact that their depletion by immunological or genetic methods reduces macrophage infiltration, the release of pro-inflammatory mediators by adipose tissue and insulin resistance (49).

In normal liver, lymphocytes are found throughout the parenchyma and in the portal tracts and they include subpopulations of both innate (NKT and NK cells) and adaptive (T and B cells) immune systems. Even if the total number of hepatic CD3+ T lymphocytes appears relatively un-modified in the livers with NASH, an imbalance of the different CD3+ cell subtypes has been observed involving an increased CD8+/CD4+ cell ratio (50). CD4+ T helper cells are a sub-group of lymphocytes that, by releasing panes of different cytokines, drive the activation of other immune cells, switch B cell to the production of different antibody classes, activate cytotoxic T cells and maximize the bactericidal activity of phagocytes. Following the initial activation by immunogenic peptides presented by antigen presenting cells (APC) in the context of major histocompatibility (MHC) molecules, naïve Th lymphocytes can differentiate into three major distinct Th cell populations, Th1, Th2 and Th-17, induce macrophage activation and they are very effective in

controlling infection with intracellular pathogens. By releasing interferon-γ (IFN- γ), TNF-α and CD40 ligand, Th1 polarized CD4+ T-cells drive macrophages to the release of pro-inflammatory mediators and are important effectors of chronic inflammation. In turn, IL-12, produced by activated monocytes/macrophages and dendritic cells, is the dominant factor promoting Th1 cell polarization in both human and mouse systems. In contrast, Th2 cells secrete IL-4, IL-5 and IL-13 as key cytokines, promote allergic reactions, defence against parasites and help B cells in producing antibodies. In that case, IL-4 and IL-6 have an important role in Th2 differentiation and GATA-3 is the most dominant transcription factor regulating Th2 cytokine production. Th-17 cells are a subtype of T helper cells that is specifically induced by the presence of TGF-β and IL-6 through the transcription factor retinoic acid receptor-related orphan receptor (ROR)-γT and by secreting IL-17 exert pro-inflammatory functions (51). Recent reports have shown that an increase in circulating IFN-γ-producing CD4+ T-cells characterizes NASH in both paediatric and adult patients in conjunction with an enhanced liver IFN-γ expression (50, 52), suggesting the possible relevance of Th-1 responses to the human disease. Furthermore, a higher number of Th-17 cells has been observed in mice with steatosis induced by feeding a high fat diet and in liver biopsies of NASH patients. Accordingly, the Th-17 related genes (ROR-γT, IL-17, IL-21, IL-23) are up-regulated in NASH patients as compared to healthy controls and neutralization of IL-17 in high fat diet fed mice ameliorates liver injury and inflammation (53). Although in some hepatotoxic and cholestatic mouse models of liver injury an increase of IL-17 has been implicated in the evolution of hepatic fibrosis (54), no evidence is so far available about the implication of Th-17 responses in NASH evolution.

Conversely, recent studies have pointed out a possible involvement of natural killer T (NKT) cells in the progression of NASH. NKT cells are a lymphocyte population expressing both NK and T markers that recognize the lipid antigens presented by the non-classical MHC class I-like molecule CD1 (55). NKT cells account for 20-35% of mouse liver lymphocytes and 10-15% of rat and human liver lymphocytes (56, 57). They have been shown to play an important role in regulating innate and adaptive immunity through the production of a variety of cytokines, such as IFN-γ and IL-4 (58, 59). In addition to classical cytokines, NKT cells also secrete osteopontin (OPN) (60), a cytokine with both pro-inflammatory and pro-fibrogenic capacities (61) and the fetal morphogen, sonic hedgehog (Shh), which activates hepatic stellate cells (HSC) into collagen secreting myofibroblasts and amplifies the repair-associated inflammatory response (62).

The role of NKT cells during the progression from NAFLD to NASH is complex. Studies conducted by different laboratories suggest that NKT cells may have a protective effect in animal model of NAFLD. For example, leptin deficiency, high fat diet consumption and high sucrose diet

cause NAFLD associated with reduction of hepatic NKT cells. This reduction may be caused by both a lowering of hepatic CD1d expression and an increase of NKT apoptosis due to a reduced production of norepinephrine and IL-15 in a combination with an up-regulation in hepatic expression of IL-12 (63-65). Indeed, the depletion of Kupffer cells decreases the expression of IL-12 and blocks the impairment of NKT cells (64, 66). On the contrary, in mice with NASH there is an increase in the number of NKT cells, which parallels the progression of the disease to fibrosis. Accordingly, NKT cell deficient mice fed MCD diet show blunted OPN expression and a reduced collagen deposition (67). In the setting of human NASH, advanced fibrosis is correlated with increased hepatic levels of OPN and Hh and elevated plasma OPN levels in comparison with early fibrosis (60, 68).

Factors involved in NASH evolution to hepatic fibrosis

Unresolved inflammation promotes pathologic repair, thus progressive fibrosis and cirrhosis represent the outcomes of NASH. NASH-related fibrosis develops primarily in the pericentral areas, where thin bindles of fibrotic tissue surround groups of hepatocytes and thicken the space of Disse, in a ‘chicken wire’ fashion (9). The main cell type responsible for extracellular matrix deposition are hepatic stellate cells (HSCs), which, under the local influence of TGF-β1 (transforming growth factor beta1), PDGF (platelet-derived growth factor) and CCL2, trans-differentiate to myofibroblast-like cells producing collagen and other extracellular matrix components (69). Furthermore, decreased hepatic matrix degradation due to a reduced production of matrix metalloproteinases (MMPs) and/or an increased production of matrix metalloproteinase inhibitors might also contribute to collagen accumulation (69). Kupffer cell activation in response to chronic inflammatory stimuli is mostly responsible for the secretion of pro-fibrogenic cytokines (69). In addition, HSC proliferation and transformation to collagen-producing myofibroblasts are influenced by lymphocyte-derived cytokines and oxidative stress (70, 71). Alterations in adipokine secretion consequent to obesity might have a specific role for the induction of fibrogenesis in this condition. Activated HSCs selectively express leptin receptors and leptin stimulates HSC survival, the expression of pro-inflammatory and angiogenic cytokines (72). The pro-fibrogenic action of leptin might be enhanced by the combined lowering of adiponectin as, beside its anti-inflammatory action, adiponectin reduces proliferation and increases apoptosis of cultured HSCs (71). Furthermore, recent work has recognized that in NASH the development of portal inflammation and fibrosis is a marker for progressive disease (9).

Aims of the study

In spite of the large amount of data generated by recent research efforts, a number of issues concerning the mechanisms involved in the evolution of NASH to fibrosis/cirrhosis are still unresolved. Furthermore, from the clinical point of view, only some NAFLD patients develop NASH and, among these, there is a large inter-individual variability in the evolution to fibrosis. The factors responsible for such an inter-individual variability represent a particular challenge as multiple interaction occur between hepatocytes, inflammatory cells hepatic stellate cells in the different phases of the disease progression. Addressing these open questions is necessary and urgent because NAFLD/NASH, as well as its main risk factors, for example obesity, diabetes and metabolic syndrome, is increasing worldwide both in adults and in children.

In my doctoral project, I focused my attention on trying to understand the role of inflammatory and immune mechanisms responsible in the evolution of NASH. In particular, macrophages and NKT cells play a central role in the maintenance of inflammation also in advanced phases of NASH.

One limitation of the studies dealing with NAFLD/NASH pathogenesis relies in the fact that at present there is no universally accepted mouse model for the disease. The methionine choline deficient (MCD) diet model is widely used as it induced in a rapid (three-four weeks) and reproducible manner steatohepatitis that reproduces several aspects of human NASH. It is also suitable to study the progression of the disease since fibrosis is detectable within eight-ten weeks (73). However, this model lacks obesity and insulin resistance that are hallmarks of the human disease and, at long stage, animals become severely malnourished. At opposite, rodent feeding a high fat diet leads, as in humans, to steatosis combined with subcutaneous and visceral obesity, insulin resistance and adipokine unbalances. However, in rodents receiving high fat diet, liver injury and inflammation are usually modest and fibrosis very limited (73). Additional models using of high fat high cholesterol diets supplemented with either cholate or high fat high sucrose diet have also been proposed. Although these diets cause more liver injury than simple high fat diet, their pathological and metabolic features are less well characterized (73).

Results – Article 1

Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice

Background and aims: It is well documented that NAFLD evolution to NASH involves the cytotoxic action of circulating free fatty acids (FFAs), hepatic oxidative stress, endoplasmic reticulum stress and the stimulation of hepatic inflammation through the activation of Kupffer cells and the recruitment of monocytes and lymphocytes. However, little is known about why some NAFLD patients progress to NASH and others do not. An increasing number of studies has taken advantage from the inter-strain variability in both innate and adaptive immunity present in mice to elucidate the pathogenesis of some inflammatory diseases, such as atherosclerosis. Moreover, in the recent years, several groups reported differences among mice strains in the susceptibility to experimental NASH: for instance, strains such as C57BL/6 and 129/SVJ develop NASH and hepatic fibrosis upon receiving a high fat diet or methionine and choline deficient diet, while other strains (C3H/HeN or Balb/c) are resistant. C57BL/6 mice have a prominent lymphocyte Th-1 bias characterized by an increased production of cytokines such as INF-γ, TNF-α and IL-2 associated with an increased susceptibility to atherosclerosis, while Balb/c mice are more biased to Th-2 responses leading to IL-4, IL-5 and IL-13 production associated with an augmented fibrosis. Independently from the Th-1/Th-2 bias, macrophages from C57BL/6 mice show a 'classical' M1 polarization, whereas Balb/C-derived macrophages have a tendency to an 'alternative' M2 activation.

In this study, we have compared liver inflammation in C57BL/6 and Balb/c mice that are characterized by a different bias in cytokine production by CD4+ T helper (Th) lymphocytes and macrophages, to investigate if the variability in the severity of NASH in mice strains can give some clues to understand the evolution of the disease in humans. To avoid possible interferences due to fat inflammation, in this study we have used an experimental model of NASH based on mice feeding methionine and choline deficient (MCD) diet that allows to reproduce the liver features of the human disease in the absence of obesity and insulin resistance.

Key results: By feeding C57BL/6 and Balb/c mice with the MCD diet for four weeks, we have observed that C57BL/6 animals develop more severe steatohepatitis than Balb/C mice, in terms of hepatic triglycerides content, lobular inflammation, focal necrosis and TNF-α expression. These differences are not dependent upon the different Th1/Th2 bias as the evaluation of the liver mRNA

expression for T-lymphocyte polarization markers (IFN-γ, IL-10 and IL-17a) and the respective transcription factors (T-bet, Gata-3 and RORγT) are not different between the two strains. In both C57BL/6 and Balb/c mice, the development of NASH is associated with an increased expression of M1 activation markers, such as iNOS, IL12p40 and CXCL10. However, these cytokines are higher in the livers of MCD-treated C57BL/6 mice, suggesting that the increased severity of hepatic inflammation in C57BL/6 mice is correlated with the M1 skewing of Kupffer cells of this strain. This result has been confirmed by analysing isolated macrophages from MCD-treated mice, which show a prominent iNOS (inducible NO synthetase) and IL-12p40 production in C57BL/6 macrophages as compared to Balb/C macrophages.

Outcome and conclusions: Collectively, the results present in this paper highlight that the liver M1 macrophage bias of C57BL/6 mice is responsible for the major severity of NASH of this strain. Therefore, the genetic and epigenetic factors that regulate macrophage activation have an important role in influencing the progression from simple steatosis to steatohepatitis. Moreover, these observations point to the utility of exploring mice strain differences in the susceptibility to experimental NASH for getting new insides in the mechanisms responsible for the inter-individual variability of NAFLD evolution in humans.

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Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice

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ABSTRACT

In humans, there is large inter-individual variability in the evolution of NAFLD (non-alcoholic fatty liver disease) to NASH (non-alcoholic steatohepatitis). To investigate this issue, NASH was induced with an MCD (methionine–choline-deficient) diet in C57BL/6 and Balb/c mice that are characterized by different biases in Th1/Th2 and macrophage (M1/M2) responses. Following 4 weeks on the MCD diet, steatosis and lobular inflammation were prevalent in C57BL/6 (Th1, M1 oriented) than in Balb/c (Th2, M2 oriented) mice. Consistently, hepatic TNFα (tumour necrosis factor α) mRNA expression and circulating TNFα levels were higher in MCD-fed C57BL/6 than in MCD-fed Balb/c mice. The Th1/Th2 bias did not account for the increased NASH severity, as in both strains MCD feeding did not significantly modify the liver mRNA expression of the Th1 markers IFNγ (interferon γ) and T-bet or that of the Th2 markers IL-4 (interleukin 4) and GATA-3. Conversely, MCD-fed C57BL/6 mice displayed higher liver mRNAs for the macrophage M1 activation markers iNOS (inducible NO synthase), IL-12p40 and CXCL10 (CXC chemokine ligand 10) than similarly treated Balb/c mice, without effects on the M2 polarization markers IL-10 and MGL-1 (macrophage galactose-type C-type lectin-1). Circulating IL-12 was also higher in MCD-fed C57BL/6 than in MCD-fed Balb/c mice. The analysis of macrophages isolated from the livers of MCD-fed animals confirmed an enhanced expression of M1 markers in C57BL/6 mice. Among all of the MCD-treated mice, liver iNOS, IL-12p40 and CXCL10 mRNA levels positively correlated with the frequency of hepatic necro-inflammatory foci. We concluded that the macrophage M1 bias in C57BL/6 mice may account for the increased severity of NASH in this strain, suggesting macrophage responses as important contributors to NAFLD progression.

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is characterized by TAG (triacylglycerol) accumulation within the liver and is, at present, the most frequent hepatic lesion in Western countries in relation to the growing prevalence of obesity and the metabolic syndrome [1]. Although fatty liver is often benign, approximately 10–25 % of NAFLD patients develop NASH (non-alcoholic steatohepatitis) characterized by parenchymal injury, inflammation and

Key words: cytokine, Kuppfer cell, liver inflammation, liver injury, non-alcoholic fatty liver disease, strain difference.

Abbreviations: ALT, alanine aminotransferase; CCL2, CC chemokine ligand 2; CXCL10, CXC chemokine ligand 10; IFNγ, interferon γ; iIL, interleukin; iNOS, inducible NO synthase; MCD, methionine–choline-deficient; MGL-1, macrophage galactose-type C-type lectin-1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; RORγT, retinoic acid-receptor-related orphan receptor γT; ROS, reactive oxygen species; TAG, triacylglycerol; TNFα; tumour necrosis factor α; TLR, Toll-like receptor.

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fibrosis that can progress to cirrhosis and, in some case, to hepatocellular carcinoma [1]. A still open question in this field concerns the mechanisms responsible for NAFLD progression to more severe liver injury [2]. NAFLD is characterized by an increase in circulating NEFAs (non-esterified fatty acids), hepatic oxidative damage, endoplasmic reticulum stress and adipokine inbalances [3–5]. The current view suggests that all these events promote hepatic inflammation by stimulating Kupffer cells to secrete pro-inflammatory mediators and to recruit leucocytes within the liver [3–5]. Consistently, NASH patients show an increased hepatic expression of cytokine genes that correlates with the severity of liver lesions [6]. However, little is known about the factors determining why some NAFLD patients progress to NASH and others not.

Inter-strain variability in both innate and adaptive immunity is well documented in mice and has been exploited for elucidating the pathogenesis of several inflammatory diseases [7,8] as well as of multifactorial diseases with an inflammatory component, such as atherosclerosis [9]. In recent years, several groups reported differences among mice strains in the susceptibility to experimental NASH [10–14]. In particular, C57BL/6 and 129/SVJ develop NASH and hepatic fibrosis upon receiving a high fat diet, whereas A/J mice are resistant [12]. Steatohepatitis induced by feeding an MCD (methionine–choline-deficient) diet is also more severe in A/J and C57BL/6 than in C3H/HeN or Balb/c mice [10,11]. So far, the mechanisms responsible for these differences have not been investigated in detail. In particular, little is known about the influence of the genetic background on the multiple mechanisms controlling hepatic inflammation. Studies in obese subjects have shown that chemokines released by fat-resident macrophages recruit to the adipose tissue T-lymphocytes and NKT cells (natural killer T-cells) [15]. In turn, Th1-activated $CD4^+$ stimulate macrophage M1 polarization with the production of pro-inflammatory mediators [TNFα (tumour necrosis factor α), IL (interleukin)-12, CCL2 (CC chemokine ligand 2) and NO] causing insulin resistance and further recruitment of inflammatory cells into the adipose tissue [15,16]. Interestingly, an increase in M1 cytokines is also associated with the development of NASH in both experimental animals and humans [4–6].

To investigate whether the variability in the severity of NASH among mice strains might give some clue to understand the factors influencing the progression of the human disease, we compared liver inflammation in C57BL/6 and Balb/c mice that are characterized by a different bias in cytokine production by $CD4^+$ Th and macrophages. In fact, C57BL/6 mice show a prominent Th1 cytokine production [IL-2, TNFα and IFNγ (interferon γ)] as opposed to a prevalence of Th2 cytokine response (IL-4, IL-5 and IL-13) in Balb/c mice [17]. Such a Th1 bias has a major role in determining the increased susceptibility to atherosclerosis of C57BL/6 mice, being the driving force for plaque inflammation [17,18]. Conversely, a Th2 bias is associated with an increased fibrosis in Balb/c mice [19]. Independently from the Th1/Th2 responses, C57BL/6-derived macrophages also show a bias to M1 polarized activation, whereas macrophages from Balb/c mice have a tendency to the so-called 'alternative' M2 activation [20]. This latter condition is characterized by anti-inflammatory, pro-fibrogenic and immunosuppresive responses [21]. Since Th1 lymphocytes and M1 macrophages are responsible for adipose tissue inflammation and insulin resistance in obesity [15,16], in the present study we avoided possible interferences due to fat inflammation by using the MCD model of NASH that allows to reproduce the liver features of the human disease in the absence of obesity and insulin resistance [22].

MATERIAL AND METHODS

Animals and experimental protocol

Male C57BL/6 and Balb/c mice (8-weeks old) were purchased from Harlan–Nossan, and the dietary treatment was initiated after 1 week acclimatization. Mice were fed for 4 weeks with either an MCD diet or a control diet supplied by Laboratorio Dottori Piccioni. Body weight was recorded weekly throughout the experiment. Mice were anaesthetized with sevofluorane and blood was collected by cardiac puncture. Livers were rapidly removed and, after rinsing in ice-cold saline, cut in pieces. Aliquots were immediately frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until analysed. Two portions of each liver were fixed in 10% formalin or snap-frozen in OCT (optimal cutting temperature) compound for histology respectively.

Animal experiments were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

Biochemical analysis

Plasma ALT (alanine aminotransferase) and total liver TAG content were determined by spectrometric kits supplied by Radim and Sigma Diagnostics respectively. Circulating TNFα and IL-12 levels were evaluated in the sera using commercial ELISA kits (Peprotech).

Isolation and purification of liver macrophages

Liver macrophages were isolated from the livers of MCD-fed mice by collagenase perfusion according to Froh et al. [23]. The cells were suspended in serum-free RPMI 1640 medium and purified by 1 h of adhesion on plastic disks. Cell purity, as estimated by immunofluorescence and flow cytometry,

was above 80% (see Supplementary Figure S1 at http://www.clinsci.org/cs/122/cs1220545add.htm). The cells were then processed for mRNA and protein extraction as outlined below.

mRNA extraction and real-time PCR

RNA was extracted with TRI reagent (Applied Biosystems) according to the manufacturer's instructions and was quantified at an absorbance of 270 nm with a Nanodrop spectrometer (Thermo Scientific). RNA (1 μg) was retro-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. Real-time PCR was performed in a TC-312 thermalcycler (Thecne), using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNFα, IL-12p40, IL-4, IFNγ, IL-17a, IL-10, T-bet, GATA-3, RORγT (retinoic acid-receptor-related orphan receptor γT), iNOS (inducible NO synthase), CXCL10 (CXC chemokine ligand 10), MGL-1 (macrophage galactose-type C-type lectin-1), argininase-1, 18S and β-actin (Applied Biosystems). The data were processed using 7000 System Software and normalized to the β-actin or 18S gene expression. All samples were run blind in duplicate and the results were expressed as arbitrary units.

Western blotting

Liver macrophages were homogenized in ice-cold lysis buffer [10 mM Tris/HCl (pH 7.4) containing 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 20 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM DTT (dithiothreitol), 1 mM PMSF, 10 mg/ml leupeptin, 1 mg/ml pepstatin and 60 mg/ml aprotinin], and protein extracts (100 μg) were electrophoresed by SDS/PAGE on 10% polyacrylamide gels. Nitrocellulose membranes were probed with monoclonal antibodies against mouse iNOS and argininase-1 (BD Biosciences and Genetex) and revealed with Western Lightning Chemiluminescence Reagent Plus [ECL (enhanced chemiluminescence); PerkinElmer] using the VersaDoc 3000 quantitative imaging system and Quantity One software (Bio-Rad Laobratories).

Histology and immunohistochemistry

Liver pathology was assessed in haematoxylin/eosin and Masson's trichrome stained sections. The severity of steatosis and lobular inflammation was scored by an experienced pathologist, according to the method described by Kleiner et al. [24]. The number of necro-inflammatory foci was counted in ten different high-magnification microscopic fields. Kupffer cells and liver infiltrating T-cells were detected in frozen sections using anti-mouse F4/80 (eBioscience) or anti-mouse CD3 monoclonal antibodies (R&D Systems) in combination with peroxidase-linked goat anti-rat IgG and HRP (horseradish peroxidase) polymer kit (Biocare Medical) respectively. The numbers of F4/80-positive macrophages were counted in ten different microscopic fields. CD3-positive cells are expressed as a percentage of the inflammatory cells infiltrating liver parenchyma in ten different microscopic fields.

Data analysis and statistical calculations

Statistical analyses were performed by SPSS statistical software using a one-way ANOVA test with Tukey correction for multiple comparisons, or a Kruskal–Wallis test for non-parametric values. A Pearson's r value was used for verifying correlations. Significance was taken at the 5% level. Normality distribution was preliminary assessed by the Kolmogorov–Smirnov test.

RESULTS

Feeding C57BL/6 and Balb/c mice with an MCD diet for 4 weeks resulted in the development of steatohepatitis characterized by liver TAG accumulation and ALT release (Figure 1). Liver histology revealed the presence of macrovescicular steatosis accompanied by lobular inflammation, lipogranulomas, hepatocyte apoptosis and focal necrosis (Figures 2A–2D). By comparing MCD-fed animals, C57BL/6 mice had higher ($P < 0.05$) hepatic TAGs than Balb/c mice, whereas no significant differences were appreciable in ALT release (Figure 1). The two strains also suffer similar weight loss upon the administration of the MCD diet (results not shown). Following blind semi-quantitative evaluation of hepatic histology, MCD-treated C57BL/6 mice had higher scores for steatosis ($P < 0.05$) and lobular inflammation ($P < 0.002$) and an increased prevalence of focal necrosis ($P < 0.001$) as compared with similarly treated Balb/c mice (Figures 2E–2G). Collagen deposition in MCD-fed mice was very modest and mostly localized to the peri-sinusoidal spaces of the centrilobular areas without appreciable strain differences (results not shown). In accordance with the histopathology, liver mRNA expression and circulating levels of TNFα were significantly higher in C57BL/6 than in Balb/c MCD-treated mice (Figure 2).

Immunohistochemistry for CD3-positive cells showed that inflammatory infiltrates in MCD-treated C57BL/6 livers were characterized by an increased prevalence of T-lymphocytes (19.8 ± 7.0 compared with 13.3 ± 5.1%; $P < 0.02$) that were mostly localized around the necrotic foci (Figure 3). By measuring the liver mRNA content of IFNγ and IL-4 as representative for $CD4^+$ T-cells Th1 and Th2 responses respectively, we observed that IFNγ and IL-4 expression were higher in control C57BL/6 than in Balb/c mice (Figure 3). The MCD diet did not significantly modify IFNγ expression in C57BL/6, whereas it lowered IFNγ mRNA in Balb/c mice (Figure 3). In both strains MCD feeding did not significantly affect IL-4 mRNA pattern (Figure 3).

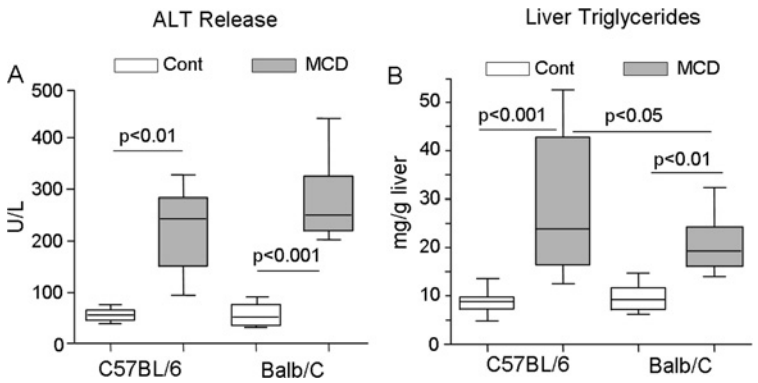

Figure 1 Liver ALT release (A) and TAG content (B) evaluated in C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet

The values refer to 12–15 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

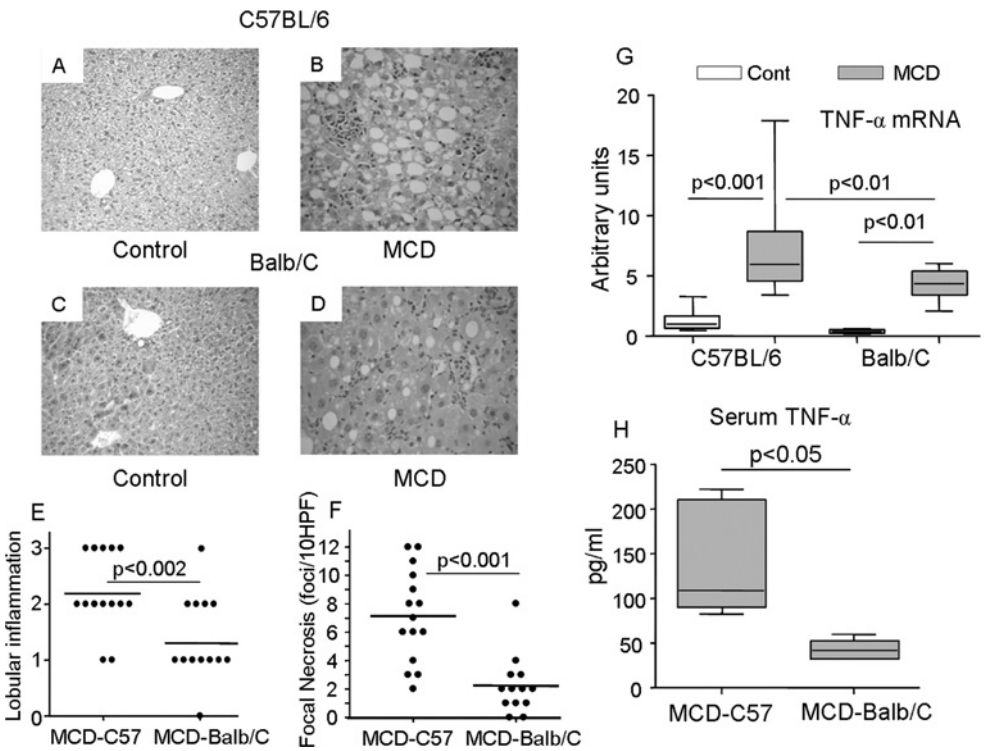

Figure 2 Hepatic inflammation in C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet

Liver histology was revealed by haematoxylin/eosin staining (A–D: magnification, ×200 and ×400). Lobular inflammation was scored semi-quantitatively according to Kleiner et al. [24] (E), while the number of necro-inflammatory foci (F) were counted in ten different high-magnification microscopic fields. The liver mRNA expression of TNFα (G) was measured by real-time PCR and expressed as arbitrary units after normalization to the actin gene expression. The circulating levels of TNFα were determined in the sera of the same animals (H). The values refer to 12–15 animals in each group and the boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

No significant changes were also evident in the liver mRNAs of the Th1 regulator T-bet and of the Th2 transcription factor GATA-3 (Figure 3). IL-17-producing Th17 lymphocytes are a newly identified subset of effector helper T-cells, distinct from Th1 and Th2 $CD4^+$ T-cells, which increasingly recognized to play a role in driving inflammation in chronic liver diseases [25]. As Th17 lymphocytes have been implicated in human alcoholic hepatitis [26], we investigated possible signs of Th17 response in experimental NASH. IL-17a mRNA was almost undetectable in the livers of control mice and increased just above detection limits

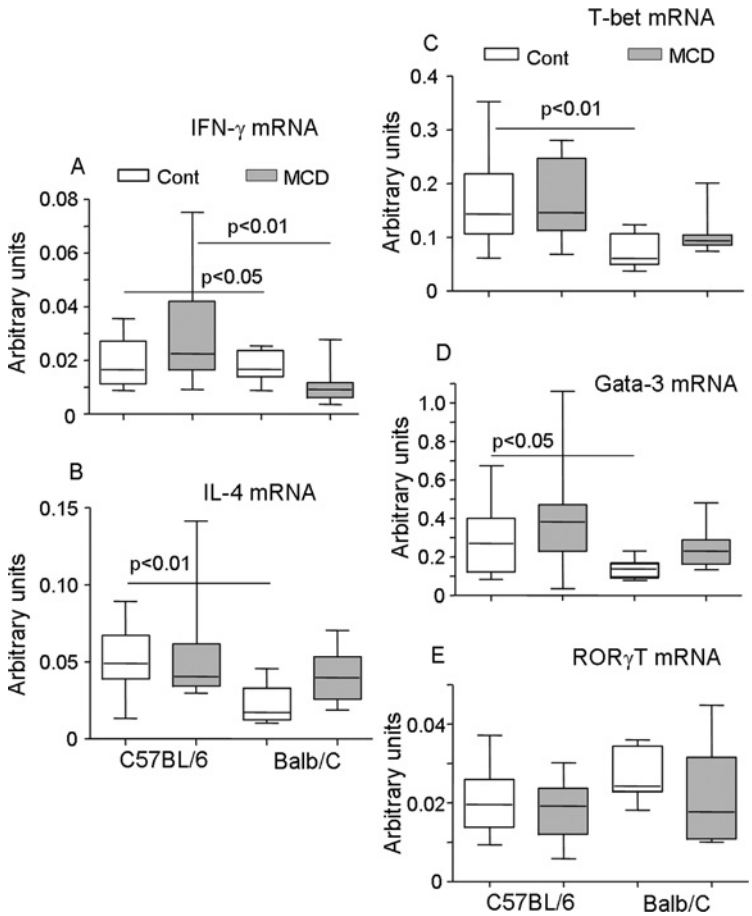

Figure 3 Evaluation of T-lymphocyte polarization markers in the liver of C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet

The mRNA expression of IFNγ and IL-4 and of the transcription factors T-bet, GATA-3 and RORγT were measured by real-time PCR. The values refer to 12–15 animals in each group and are expressed as arbitrary units after normalization to the actin gene expression. The boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

upon MCD feeding, but because of the very low values the data were unreliable (results not shown). However, MCD-diet feeding did not modify the mRNA expression of RORγT, a transcription factor controlling Th17 lymphocyte differentiation (Figure 3). Taken together these findings suggest that lymphocyte polarized responses did not primarily account for strain differences in the onset of MCD-induced NASH in mice.

The possible influence of strain bias in macrophage activation was then investigated. Immunohistochemistry using anti-F8/40 antibodies did not reveal appreciable differences in the number of liver macrophages between MCD-fed C57BL/6 and Balb/c mice (results not shown). In both strains, the development of NASH was associated with an increased mRNA expression of M1 activation markers such as iNOS, IL-12p40 and CXCL10 (Figure 4). However, in the livers of MCD-treated C57BL/6 mice iNOS, IL-12p40 and CXCL10 mRNAs were respectively 3.3, 3.5 and 2.5 times higher than in MCD-treated Balb/c mice (Figure 4). Measurement of circulating IL-12 confirmed an increased production of this cytokine in MCD-fed C57BL/6 mice (Figure 4). On the other hand, despite the hepatic mRNAs for the M2 markers MGL-1 (CD301) and IL-10 were higher in control C57BL/6 than Balb/c mice, the MCD diet did not appreciably influence these markers (Figure 4). To further substantiate these observations, macrophages were isolated from the livers of MCD-fed mice of both strains. Figure 5 shows that the macrophages from C57BL/6 livers displayed an enhanced expression of iNOS and IL-12p40 as compared with those from Balb/c mice. Conversely, the M2 marker argininase-1 was greatly increased in Balb/c-derived macrophages, whereas no change was observed in MGL-1 expression (Figure 5).

The relationship between macrophage M1 bias and NASH severity was supported by the observation that, among all MCD-fed mice, the liver mRNA expression of iNOS ($r = 0.70$, $P = 0.017$), IL-12p40 ($r = 0.60$, $P = 0.009$) and CXCL10 ($r = 0.51$, $P = 0.018$) positively correlated with the frequency of hepatic necro-inflammatory foci.

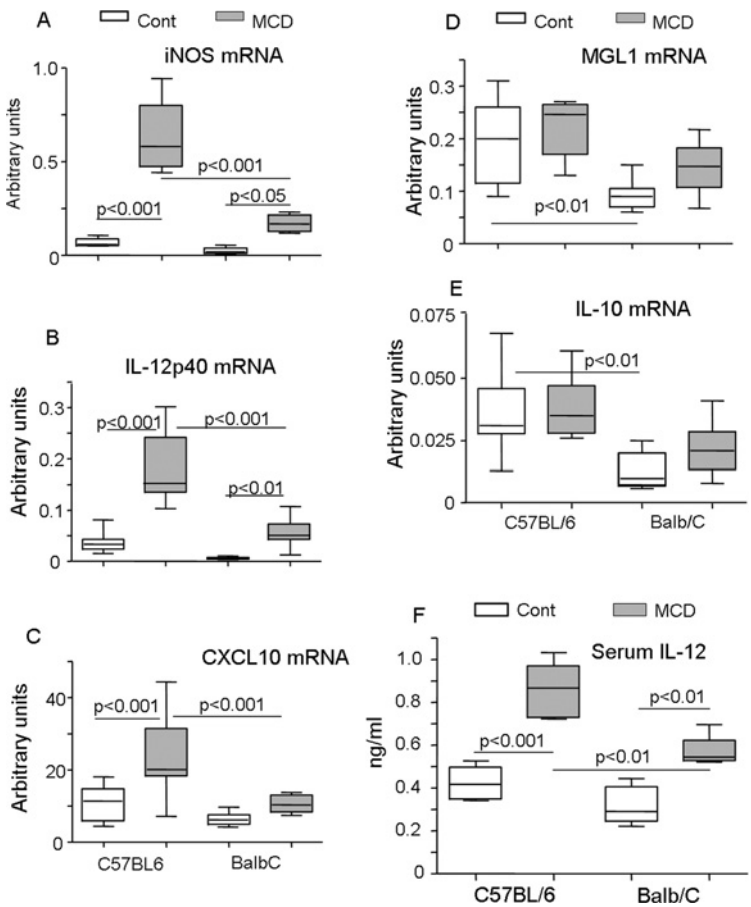

Figure 4 Changes in macrophage M1/M2 polarization markers in the livers of C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet

The mRNA expression of M1 (iNOS, IL-12p40 and CXCL10) and M2 (MGL-1 and IL-10) polarization markers were measured by real-time PCR (A–E). The values refer to 12–15 animals in each group and are expressed as arbitrary units after normalization to the actin gene expression. The circulating levels of IL-12 were determined in the sera of the same animals (F). The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians; 80% of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

DISCUSSION

To date, only a few studies have investigated the inter-strain differences in mice susceptibility to experimental NASH. Despite some variability related to the different dietary protocols, the following picture emerges: by using the MCD diet, the apparent rank in transaminase release is A/J>C57BL/6>C3H/HeN=Balb/c=DBA/2J [10,11], whereas long-term feeding of a diet deficient only in methionine causes more liver injury and hepatocarcinogenesis in DBA/2J than in C57BL/6 mice [14]. Conversely, C57BL/6 and 129/SVJ, but not A/J, mice develop NASH and hepatic fibrosis upon receiving a high fat diet [12,13]. At present, the factors responsible for such differences have been poorly characterized. QTL (quantitative trait locus) analysis has identified several loci in chromosomes 1, 2 and 7 that influence the extent of liver injury (ALT release) in seven mice strains receiving the MCD diet [10]. Furthermore, epigenetic mechanisms controlling DNA and histone methylation have been proposed to account for the differences in NASH progression between DBA/2J and C57BL/6 mice [14]. Our present results confirm and extend the above observations, showing that after 4 weeks on the MCD diet C57BL/6 mice developed more severe NASH than Balb/c mice.

Recent evidence indicates that the inflammatory process in NASH and atherosclerosis may share common mechanisms [27]. Interestingly, in experimental models of atherosclerosis C57BL/6 mice develop more extensive plaques than Balb/c mice [28,29]. Such a different behaviour depends upon a prevalent expression in the C57BL/6 strain of specific class II MHC molecules that regulate Th1 $CD4^+$ T-cell activation [28]. In turn, IFNγ, TNFα and CD40 ligand produced by $CD4^+$ T-cells drive plaque macrophages to produce ROS (reactive oxygen species), NO and pro-inflammatory cytokines [30]. Consistently, blunting $CD4^+$ T-cells or their

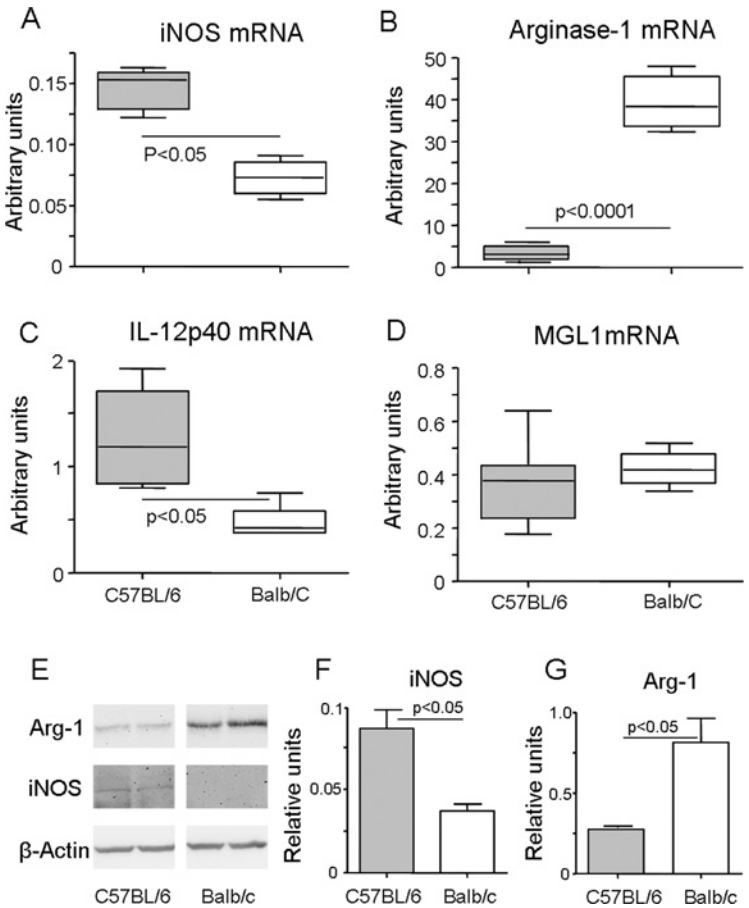

Figure 5 Evaluation of M1/M2 polarization markers in macrophages isolated from the livers of C57BL/6 and Balb/c mice fed for 4 weeks with an MCD diet

Macrophages were purified as reported in the Materials and methods section and the mRNAs of M1 (iNOS and IL-12p40) and M2 (argininase-1 and MGL-1) polarization markers were measured by real-time PCR (A–D). The values refer to four to five cell preparations in each group and are expressed as arbitrary units after normalization to the 18S gene expression. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians; 80% of the values are between the extremes of the vertical bars (10–90th percentile). The iNOS and argininase-1 (Arg-1) protein expression was evaluated by Western blotting in the extracts from the same cell preparations (E–G). The results are expressed as ratios of, respectively, iNOS and Arg-1 band densities and those of β-actin and are means $\pm$ S.D. from four to five cell preparations.

Th1 responses decreases atherosclerosis in ApoE$^{-/-}$ (apolipoprotein E-deficient) C57BL/6 mice fed on a high-cholesterol diet [30]. C57BL/6 Th1 bias does not appear to have a major influence on the severity of MCD-induced NASH. This result is rather unexpected, since previous studies have shown that fatty liver in mice fed on hypercaloric or choline-deficient diets promotes IFNγ production [31,32]. Nonetheless, it should be noted that in these latter studies IFNγ expression requires extensive T-cell activation by concanavalin A [31] or long-term (10–20 weeks) choline deficiency [32]. Thus it is possible that CD4$^+$ T-cell recruitment might require longer than the 4-week treatment used in the present experiments.

During inflammation, macrophages can express different functional differentiation patterns in response to environmental stimuli such as bacterial products and cytokines [21]. Bacterial LPS (lipopolysaccarides) and IFNγ promote classic M1 activation characterized by the production of ROS, NO and pro-inflammatory cytokines and chemokines (IL-1β, TNFα, IL-12, CXCL9 and CXCL10); conversely IL-4, IL-10 and TGFβ (transforming growth factor β) induce alternative M2 macrophage activation associated with anti-inflammatory, pro-fibrogenic, angiogenetic and immuno-suppresive activities [21]. M1 activation characterizes not only bacterial infections, but is also evident in macrophages of atherosclerotic plaques and in those from the adipose tissue of obese subjects [16,33]. Our present results point to the importance of M1 responses in the development of NASH by showing that a prevalent liver expression of M1 markers correlates with increased hepatic inflammation in C57BL/6 mice with MCD-induced NASH. On the same line, a recent study in morbidly obese patients demonstrates that NASH, but not NAFLD, is associated

with a specific increase in the liver expression of a wide array of pro-inflammatory M1 cytokines/chemokines (IL-1β, IL-18, CCL2–5 and CXCL9–11) [34]. Several mechanisms might contribute in promoting M1 responses in the early phase of NASH. For instance, TLR (Toll-lik receptor)-4, TLR-2 and TLR-9 have been shown to trigger pro-inflammatory responses in fatty livers [35,36], being activated by dietary lipids, oxidation products and molecules released from damaged hepatocytes [5,37]. The complement cascade can also stimulate macrophage activation through C3b and C5b receptor interactions and the release of anaphylotoxins. In this latter context, Rensen et al. [38] have recently reported an extensive deposition of different complement fractions in liver biopsies from NASH patients that associates with increased hepatocyte apoptosis, granulocyte infiltration and higher liver expression of IL-1β, IL-6 and IL-8 mRNAs.

Altogether these results indicate that C57BL/6 mice M1 bias in liver macrophages responses contributes to the increased susceptibility to NASH of this strain, suggesting the importance of genetic/epigenetic factors regulating macrophage activation in influencing NAFLD progression to steatohepatitis. These observations point to the usefulness of exploring mice strain differences in the susceptibility to experimental NASH for obtaining new insights into the mechanisms responsible for inter-individual variability of NAFLD evolution in humans.

AUTHOR CONTRIBUTION

Virginia Maina and Salvatore Sutti designed the study and performed the experiments; Irene Locatelli, Matteo Vidali and Cristina Mombello contributed to the experiments and data analysis; Cristina Bozzola performed the histological analysis; and Emanuele Albano supervised the research and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice

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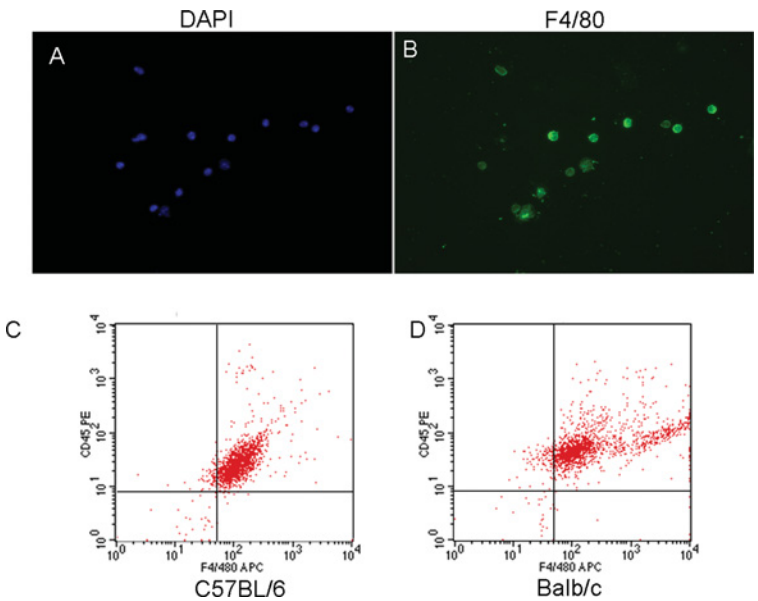

Figure S1 **Purity of macrophages isolated from the livers of C57BL/6 and Balb/c mice fed on an MCD diet for 4 weeks was estimated by immunofluorescence using an anti-mouse F4/80 rat IgG and FITC-labelled anti-rat IgG serum (A and B) as well as by flow cytometry using allophycocyanin-labelled anti-mouse F4/80 and phycoerythrin-labelled anti-mouse CD45 antibodies (C and D)**

In the immunofluorescence assays, nuclei were counterstained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole). Magnification, $\times$40.

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Results – Article 2

NF-kB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses

Background and aims: Recent studies have demonstrated that the p50 subunit of the transcription factor NF-kB (nuclear factor kB), codified by NF-kB1 gene, shows important regulatory activities that contribute in down-modulating NF-kB-mediated responses and is involved in the regulation of macrophage activation. In particular, p50 homodimers down-regulate pro-inflammatory cytokines production by macrophages and hepatic stellate cells and drive alternative M2 polarization, producing Arginase-1 and CCL17 and inhibiting iNOS and TNF-α production. Accordingly, it has been observed that NF-kB1-knockout mice (NF-kB1$^{-/-}$) develop more inflammation and parenchyma injury as compared to wild type animals in a model of *Escherichia Coli*-induced pneumonia and present a delayed resolution in glomerulonephritis. At hepatic level, NF-kB1 deficiency in mice increases inflammation and fibrosis after chronic carbon tetrachloride (CCL_4) intoxication. In a previous study, we have observed that liver M1 macrophage bias of C57BL/6 mice is responsible for the major severity of NASH of this strain and this suggests that genetic and epigenetic factors regulating macrophage activation might have an important role in influencing the progression of NAFLD from simple steatosis to steatohepatitis. In this contest, recent studies have characterized a new functional polymorphism in the promoter region of the human NF-kB1 gene (-94 insertion/deletion ATTG, rs28720239) that reduces protein production and is associated with a higher prevalence of inflammatory and autoimmune diseases. The same polymorphism also increases the risk of cirrhosis in alcoholic patients by an up-regulation of hepatic inflammation.
Starting from this background, in the present study we have investigated whether NF-kB1 deficiency in mice might affect macrophage regulation and influence the progression of NASH. To this aim, NASH has been induced by treating NF-kB1$^{-/-}$ and wild type C57BL/6 mice with methionine and choline deficient (MCD) diet because NF-kB1$^{-/-}$ mice are protected against insulin resistance and obesity induced by high fat diet in relation to an increase in hepatic insulin response and an elevated energy expenditure.

Key results: Even if both wild type and NF-kB1$^{-/-}$ mice receiving the MCD diet for four weeks develop NASH, the knockout mice have more steatosis, lobular infiltration, increased hepatocyte apoptosis, ALT release and TNF-α production as compared to the wild type animals. Moreover,

NF-kB1 deficiency induces centrilobular collagen deposition after only four weeks of MCD diet. Surprisingly, we have found that NF-kB1 deficiency does not alter the macrophage response in this model: in fact, there are no differences between the two strains of mice in the number of liver infiltrating F4/80 positive macrophages and in the up-regulation of macrophage M1 markers IL-12p40 and iNOS. Conversely, NF-kB1$^{-/-}$ mice show an increased liver infiltration of T-cells, in particular of CD8+ lymphocytes and NKT cells, that is associated to the rapid progression of NASH. Such an increase in liver NKT cells is associated with an up-regulation of IL-15, a cytokine involved in the NK and NKT cells survival and maturation, which is evident after only two weeks of treatment with MCD diet. In line with an increased recruitment of NKT cells, NF-kB1$^{-/-}$ mice have a higher liver content of IFN-γ and osteopontin, two NKT cells-derived cytokines, than wild type animals and around the 40% of osteopontin-expressing cells isolated from NF-kB1$^{-/-}$ with NASH are NKT cells.

Outcome and conclusions: In this paper, we have demonstrated that in spite NF-kB1 coded p50 is involved in regulating macrophage polarization, NF-kB1 deficiency does not influence macrophage response in NASH. However, the more rapid progression of NASH in NF-kB1$^{-/-}$ mice as compared to wild type animals involves an increased recruitment and activation of NKT cells likely in relation to the fact that in knockout mice there is an augmented production of IL-15 already at early stages of the disease. Moreover, NASH in NF-kB1$^{-/-}$ mice is characterized by a specific increase in the liver production of IFN-γ and osteopontin. The elevation of osteopontin in knockout animals can explain the worsening of fibrosis in these mice as osteopontin is able to stimulate collagen production by hepatic stellate cells. These observations support the importance of NKT cells in the evolution of NASH to fibrosis and point to possible importance of NF-kB1 polymorphisms as risk factor in the progression of human NASH.

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Original Paper

NF-κB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses

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Abstract

Growing evidence indicates that NF-κB (nuclear factor κB) activation contributes to the pathogenesis of NASH (non-alcoholic steatohepatisis). Among the NF-κB subunits, p50/NF-κB1 has regulatory activities down-modulating NF-κB-mediated responses. In the present study, we investigated the effects of NF-κB1 deficiency on the progression of NASH induced by feeding mice on an MCD (methionine/choline-deficient) diet. Following 4 weeks on the MCD diet, steatosis, ALT (alanine aminotransferase) release, hepatocyte apoptosis, lobular inflammation and TNFα (tumour necrosis factor α) production were higher in NF-κB1$^{-/-}$ (NF-κB1-knockout) mice than in WT (wild-type) mice. NF-κB1$^{-/-}$ mice also showed appreciable centrilobular collagen deposition, an increased number of activated hepatic stellate cells and higher type-I procollagen-α and TIMP-1 (tissue inhibitor of metalloproteases-1) mRNA expression. Although NF-κB p50 homodimers regulate macrophage activation, the number of hepatic macrophages and liver mRNAs for iNOS (inducible NO synthase), IL (interleukin)-12p40, CCL2 (CC chemokine ligand 2) and CXCL10 (CXC chemokine ligand 10) were comparable in the two strains. NASH was associated with an increase in liver infiltrating T-cells that was more evident in MCD-fed NF-κB1$^{-/-}$ than in similarly treated WT mice. Flow cytorimetry showed that T-cell recruitment involved effector CD8^{+} T-cells without changes in the helper CD4^{+} T-cell fraction. Furthermore, although NASH lowered hepatic NKT cells [NK (natural killer) T-cells] in WT mice, the NKT cell pool was selectively increased in the livers of MCD-fed NF-κB1$^{-/-}$ mice. Such NKT cell recruitment was associated with an early overexpression of IL-15, a cytokine controlling NKT cell survival and maturation. In the livers of MCD-fed NF-κB1$^{-/-}$ mice, but not in those of WT littermates, we also observed an up-regulation in the production of NKT-related cytokines IFN (interferon)-γ and osteopontin. Taken together, these results indicate that NF-κB1 down-modulation enhanced NASH progression to fibrosis by favouring NKT cell recruitment, stressing the contribution of NKT cells in the pathogenesis of NASH.

Key words: liver fibrosis, liver inflammation, natural killer T-cell (NKT cell), non-alcoholic fatty liver disease

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is becoming one of the most common liver diseases worldwide [1]. One still open issue in NAFLD pathogenesis concerns the mechanisms responsible for the switching from simple steatosis to NASH (non-alcoholic steatohepatitis). This aspect is clinically relevant because parenchymal injury and inflammation that characterize NASH are the driving forces for the disease evolution to fibrosis/cirrhosis [2,3]. Growing evidence indicates that the activation of the NF-κB (nuclear factor κB) plays an critical role in the onset of adipose tissue inflammation in obesity [4]. Furthermore, studies in rodent models of NAFLD/NASH show that an increased NF-κB activity is also associated with the development of steatosis, hepatic insulin resistance and inflammation [5,6]. A similar NF-κB stimulation is also evident in the liver biopsies from NASH patients [7]. On the other hand, interfering with NF-κB nuclear translocation ameliorates liver insulin resistance, steatosis and inflammation in

Abbreviations: ALT, alanine aminotransferase; CCL2, CC chemokine ligand 2; CXCL10, CXC chemokine ligand 10; GM-CSF, granulocyte/macrophage colony-stimulating factor; HSC, hepatic stellate cell; HDAC-1, histone deacetylase-1; IFN, interferon; IL, interleukin, iNOS, inducible NO synthase; MCD, methionine/choline-deficient diet; MGL1, macrophage galactose-type C-type lectin 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor κB; NF-κB1$^{-/-}$, NF-κB1-knockout; NK, natural killer; NKT cell, NK T-cell; α-SMA, α-smooth muscle actin; TIMP-1, tissue inhibitor of metalloproteases-1; TNFα, tumour necrosis factor α; WT, wild-type.

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NAFLD [6,8]. NF-κBs are a family of dimeric proteins consisting of five Rel subunits (p50/NF-κB1, p52/NF-κB2, p65/RelA, p68/RelB and p75/c-Rel) that by binding with each other can form a variety of hetero- and homodimers regulating genes involved in immunity, inflammation and cell survival [9]. Among the different NF-κB units, p50/NF-κB1 and its precursor molecule p105 have been show to have important regulatory activities that contribute in down-modulating NF-κB-mediated responses [10,11]. Accordingly, NF-κB1-deficient animals show impaired macrophage M2 polarization, develop more severe colitis and pneumonia and have a delayed resolution of glomerulonephritis [11,12]. Moreover, a functional polymorphism of human NF-κB1 gene ($-$94ins/delATTG, rs28720239) that reduces the protein production has been associated with a higher prevalence of inflammatory and autoimmune diseases [13–15]. The same polymorphism also increases the risk of cirrhosis in alcoholic patients [16]. From this background we investigated whether NF-κB1 deficiency might influences the progression of NASH. So far, experimental data regarding the role of NF-κB1 in liver injury have given conflicting results, as NF-κB1 deletion in mice does not affect acute liver injury and regeneration, while it enhances inflammation and fibrogenesis following chronic CCl_4 (carbon tetrachloride) intoxication [17–19]. In the present study, NASH was induced by feeding mice on an MCD (methionine–choline-deficient) diet because NF-κB1$^{-/-}$ (NF-κB1-knockout) mice are protected against insulin resistance and obesity induced by feeding a high-fat diet in relation to an increase in hepatic insulin response and an elevated energy expenditure [20].

MATERIALS AND METHODS

Animals and experimental protocol

NF-κB1$^{-/-}$ mice on C57BL/6 background [28] were a gift from Dr Antonio Sica (Department of Pharmaceutical Science, University of East Piedmont, Novara, Italy). WT (wild-type) C57BL/6 mice were bred at the same facility. Mice at 8 weeks of age were used for all the experiments. MCD and control diets were supplied by Laboratorio Dottori Piccioni, and mice were fed for 2 or 4 weeks. Body weight was recorded weekly throughout the experiment. At the end of the study protocol, mice were anaesthetized with sevofluorane and blood was collected by cardiac puncture. Livers were rapidly removed, weighed, and cut in pieces that were immediately frozen in liquid nitrogen and kept at -80°C until analysed. Two portions of each liver were, respectively, fixed in buffered, pH 7.4, 10% formalin or snap-frozen in OCT for histology. The experiments were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

Biochemical analysis

Plasma ALT (alanine aminotransferase) and liver triacylglycerol (triglyceride) levels were determined by spectrometric kits (Radim and Sigma Diagnostics respectively). Circulating TNFα (tumour necrosis factor α) as well as liver IFN (interferon)-γ and osteopontin levels were evaluated by commercial ELISA kits (Peprotech and R&D Systems respectively).

mRNA extraction and real-time PCR

RNA was extracted from mouse livers with TRI reagent and retro-transcripted using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia) according to the manufacturer's instruction. Real-time PCR was performed in a Techne TC-312 termalcycler (Tecne), using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNFα, IL (interleukin)-12p40, IFN-γ, IL-10, IL-15, CCL2 (CC chemokine ligand 2), CXCL10 (CXC chemokine ligand 10), NK1.1, iNOS (inducible NO synthase), osteopontin, MGL1 (macrophage galactose-type C-type lectin 1), α1-procollagen, TIMP-1 (tissue inhibitor of metalloproteases-1), Bcl-XL, A20 and β-actin (Applied Biosystems Italia). All samples were run blind in duplicate and the relative gene expression was calculated as $2^{-\Delta C_t}$ ($\Delta C_t = C_t$ of the target gene $- C_t$ of β-actin, taken as housekeeping gene) using the 7000 System Software. The results are expressed as the fold increase over control samples.

Histology and immunohistochemistry

Liver pathology was assessed in haematoxylin/eosin and Masson's trichrome stained sections. Steatosis and lobular inflammation were scored blind by an experienced pathologist as described by Kleiner et al. [21]. The number of necro-inflammatory foci was counted in ten different high-magnification microscopic fields. TUNEL (terminal deoxyribonucleotide transferase-mediated dUTP nick-end labelling) staining was performed on paraffin liver sections using Apoptags Kit (Intergen), according to the manufacturer's instructions. Hepatic macrophages and activated HSCs (hepatic stellate cells) were evidenced in formalin-fixed sections using respectively, anti-mouse F4/80 (eBiosciences) or α-SMA (α-smooth muscle actin) polyclonal antibodies (Labvision; Bio-Optica) in combination with a peroxidase-linked goat anti-(rat IgG) and horseradish peroxidase polymer kit (Biocare Medical). F4/80- or α-SMA-positive cells were counted in ten different microscopic fields.

Intrahepatic lymphocyte isolation and flow cytometry analysis

Lymphocytes were isolated from the livers of WT and NF-κB1$^{-/-}$ mice and purified on a density gradient (Lympholyte®-M; Cedarlane Laboratories) as described in [22]. Cells were then washed in Hanks medium, incubated 30 min with decomplemented mouse serum to block unspecific immunoglobulin binding. The cells were than stained with fluorochrome-conjugated antibodies for CD45, CD3, CD4, CD8, NK1.1 (eBiosciences) and their relative numbers were quantified with a FACScalibur (Becton Dickinson) flow cytometer. A polyclonal anti-osteopontin rabbit antiserum (Millipore) and phycoerythrin-conjugated anti-rabbit IgG (Sigma) were used for the evaluation of osteopontin-producing cells.

Data analysis and statistical calculations

Statistical analyses were performed by SPSS statistical software (SPSS Inc.) using one-way ANOVA test with Tukey's

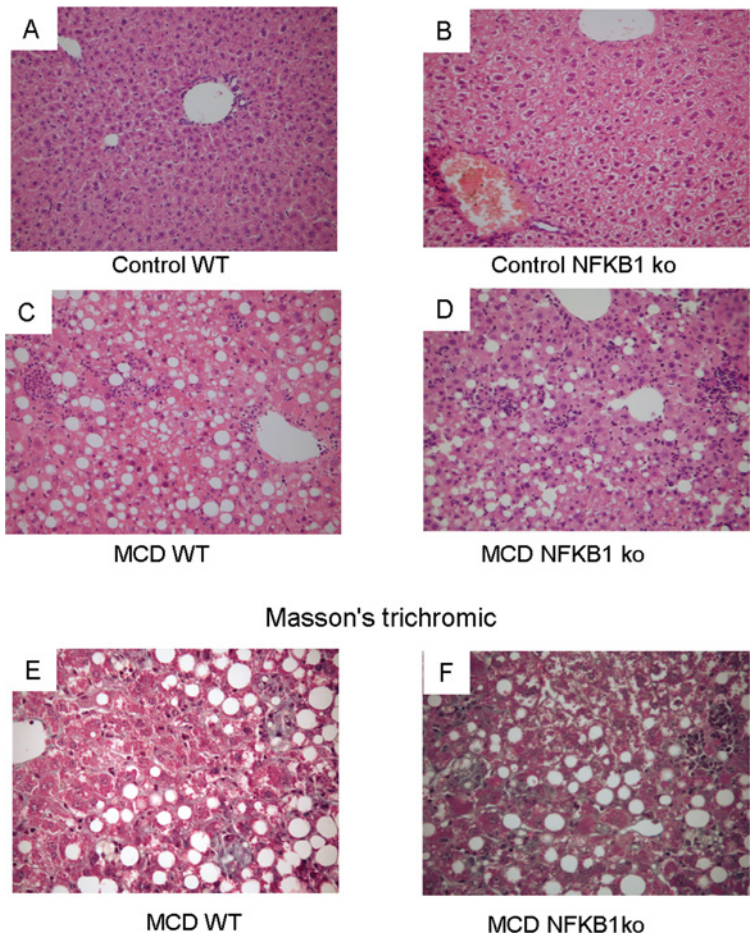

Figure 1 **NF-κB1 deletion enhances liver injury and fibrosis in mice with NASH**
WT and NF-κB1$^{-/-}$ (NFKB1 ko) mice were fed 4 weeks with either a control or a MCD diet. The livers were stained with haematoxylin/eosin (**A**–**D**: magnification ×200) or Masson's trichrome (**E**, **F**; magnification ×400).

correction for multiple comparisons or Kruskal–Wallis test for non-parametric values. Significance was taken at the 5% level. Normality distribution was preliminarily assessed by the Kolmogorov–Smirnov test.

RESULTS

NF-κB1 deficiency worsens steatohepatitis and promotes liver fibrosis in mice fed on the MCD diet

The livers of NF-κB1$^{-/-}$ mice fed on the control diet had normal histological appearance, except for the presence of sporadic aggregates of mononuclear cells surrounding apoptotic hepatocytes (Figure 1). Feeding for 4 weeks on the MCD diet caused an appreciable weight loss in rodents. In line with the higher energy expenditure [20], NF-κB1$^{-/-}$ mice suffered more severe weight loss than the WT littermates (39.4 ± 5.7% compared with 27.5 ± 3.0; $P = 0.0002$). Both WT and NF-κB1$^{-/-}$ mice fed on the MCD diet developed NASH, which was characterized by macrovesicular steatosis accompanied by lobular infiltration of inflammatory cells, hepatocytes ballooning and focal necrosis (Figure 1). In addition, granulomas consisting in aggregates of mononucleated cells surrounding fat-laden hepatocytes were also observed. (Figure 1). Blinded semi-quantitative scoring for steatosis and lobular inflammation showed that MCD-fed NF-κB1$^{-/-}$ mice had more steatosis, lobular infiltration and increased hepatocyte apoptosis as compared with the WT littermates, whereas the prevalence of necrotic foci was comparable. The frequency of granulomas was also higher in NF-κB1$^{-/-}$ mice (Table 1). In accordance with histology, intrahepatic triacylglycerol accumulation, ALT release and oxidative stress were significantly higher in MCD-fed NF-κB1$^{-/-}$ mice than in MCD-fed WT animals (Figure 2). Furthermore, NF-κB1$^{-/-}$ mice fed on the MCD diet had a 2-fold increase in both liver TNFα mRNA and circulating TNFα levels compared with similarly treated WT animals (Figure 2). The stimulation in apoptosis observed in livers from MCD-fed NF-κB1$^{-/-}$ mice was not due to an impaired regulation of NF-κB-dependent anti-apoptotic factors, as the hepatic expression of Bcl-XL and A20 genes was not affected by NF-κB1 deficiency (results not shown). The steatohepatitis induced by the MCD diet is known to triggers hepatic fibrosis. However, in mice fibrillar matrix, accumulation becomes evident only after 8 weeks of treatment. Unexpectedly,

Table 1 NF-κB1 deletion worsens NASH histology in mice
Steatosis and lobular inflammation were scored semi-quantitatively as described by Kleiner et al. [23], while the number of necro-inflammatory foci, apoptotic hepatocytes and granulomas were counted in ten different high magnification microscopic fields. The values refer to 8–10 animals in each group.

	MCD-fed		
Parameter	**WT mice**	**NF-κB1$^{-/-}$ mice**	***P* value**
Steatosis (*n*)	1.9 ± 0.6	2.6 ± 0.5	0.02
Lobular inflammation (*n*)	1.5 ± 0.5	2.2 ± 0.7	0.02
Hepatocyte apoptosis (*n*)	2.5 ± 1.5	4.7 ± 2.2	0.03
Necrotic foci (*n*)	5.0 ± 2.3	5.3 ± 3.0	0.79
Granulomas (*n*)	2.8 ± 0.75	5.2 ± 1.2	0.003

Masson's trichromic staining for liver collagen revealed that already after 4 weeks on the MCD diet NF-κB1$^{-/-}$ mice developed appreciable centrilobular collagen deposition (Figure 1). Conversely, MCD-fed WT mice had a very modest increase in fibrillar matrix mainly localized in the peri-sinusoidal spaces (Figure 1). Immunohistochemisty for α-SMA also showed an increased number of α-SMA-positive activated myofibroblast-like HSCs only in the livers of MCD-fed NF-κB1$^{-/-}$ mice (Figure 2). Supporting these observations, the hepatic mRNAs for type 1 procollagen α and the TIMP-1 were expressed significantly more in NF-κB1$^{-/-}$ mice than in WT mice (Figure 2). Interestingly, an increased severity of NASH in NF-κB1$^{-/-}$ mice was already appreciable after 2 weeks of feeding on the MCD diet, as knockout animals had more extensive lobular inflammation and an increase in ALT release and TNFα and procollagen Iα mRNA expression than their WT littermates (see Supplementary Figure S1 at http://www.clinsci.org/cs/124/cs1240279add.htm).

NF-κB1 deficiency does not influence macrophage responses in NASH

NF-κB1-derived p105 and p50 proteins are known to be involved in regulating macrophage and lymphocyte responses [10,11]. In particular, p50 homodimers have an important role in down-modulating pro-inflammatory cytokine production in macrophages and HSCs as well as in driving macrophage 'alternative' M2 polarization [23–25]. Macrophage immunostaining with anti-F4/80 antibodies revealed that in both strains the development of NASH was associated with an increase in the number of F4/80-positive cells, but without significant differences between WT and NF-κB1$^{-/-}$ mice (see Supplementary Figure S2 at http://www.clinsci.org/cs/124/cs1240279add.htm). Furthermore, NF-κB1 deficiency did not influence the up-regulation of macrophage M1 markers IL-12p40 and iNOS promoted by NASH (Supplementary Figure S2). As expected, hepatic mRNAs for the M2 markers MGL1 (CD301) and IL-10 were lower in control NF-κB1$^{-/-}$ mice compared with the WT littermates. MDC feeding did not affect MGL1 and IL-10 expression, although IL-10 mRNA remained significantly lower in MCD-fed NF-κB1$^{-/-}$ mice (Supplementary Figure S2). Furthermore, despite the loss of NF-κB1 that has been reported to enhance the production of CCL2 and CXCL10 in HSCs [24], liver mRNAs for these chemokines were comparable in the MCD-fed WT and NF-κB1$^{-/-}$ animals (Supplementary Figure S2).

NKT cell [NK (natural killer) T-cell] recruitment characterizes NASH in NF-κB1-deficient mice

Lymphocytes are a common feature in NASH inflammatory infiltrates. Immunohistochemical staining for CD3 showed an increased prevalence of T-cells in the lobular infiltrates of MCD-fed NF-κB1$^{-/-}$ (27.9 ± 2.4 compared with 20.1 ± 4.7; $P = 0.002$). Flow-cytorimetry confirmed that CD3$^+$ T-cells were increased in NASH livers and that T-cell recruitment was more pronounced in NF-κB1$^{-/-}$ mice (Figure 3). Such an increase involved effector CD8$^+$ T-cells, without changes in the helper CD4$^+$ T-cell fraction (Figure 3). We also observed that, although NASH in WT animals was characterized by the lowering of both liver NK (CD3$^-$, NK1.1$^+$) and NKT (CD3$^+$, NK1.1$^+$) cell populations (Figure 3), the NK cell fraction was unchanged in the livers of MCD-fed NF-κB1$^{-/-}$ mice (Figure 3). Furthermore, in these latter mice the development of NASH was associated with an increase in the NKT cell pool (Figure 3). The increase in liver NKT cells observed NF-κB1$^{-/-}$ mice fed on the MCD diet was associated with a specific up-regulation in the hepatic mRNA expression of IL-15, a cytokine involved in NK and NKT cell survival and maturation [26] (Figure 4). IL-15 overexpression was evident already after 2 weeks of treatment (Figure 3) concomitantly with an increase in the mRNA of the NK/NKT cell marker NK1.1 (Supplementary Figure S1), suggesting that NF-κB1 deficiency might stimulate NKTcell recruitment by promoting IL-15 activity. Recent studies have proposed a role for NKT-cell-derived osteopontin in the progression of NASH [27,28]. In line with this view, MCD-fed NF-κB1$^{-/-}$ mice had a specific increase in the liver mRNAs for IFN-γ and osteopontin, two cytokines produced by NKT cells. The hepatic content of these cytokines was also 3- and -2-fold higher in NF-κB1$^{-/-}$ than in the WT mice. Moreover, approximately 40 % of the osteopontin-expressing leucocytes isolated from NF-κB1$^{-/-}$ NASH livers were positive for NK1.1 (Figure 4).

DISCUSSION

Growing evidence indicates that the NF-κB1-gene-coded proteins p105 and p50 have important regulatory activities of inflammatory responses [10,11]. In particular, p105 forms complexes with p50 and the protein kinase TPL2 (tumour progression locus-2) blocking their functions, whereas p50 homodimers act as transcriptional activators promoting memory T-cell activation and macrophage M2 polarization [10,11,25]. Furthermore, p50 in combination with HDAC-1 (histone deacetylase-1) regulates pro-inflammatory genes [TNFα, CCL2, CXCL10 and GM-CSF (granulocyte/macrophage colony-stimulating factor)] expression in activated HSCs [24]. In the present study, we show that steatohepatitis induced by the MCD diet is more severe in NF-κB1$^{-/-}$ mice being characterized by extensive lobular infiltration by mononucleated cells, frequent lipogranulomas, higher

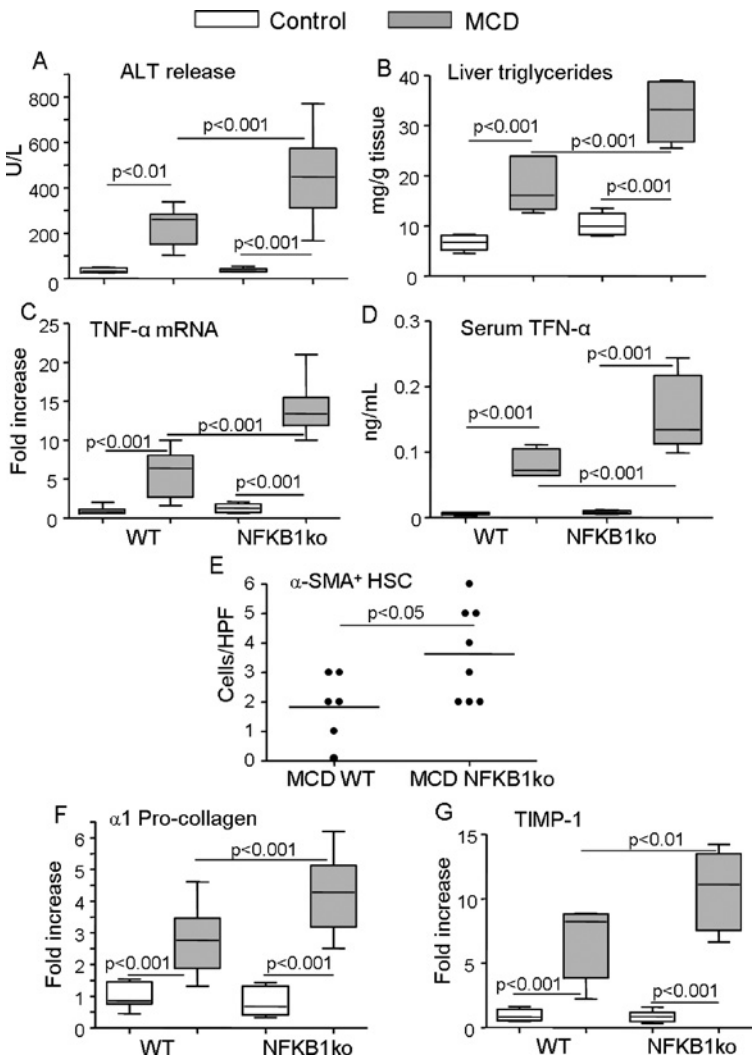

Figure 2 **NF-κB1 deletion enhances markers of liver injury and fibrosis in mice with NASH**

WT and NF-κB1$^{-/-}$ (NFKB1 ko) mice were fed for 4 weeks on either a control or an MCD diet. ALT release (**A**) and liver triacylglycerol content (**B**) were evaluated by enzymatic methods. The liver mRNA expression of TNFα, α1-procollagen and TIMP-1 (**C**, **F**, **G**) were measured by RT–PCR (reverse transcription–PCR) and expressed as the fold increase after normalization to the β-actin gene. The circulating levels of TNFα were determined in the sera of the same animals (**D**). The values refer to 8–10 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile). Activated HSCs expressing α-SMA + HSC were evidenced by immunohistochemistry and counted in ten different high-magnification microscopic fields (**E**).

circulating TNFα levels and increased hepatocyte apoptosis. Furthermore, NASH in NF-κB1$^{-/-}$ mice associates with a more rapid progression of centribolular fibrosis that is already evident after 4 weeks of treatment. Previous observations concerning the factors promoting chronic liver injury in CCl_4-treated NF-κB1$^{-/-}$ mice have implicated an enhanced recruitment of α-SMA-positive activated HSCs that, beside producing procollagen 1α and TIMP-1, overexpress pro-inflammatory mediators such as TNFα, CCL2, CXCL10, GM-CSF in relation with an impaired down-modulation of these genes by NF-κB1/p50 and HDAC-1 [24]. Although an increase in activated HSCs characterizes NASH in NF-κB1$^{-/-}$ mice, the expression of CCL2 and CXCL10 genes in these animals is comparable with the WT mice, thus excluding that HSC-derived cyto/chemokines account for the promotion of liver injury.

Studies using mice models of glomerulonephritis have shown that a diffuse T-cell infiltration characterize delayed resolution of kidney inflammation in NF-κB1$^{-/-}$ mice [12]. In accordance with these findings, we have observed that T-cell recruitment is associated the rapid progression of NASH in NF-κB1$^{-/-}$ mice. Such an increase in T-cells specifically involves cytotoxic CD8$^+$ T-cells and NKT cells, whereas CD4 helper T-cells are unaffected. The role of NKT cells in NASH is complex, as steatosis and the early phase of steatohepatitis are characterized by the lowering of the liver NKT cell pool as a consequence of IL-12 production [29–30], whereas an expansion of the NKT

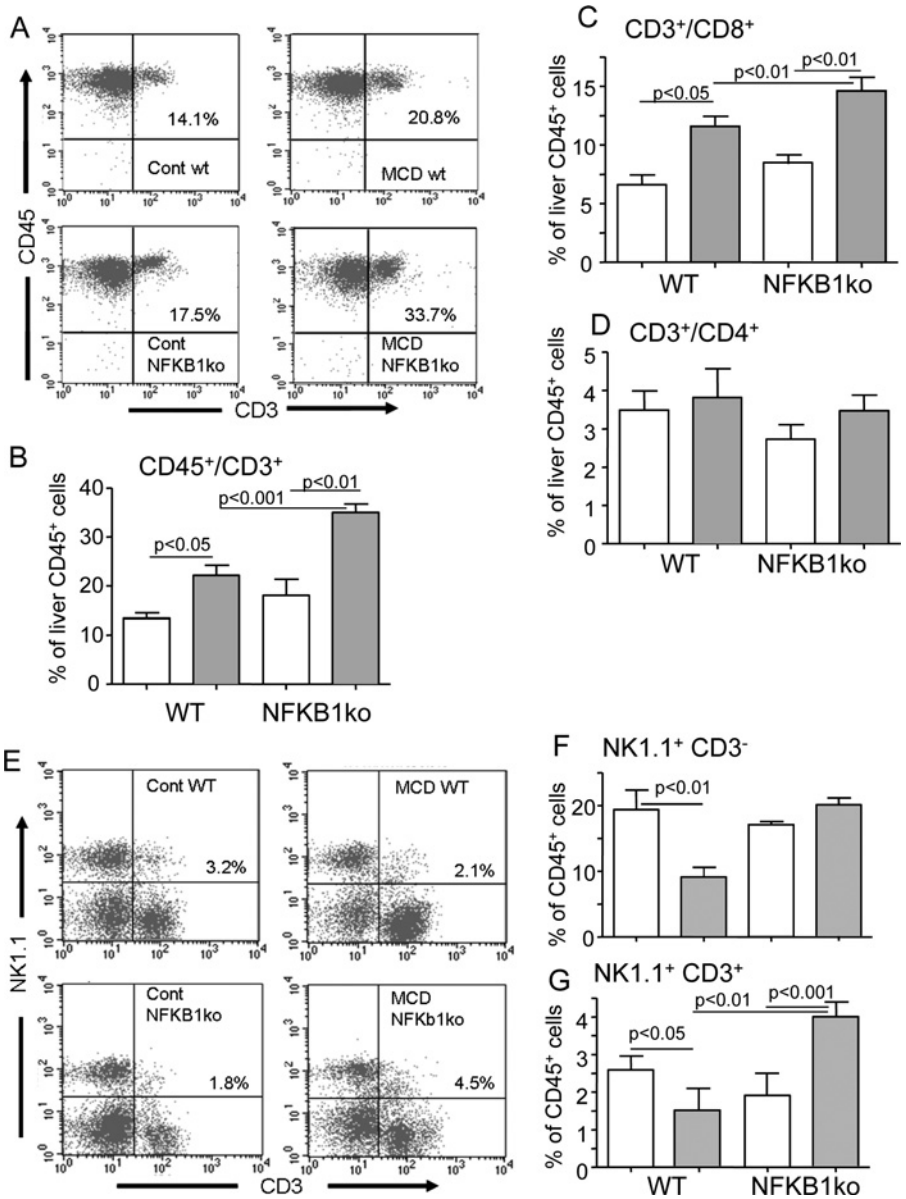

Figure 3 **NASH in NF-κB1-deficient mice associates with an increased recruitment of hepatic T-cells**
Liver leucocytes were isolated from the livers of WT and NF-κB1$^{-/-}$ (NFKB1 ko) mice fed for 4 weeks on either a control or a MCD diet (**B**) and analysed by FACS. Representative plot of CD3-positive T-cells (**A**) and statistical analysis of total CD3$^+$ and CD8$^+$ or CD4$^+$ T-cells subsets (**B**–**D**). Representative FACS plot of NK1.1 and CD3-positive lymphocytes (**E**) and statistical analysis of CD3$^-$ NK1.1$^+$ (NK) and CD3$^+$ NK1.1$^+$ (NKT) cells (**F**, **G**). The values refer to 5–6 animals in each group and the bars represent medians $\pm$ S.D.

cell population is evident in mice with more advanced NASH [27]. Furthermore, NKT cell depletion prevents hepatic inflammation and fibrosis induced by the MCD diet [28], but enhances the liver expression of inflammatory markers in mice fed on a high-fat diet [31]. We observed that an increase in NKT cells characterizes advanced NASH (steatohepatitis plus fibrosis) in MCD-fed NF-κB1$^{-/-}$ mice as opposed to NKT cell depletion present in WT animals that only show steatohepatitis. This is consistent with the correlation between the NAS score and hepatic NKT cell prevalence reported in NASH patients [32], supporting the importance of NKT cells in driving the progression of NASH towards fibrosis. So far, little is known about how NF-κB1 deficiency influences NKT cell responses. Stankovic et al. [33] recently reported that NF-κB1 loss moderately reduces hepatic NKT cell pool in C57BL/6 mice by affecting their maturation. However, we observed that already in the early phases of NASH NF-κB1$^{-/-}$ mice up-regulate the hepatic expression of IL-15. IL-15 is a pleiotropic cytokine belonging to the four α-helix bundle cytokine family and is responsible for macrophage, T-cell, NK cell and NKT cell survival and maturation [26].

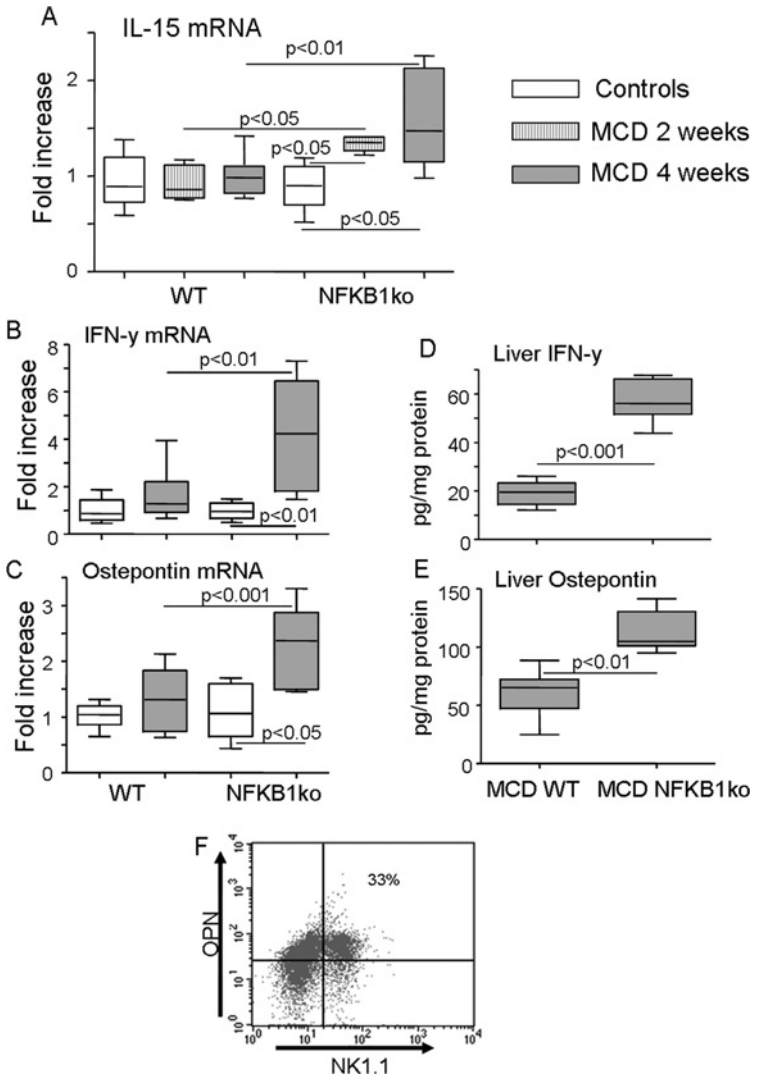

Figure 4 **Production of NKT-cell-derived cytokines characterizes NASH in NF-κB1-deficient mice**

NASH was induced by feeding mice on an MCD diet for either 2 or 4 weeks. Liver mRNA expression of IL-15, IFN-γ and osteopontin (**A**–**C**) were evaluated by RT–PCR and expressed as the fold increase after normalization to the β-actin gene. The intrahepatic levels of IFN-γ (**D**) and osteopontin (**E**) were evaluated by ELISA. The values refer to 6–10 animals in each group and the boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile). Representative FACS plot of osteopontin- and NK1.1-expressing leucocytes isolated from the livers of NF-κB1$^{-/-}$ (NFKB1 ko) mice fed on the MCD diet (**F**).

In the liver, IL-15 produced by hepatocytes has a key role in creating a T-cell-favourable microenvironment and it also induces the expression of NK cell markers on T-cells [34]. Although IL-15 is constitutively produced in the liver, its expression by hepatocytes and hepatic progenitor cells increases in response to injury [35]. Indeed, in WT mice IL-15 up-regulation is evident in advanced NASH induced by 8 weeks feeding on the MCD diet concomitantly with hepatic NKT cells expansion [27]. Thus we propose that NF-κB1 loss might favour IL-15 up-regulation at the onset of NASH, preventing NKT cell depletion caused by IL-12 [30] and leading to a more rapid increase in their number through a more efficient hepatic differentiation.

Concerning the mechanisms by which NKT cells could favour the progression of liver injury in NASH, we observed that MCD-fed NF-κB1$^{-/-}$ mice display a specific increase in the liver production of IFN-γ and osteopontin. IFN-γ can originate from both CD8^{+} T- and NKT cells and is potent inducer of TNFα and ROS (reactive oxygen species) generation by macrophages. Osteopontin is a glucosylated cytokine produced by both immune and parenchymal cells that is increasingly recognized to play important roles in inflammation and tissue healing [36]. NKT cells are a relevant source of osteopontin in lymphocyte-mediated hepatic injury [37], and osteopontin-expressing NKT cells are evident in the livers of NF-κB1$^{-/-}$ mice with NASH. This does not

exclude the possibility that other cells such as T-cells, macrophages or cholangiocytes [38] might also contribute to osteopontin formation. A role for osteopontin in stimulating NASH evolution is in line with the observation that osteopontin-deficient mice are protected against steatohepatitis and fibrosis induced by feeding on an MCD diet [28,39]. Furthermore, osteopontin has been implicated as an important modulator of fat inflammation during obesity [40]. However, in comparision with that reported by Sahai et al. [39] using A/J mice we did not observe changes in hepatic osteopontin expression in WT C57BL/6 mice fed on the MCD diet for 4 weeks. This discrepancy might reflect strain differences in hepatic inflammatory responses involved in the onset of NASH [41]. It is noteworthy that NF-κB1-deficient mice develop appreciable liver fibrosis despite the presence of elevated IFN-γ production that should antagonize fibrogenesis. In this context, the increase in liver osteopontin observed in these animals might have a relevant role, as osteopontin can efficiently stimulate HSCs to collagen production by engaging integrin $\alpha_V\beta_3$ and stimulating inositide 3-phosphate kinase and NF-κB signalling [42].

Altogether these results indicate that NF-κB1 down-modulation speed up NASH progression to fibrosis by stimulating the liver recruitment of osteopontin-producing NKT cells, thus supporting the importance of these cells in the evolution of NAFLD. Moreover, our results point to the possible relevance of NF-κB1 gene polymorphisms as a risk factor for the progression of the human disease.

CLINICAL PERSPECTIVES

- NF-κB activation is involved in the pathogenesis of NASH. Among the NF-κB subunits, p50/NF-κB1 has regulatory activities down-modulating NF-κB-mediated responses. Recently, a functional polymorphism in NF-κB1 gene (rs28720239) has been associated with a higher prevalence of inflammatory/autoimmune diseases as well as with an increased risk of alcoholic cirrhosis.
- By inducing experimental NASH feeding, we observed that liver injury, lobular inflammation and fibrosis developed more rapidly in NF-κB1$^{-/-}$ mice than in WT mice. Such effects were dependent on an increased liver recruitment of osteopontin-producing NKT cells through the up-regulation of IL-15 expression.
- These results stress on the importance of NKT cells in the evolution of NASH and point to NF-κB1 gene polymorphisms as a possible risk factor for the progression of NASH in humans.

AUTHOR CONTRIBUTION

Irene Locatelli and Salvatore Sutti conceived and carried out the experiments and analysed the data. Marco Vacchiano contributed to the experiments. Cristina Bozzola performed the histological analysis. Emanuele Albano wrote the paper. All authors had final approval of the submitted and published versions.

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Original Paper

SUPPLEMENTARY ONLINE DATA

NF-κB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses

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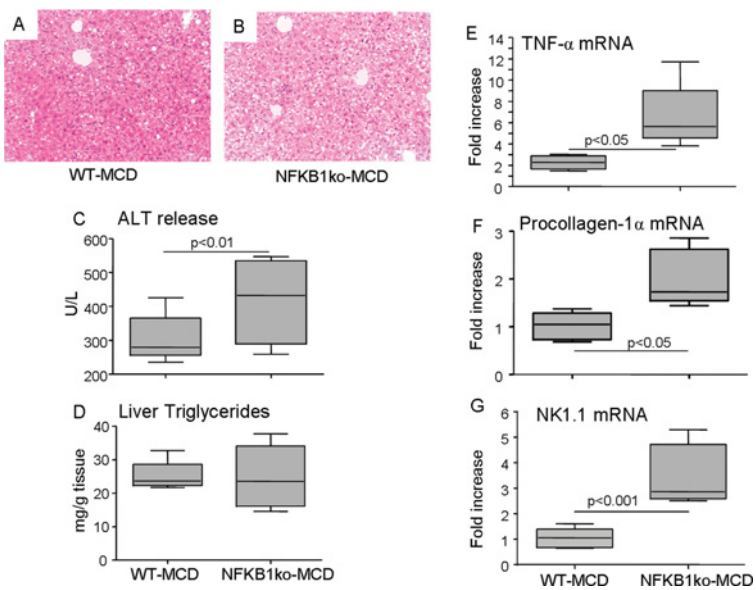

Figure S1 **Increased severity of NASH in NF-κB1-deficient mice fed for 2 weeks on an MCD diet**

Liver histology was evidenced by haematoxilin/eosin staining in MCD-fed WT and NF-κB1$^{-/-}$ (NF-κB1-ko) mice (**A**, **B**: magnification ×200). ALT release (**C**) and liver triacylglycerol content (**D**) were evaluated by enzymatic methods. The liver mRNA expression of TNF-α, α1-procollagen and NK1.1 genes (**E**–**G**) were measured by RT–PCR and expressed as fold increase after normalization to the β-actin gene. The values refer to six animals in each group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile).

[1]**These authors equally contributed to the study.**

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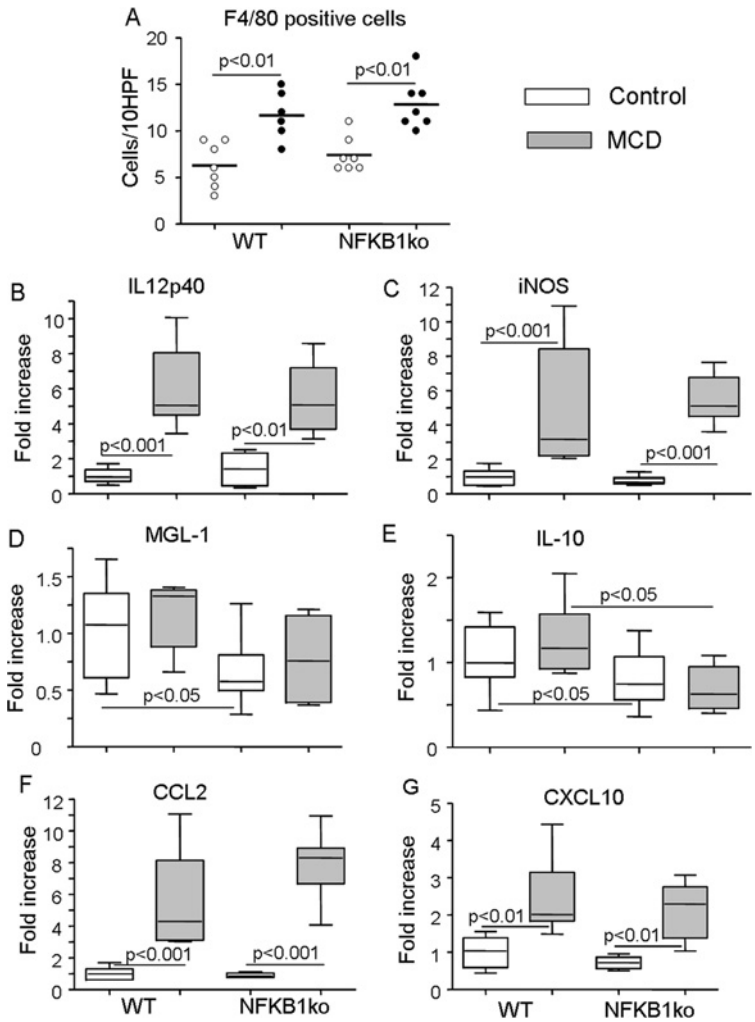

Figure S2 **The NF-κB1 deficiency did not influence macrophage responses in mice with NASH**
Wild-type and NF-κB1$^{-/-}$ (NF-κB1ko) mice were fed 4 weeks on either a control or an MCD diet. F4/80-positive macrophages were counted in ten different high-magnification microscopic fields (**A**). The liver mRNA expression of IL-12p40, iNOS, MGL1, IL-10, CCL2 and CXCL10 (**B**–**G**) were evaluated by RT–PCR and expressed as the fold increase after normalization to the β-actin gene. The values refer to 8–10 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile).

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Additional results

NASH evolution is associated with changes in macrophage functions

In order to better understand the mechanisms under-laying the evolution of NASH to fibrosis, in a series of subsequent experiments, we have compared C57BL/6 mice fed methionine and choline deficient (MCD) diet for four and eight weeks, respectively. As expected, mice treated for eight weeks present more liver damage evaluated by higher ALT release and hepatic triglyceride content than the animals fed for only four weeks (Fig. 1A-D). Histology also shows more extensive lobular inflammation that is associated with a time dependent increase in liver mRNAs for TNF-α and CD11b, an adhesion molecule expressed on activated monocytes (Fig.1E-F). This parallels with an elevation in the circulating levels of TNF-α (Fig. 1G). Although the mRNA content of hepatic fibrosis markers procollagen-1-α and α-smooth muscle actin (α-SMA) are up-regulated already after four weeks of treatment with the MCD diet, histological signs of fibrosis such as collagen deposition and increased prevalence of α-SMA-positive activated hepatic stellate cells are evident only in the animals with more advanced NASH (Fig.1H-O).

According with the worsening of lobular inflammation, we have also observed a progressive increase in the number of liver infiltrating F4/80-positive macrophages (Fig. 2A, B). Macrophages immunostaining with an anti-F4/80 antibody evidences that many of the F4/80-positive cells accumulating in the livers of mice receiving the MCD diet for eight weeks are enlarged and contain cytoplasmic vacuoles (Fig. 2A). Combined staining of frozen sections with anti-F4/80 antibodies and the lipid dye Oil Red O confirm that these cells contain lipid droplets likely derived from the phagocytosis of cell debris from fat-laden hepatocytes (Fig. 2C). The functional characterization of macrophage responses at different stages of NASH evolution shows that the liver mRNA levels for CCL2, its receptor CCR2 and the M1 activation markers inducible NO synthase (iNOS) and IL-12p40 subunit are highly upregulated in early NASH after four weeks on the MCD diet, but they significantly decrease extending the treatment up to eight weeks (Fig. 3A-D). Consistently, circulating IL-12 levels are higher in the early than in the advanced phases of NASH (Fig. 3G). These changes in macrophage M1 markers are confirmed by analysing liver macrophages isolated from control and MCD-treated mice (Fig. 3E-F). On the other hand, the macrophage expression of M2 activation markers, such as galactose-type C-type lectin-1 (MGL-1/CD301), is not appreciable affected during NASH progression (Fig. 3H). These observations indicate that during the progression of NASH the onset of fibrosis is associated with a down-modulation in inflammatory macrophage responses. This is consistent with that observed in the liver biopsies of patients with

advanced NASH where the presence of diffuse fibrosis is often combined with an improvement in the scores of lobular inflammation.

Recent studies have pointed out the importance of a variety of protein and lipid mediators involved in the resolution of acute inflammation in driving tissue healing responses (74). These factors down-modulate immune responses, control the clearance of tissue infiltrating leucocytes and promote functional changes in macrophage favouring the onset of repairing processes (75). Nonetheless, little is known about the role of these mediators in chronic inflammation.

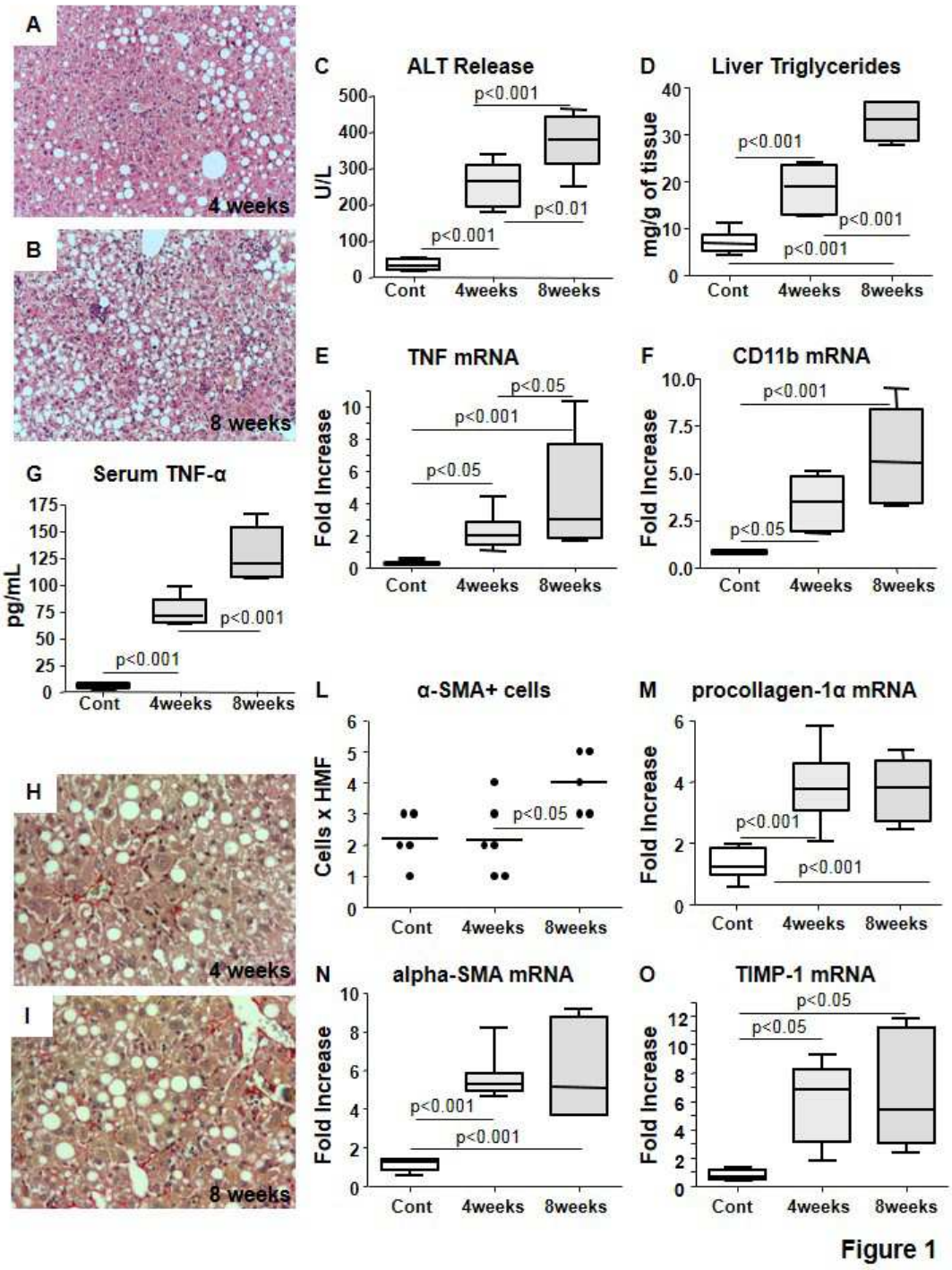

Figure 1: C57BL/6 mice fed with MCD diet for eight weeks develop NASH associated with fibrosis.
C57BL/6 mice were fed control or MCD diet up to eight weeks. Liver histology was evaluated in hematoxylin/eosin staining sections from MCD-fed animals for four and eight weeks (Panels A-B, magnification 20X). NASH severity was assessed by alanine aminotransferase (ALT) release, hepatic triglyceride content, liver TNF-α and CD11b mRNA expression and circulating TNF-α levels (Panels C-G). Liver fibrosis were evaluated by collagen deposition evidenced by Sirius Red staining (Panels H-I, magnification 20X), by activated hepatic stellate cells (HSCs) expressing alpha-smooth muscle actin (α-SMA) (Panel L) and by liver mRNA expression for procollagen-1α, alpha-SMA and TIMP-1 (Panels M-O). Hepatic mRNA was measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene. Circulating TNF-α levels were determined by ELISA in the same animals. The values refer to 6-8 animals in each group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values.

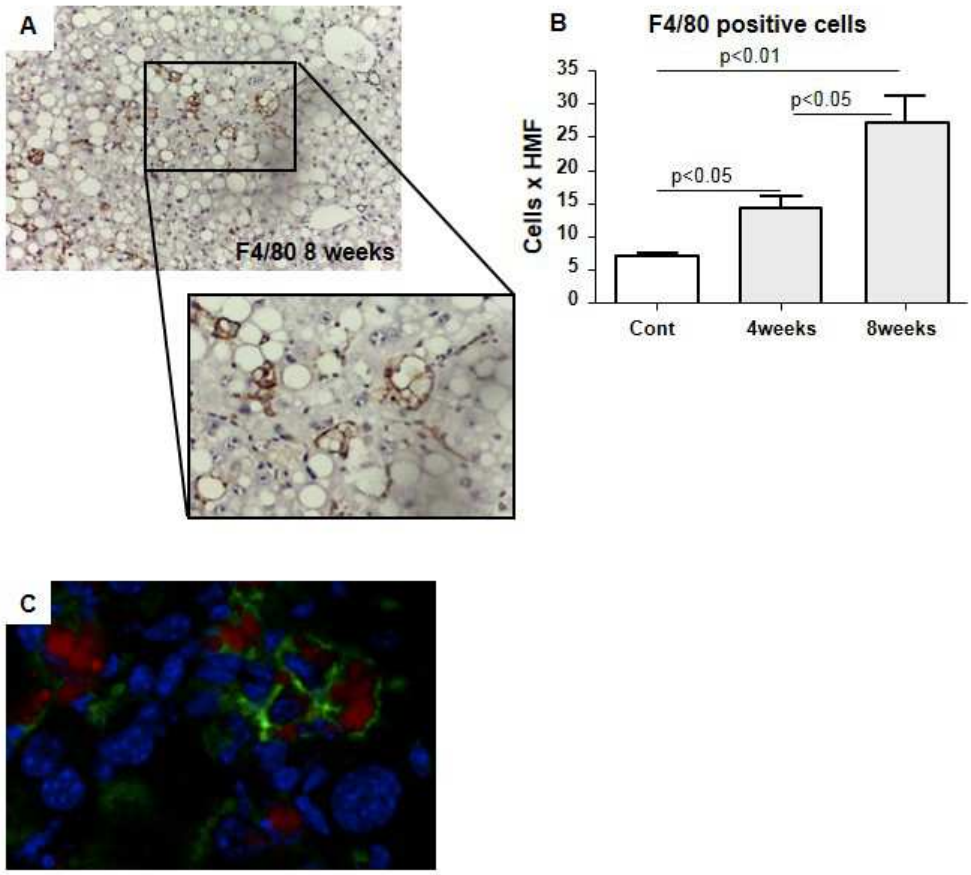

Figure 2

Figure 2: NASH development promotes macrophage recruitment.
C57BL/6 mice were fed control and MCD diet for four and eight weeks. Liver macrophages were evidenced by immunostaining with anti-F4/80 antibody and counted in 20 high magnification fields (Panels A, B). To identify macrophages containing lipid vacuoles, frozen liver sections were stained with lipid dye Oil Red O (red) and anti-F4/80 antibody (green) at immunofluorescence; cell nuclei were counter-stained with DAPI (Panel C, magnification 40X).

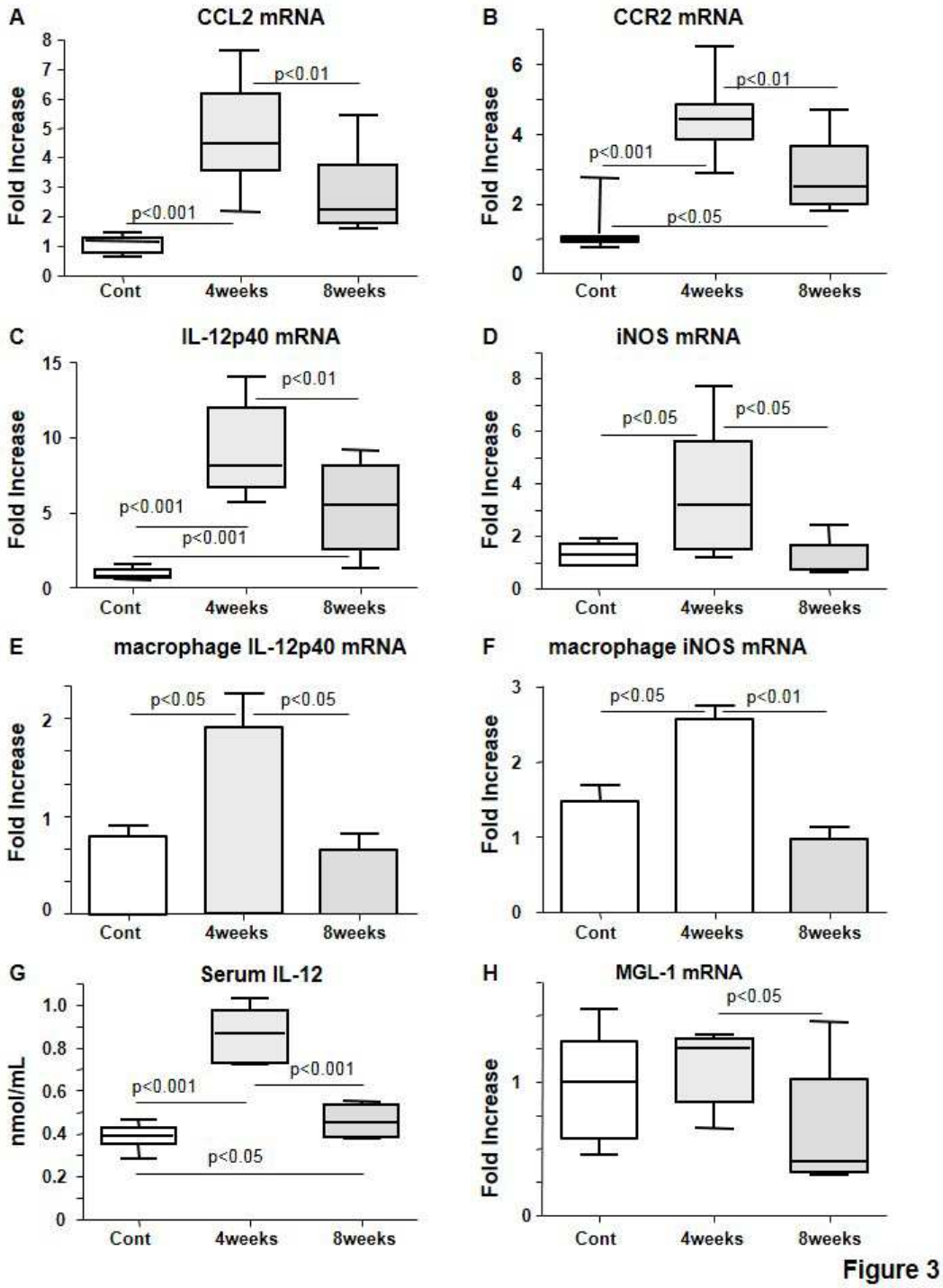

Figure 3

Figure 3: NASH evolution is associated with a change in macrophage phenotype.
C57BL/6 mice were fed control or MCD diet up to eight weeks. Liver mRNA levels for CCL2, CCR2, IL-12p40, iNOS and MGL-1 were measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene (Panels A, B, C, D, H). The liver IL-12 content was determined by ELISA in the same animals (Panel G). The values refer to 6-8 animals in each group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Hepatic macrophages were isolated from the livers of control or MCD-fed mice and analysed for the expression of IL-12p40 and iNOS (Panel E-F). The mRNA levels were measured by RT-PCR and expressed as fold increase over the values of untreated cells after normalization to the β-actin gene. The values refers to 4-5 different cell preparations ± S.D.

Results – Article 3

The role of Annexin A1 in modulating the progression of nonalcoholic steatohepatitis (NASH)

Background and aims: During the progression of experimental NASH, we have observed important changes in macrophage activity as the macrophage expression of M1 activation markers iNOS and IL-12p40 peak in the early phase of the disease and decline thereafter. This parallels with a lowering in the monocyte chemokines CCL1/CCL2 and their receptors CCR8/CCR2. The mechanisms responsible for these changes and their significance in relation to the disease evolution are still poorly characterized. The phospholipid-binding protein Annexin A1 (AnxA1) is produced by glucocorticoids and plays an important role in the resolution of inflammation with potent properties in terminating acute inflammatory responses by the stimulation of macrophage phagocytic clearance of apoptotic neutrophils. Moreover, it regulates the production of pro-inflammatory mediators such as eicosanoids, nitric oxide (NO) and IL-6. However, the role of AnxA1 in chronic setting is less investigated. A recent study has shown that the concentration of plasma AnxA1 is attenuated in obese subjects inversely correlating with the body mass index (BMI) and the increasing systemic inflammation evaluated by the measurement of C-reactive protein (CRP). Starting from these observations, we have investigated the possible role of AnxA1 in the pathogenesis of NASH, with the possible involvement of AnxA1 in modulating macrophage responses. For these experiments, we have taken advantage by the use of AnxA1 knockout ($AnxA1^{-/-}$) type C57BL/6 mice and by an experimental model of advanced NASH based on feeding animals methionine and choline deficient (MCD) diet up to eight weeks.

Key results: We have observed that in MCD-fed C57BL/6 WT mice AnxA1 increases in parallel with the progression of liver injury. At histology, AnxA1 is selectively localized in enlarged vacuolized mononucleated cells that are also positive for the macrophage marker F4/80. Analysing the liver biopsies obtained by NASH patients, we have observed that AnxA1 is also expressed by CD68+ macrophages; moreover, AnxA1 mRNA level inversely correlates with the extension of fibrosis. As compared to wild type mice, following eight weeks of MCD diet, $AnxA1^{-/-}$ animals develop more severe NASH characterized by an enhanced lobular inflammation due to increased macrophage recruitment and exacerbation of the M1 phenotype, with a sustained expression of IL-12p40, iNOS and IL-23p19. Furthermore, the incubation of isolated macrophages with recombinant AnxA1 decreases the mRNA expression of iNOS, IL-12p40, CCL2 and CCR2. These changes are

associated with AnxA1-mediated stimulation of IL-10 that implicates the activation of signalling pathway-involving p38MAPK. Interestingly, MCD-fed AnxA1$^{-/-}$ mice also display a higher degree of hepatic fibrosis than wild type mice characterized by an increase in hepatic collagen deposition and α-SMA-positive activated hepatic stellate cells (HSCs). This effect is unrelated with the stimulation of TGF-β, but involves galectin-3, a β-galactoside-binding lectin previously associated with both the pathogenesis of NASH and the activation of myofibroblasts in fibrotic livers. We have observed, in fact, that liver galectin-3 expression is higher in AnxA1$^{-/-}$ mice than in wild type animals and that galectin-3-positive macrophages and HSCs are more frequent in AnxA1-deficient livers. Furthermore, in macrophages isolated from NASH livers AnxA1-mediated signals down-modulate galectin-3 production.

Outcome and conclusions: These results suggest that in NASH progression macrophage-derived AnxA1 plays a functional role in controlling both hepatic inflammation and fibrogenesis. In particular, AnxA1 produced by macrophages acts by an autocrine signal down-modulating pro-inflammatory M1 responses and reducing the recruitment of monocytes through CCL2/CCR2 pathway, thus limiting chronic inflammation in the advanced phases of NASH. We have demonstrated that AnxA1$^{-/-}$ mice develop more fibrosis as compared to wild type animals, suggesting a possible anti-fibrogenic activity of AnxA1; this effect seems to involve the modulation of galectin-3 production, a pro-fibrotic lectin implicated in the regulation of cell adhesion, proliferation, survival and in modulation of tissue inflammation and fibrosis. Collectively, these data are a good starting point for investigating whether genetic difference in AnxA1 production might account the inter-individual variability in the evolution of NAFLD/NASH as well as for testing AnxA1 analogues as possible novel treatments to control NASH progression.

THE ROLE OF ANNEXIN A1 IN MODULATING THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS (NASH)

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Running title: Annexin A1 in NASH evolution.
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Abbeviations:
AnxA1 Annexin A1
α-SMA α-smooth muscle actin
FPR2/ALX Formyl peptide receptor 2/Lipoxin A4 receptor
MCD Methionine-choline deficient
NAFLD NonAlcoholic Fatty Liver Disease
NASH NonAlcoholic SteatoHepatitis;

Note: The authors have no conflict of interest

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Annexin A1 (AnxA1) is an effector of the resolution of inflammation highly effective in terminating acute inflammatory responses. However, its role in chronic settings is less investigated. Since changes in AnxA1 expression within the adipose tissue characterize obesity in mice or humans, we queried a possible role for AnxA1 in the pathogenesis of nonalcoholic steatohepatitis (NASH) which is commonly associated with obesity. NASH was induced in wild-type (WT) and AnxA1$^{-/-}$ C57BL/6 mice by feeding a methionine-choline deficient (MCD) diet up to 8 weeks. In MCD-fed WT mice, AnxA1 increased in parallel with the progression of liver injury. AnxA1 was also detected in liver biopsies from NASH patients and its degree of expression inversely correlated with the extent of fibrosis. In both humans and rodents, AnxA1 production was selectively localized in liver macrophages. NASH in AnxA1$^{-/-}$ mice was characterized by enhanced lobular inflammation due to increased macrophage recruitment and the exacerbation of the M1 phenotype. Consistently, in vitro addition of recombinant AnxA1 to macrophages isolated from NASH livers down-modulated M1 polarization through stimulation of IL-10 production. Furthermore, the degree of hepatic fibrosis was enhanced in MCD-fed AnxA1$^{-/-}$ mice, an effect associated with augmented liver production of the pro-fibrotic lectin galectin-3. Accordingly, AnxA1 addition to isolated hepatic macrophages reduced galectin-3 expression.
In conclusion, macrophage-derived AnxA1 plays a functional role in modulating hepatic inflammation and fibrogenesis during NASH progression, suggesting the possible use of AnxA1 analogues for the therapeutic control of this disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is regarded as the hepatic feature of the so-called "Metabolic Syndrome" and is becoming the most common form of liver injury worldwide due to the diffusion of over-weight and obesity [1]. In about 10-20% of the patients with NAFLD, the disease evolves with the development of hepatocellular damage and lobular inflammation often leading to hepatic fibrosis and cirrhosis [1]. Available evidence indicates that the mechanisms responsible for the evolution of NAFLD to nonalcoholic steatohepatitis (NASH) may involve lipotoxicity caused by increased circulating free fatty acids, oxidative damage and endoplasmic reticulum stress [2, 3]. These factors not only cause hepatocyte death, but can stimulate Kupffer cells to secrete inflammatory mediators that, in turn, recruit and activate leucocytes within the liver [2, 3]. Nonetheless, the mechanisms responsible for the persistence of hepatic inflammation along with those leading to the evolution of NASH to fibrosis are still incompletely characterized. It is increasingly evident that a failure in the mechanisms responsible for terminating inflammatory responses might result in chronic inflammation [4].

The resolution of an acute inflammation is orchestrated by a variety of protein and lipid mediators that down-modulate leukocyte recruitment, promote clearance of tissue leucocytes and switching macrophage phenotype favoring tissue healing [5]. Among these pro-resolving factors, annexin A1 (AnxA1), also known as lipocortin-1, is receiving increasing attention. AnxA1 is a 37 kDa calcium-phospholipid-binding protein highly expressed in myeloid cells and regulated by glucocorticoids [6]. By interacting with its receptor formyl peptide receptor 2/Lipoxin A_4 receptor (FPR2/ALX) AnxA1 down-regulates production of pro-inflammatory mediators such eicosanoids, NO and IL-6, reduces neutrophil migration to inflammatory sites and promotes the clearance of apoptotic granulocytes [6, 7]. Furthermore, recent works suggest that endogenous AnxA1 may orchestrate epithelial repair [8, 9] and even counteract the development of fibrosis [10].

The interest for a possible involvement of AnxA1 in the evolution of NAFLD originates from the observation that plasma AnxA1 levels are decreased in obese subjects inversely correlating with the body mass index (BMI) as well as with the inflammation marker C-reactive protein (CRP) [11], while an increased AnxA1 expression was observed in the adipose tissue of obese mice [12]. Furthermore, AnxA1 deficiency promotes adiposity and insulin resistance in Balb/c mice on a high fat diet [12]. As AnxA1-null mice also display inappropriate experimental inflammatory responses [7], we have investigated the possible role of AnxA1 in the evolution of experimental NASH induced by feeding mice with a methionine-choline deficient (MCD) diet.

Material and Methods

Animal and Experimental protocol. Eight weeks old male AnxA1 knockout (KO) mice on C57BL/6 background and wild type (WT) animals were purchased from Charles Rivers (Charles River, Margate, UK) and fed for 4 or 8 weeks with either methionine-choline deficient (MCD) or control diets (Laboratorio Dottori Piccioni, Gessate, Italy). The experiments were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

Human specimen collection and analysis. We analysed liver biopsies from 28 consecutive patients with NAFLD or NASH referring to the Division of Gastro-Hepatology of the University of Turin in the period April-November 2011. Liver specimens were collected at the time of first diagnosis and processed for histophatology and extraction of nucleic acid. Patients were characterized by anthropometric, clinical and biochemical data and liver biopsies were evaluated for the severity of steatohepatitis fibrosis [13]. The major clinical and biochemical parameters are reported in Supplementary Table 1.

Biochemical analysis. Plasma ALT and liver triglycerides were determined by spectrometric kits supplied by Radim S.p.A. (Pomezia, Italy) and Sigma Diagnostics (Milano, Italy), respectively. Circulating TNF-α and liver IL-12 levels were evaluated by commercial ELISA kits supplied by Peprotech (Milano, Italy) and R&D Systems (Abingdon, UK), respectively.

Histology and immunohistochemistry. Lobular inflammation was scored blind according to Kleiner et al. [13] in hematoxilin/eosin stained sections. Liver macrophages and activated hepatic stellate cells were evidenced in formalin-fixed sections using, respectively, anti-mouse F4/80 or anti-human CD68 (eBioscience, San Diego CA, USA) and α-smooth muscle actin (α-SMA) polyclonal antibodies (Labvision, Bio-Optica, Milan, Italy) in combination with peroxidase-linked goat anti-rat IgG or horse-radish peroxidase polymer kit (Biocare Medical, Concord, CA, USA). AnxA1, FPR2/ALX and galectin-3 were detected using specific antibodies from Zymed Laboratories-Invitrogen (Carlsbad, CA, USA), Santa Cruz (Dallas, TX, USA) and R&D Systems (Minneapolis,MN, USA), respectively.

Real time PCR analyses. Liver RNA was retro-transcripted with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy). RT-PCR was performed in a Techne TC-312 termalcycler (TecneInc, Burlington NJ, USA) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNF-α, IL-12p40, IL-23p19, IL-10, CCL2, CCR2, iNOS, arginase-1, MGL-1, AnxA1, Fpr2/3 (orthologues of human FPR2/ALX) [14], galectin-3, α1-procollagen, TGF-β1, α-SMA, and β-actin (Applied

Biosystems Italia, Monza, Italy). All samples were run in duplicate and the relative gene expression calculated as $2^{-\Delta Ct}$ was expressed as fold increase over control samples. Human sample analysis was performed using SsoFast™ EvaGreen® Supermix (Biorad, Hercules, CA, USA) following manufacturer's instructions. The sequence of primers used was: sense, 5'-GCAGGCCTGGTTTATTGAAA-3'; reverse 5'-GCTGTGCATTGTTTCGCTTA-3'. The values were normalized to those of β-actin and expressed by using comparative $2^{\Delta\Delta Ct}$ method.

AnxA1 recombinant protein purification. cDNA of human AnxA1 carrying a cleavable N-terminal poly-His tag was expressed in *E. Coli* and purified previously reported [9]. Purity of recombinant AnxA1 as assesed by SDS-PAGE and MALDI-TOF/TOF was >95%.

Isolation and purification of liver macrophages. Liver macrophages were purified from the livers of either controls or 4 weeks MCD-fed mice as previously described [15]. Cell purity was above 80%, as estimated by flow cytometry following immunostaining for CD45 and F4/80. Cells were incubated overnight in the presence of AnxA1 (100 nM) or the p38MAPK inhibitor SB203880 (10 μM) (Sigma-Aldrich, Milan, Italy) and processed for mRNA extraction as outlined above. In some experiments liver non-parenchymal cells were separated on a Ficoll density gradient, stained with fluorochrome-conjugated antibodies for CD45, F4/80, IL-10 (eBiosciences, San Diego CA, USA) and analyzed with a FACScalibur (Becton Dickinson) flow cytometer. Unspecific immunoglobulin binding was blocked by incubation with de-complemented mouse serum. AnxA1-producing cells were detected using a polyclonal anti-AnxA1 rabbit antiserum (Millipore, Temecula, CA, USA) and phycoerythrin-conjugated anti-rabbit IgG (Sigma-Aldrich, Milan, Italy).

Western blotting. Liver fragments were homogenized in ice-cold 10 in lysis buffer as previously reported [13] and protein extracts (100 μg) were electrophoresed on a 10% SDS-polyacrylamide gel. Nitrocellulose membranes probed with monoclonal antibodies against mouse AnxA1 and galectin-3 were revealed with Western Lightning Chemiluminescence Reagent Plus (ECL) (Perkin-Elmer, Boston, MA, USA) using the VersaDoc 3000 quantitative imaging system and Quantity One software (BioRad, Hercules, CA, USA).

Data analysis and statistical calculations. Statistical analyses were performed by SPSS statistical software (SPSS Inc. Chicago IL, USA) using one-way ANOVA test with Tukey's correction for multiple comparisons or Kruskal-Wallis test for non-parametric values. Significance was taken at the 5% level. Normality distribution was preliminary assessed by the Kolmogorov-Smirnov.

Results

The progression of NASH is associated with a stimulation in liver AnxA1.

AnxA1 was expressed at low extent in the livers of naïve mice, with both mRNA and protein content increasing in a time-dependent manner in the livers of animals with NASH induced by feeding the MCD diet (Fig. 1). AnxA1 expression in NASH livers was selectively localized in enlarged vacuolized mononucleated cells that were positive for the macrophage marker F4/80 (Fig. 1). Double staining of frozen liver sections with an anti-AnxA1 antibody and the lipid dye Oil Red O confirmed that AnxA1-positive macrophages contained lipid droplets (Fig. 1), likely derived from the scavenging of dying fat-laden hepatocytes. Histology analysis indicated that AnxA1-producing cells were more frequent in the livers of animals with advanced NASH (8 weeks on the MCD diet) than in those with less severe steatohepatitis (4 weeks of treatments; 4.7± 0.5 vs 2.8 ± 0.8 cells/high magnification field (hmf); p=0.03). Animals with advanced NASH showed an increase in the hepatic mRNA content of the AnxA1 receptor Fpr2/3 and presence Fpr2/3-expressing cells in liver sections (Supplementary Fig. 1).

In line with the results obtained in rodents, liver sections from subjects with NASH showed an increased prevalence of AnxA1-producing cells, mainly CD68+ macrophages (Fig. 2). A further evaluation of AnxA1 mRNA levels in 28 liver biopsies from NAFLD/NASH patients showed that AnxA1 expression was not related to the degree of insulin resistance or the severity of liver injury (not shown), but inversely correlated with the extension of fibrosis ($r=-0.59$, $p<0.003$). In particular, AnxA1 mRNA was significantly lower in the subjects with bridging fibrosis as compared to those with mild/moderate pericentral or periportal fibrosis or without fibrosis (Fig. 2).

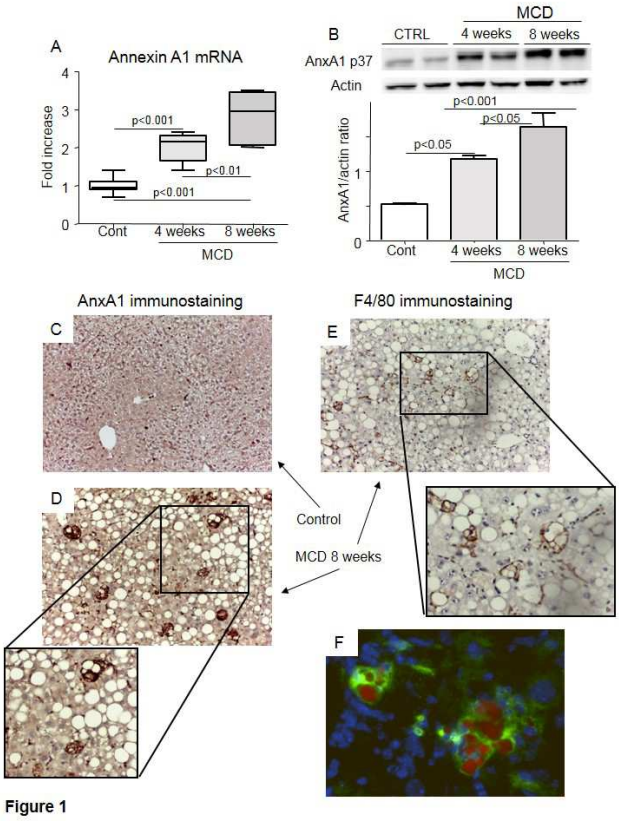

Figure 1

Figure 1. Hepatic AnxA1 expression in mice with NASH.

Mice were fed a methionine-choline deficient (MCD) diet over an 8-week time period. (A, B) AnxA1 mRNA and protein levels as measured by RT-PCR and Western blot analyses, respectively, in liver extracts of mice receiving control or MCD deficient. Hepatic mRNA data are expressed as fold increase over control values after normalization to the β-actin gene. Data are from 8-12 animals per group; boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons. (C, D) Localization of AnxA1 expression by immunohistochemistry in the liver of MCD-fed animals (magnification, 20x). (E) Detection of macrophages positive for F4/80 immunostaining (magnification 20x). (F) Co-localization of AnxA1 in macrophages containing lipid vacuoles was evidenced by double staining of frozen liver sections with the lipid dye Oil Red O (red) and anti-AnxA1 antibody (green immunofluorescence; magnification 40x). Cell nuclei were courter-stained with DAPI. Images are representative of 3-4 distinct samples.

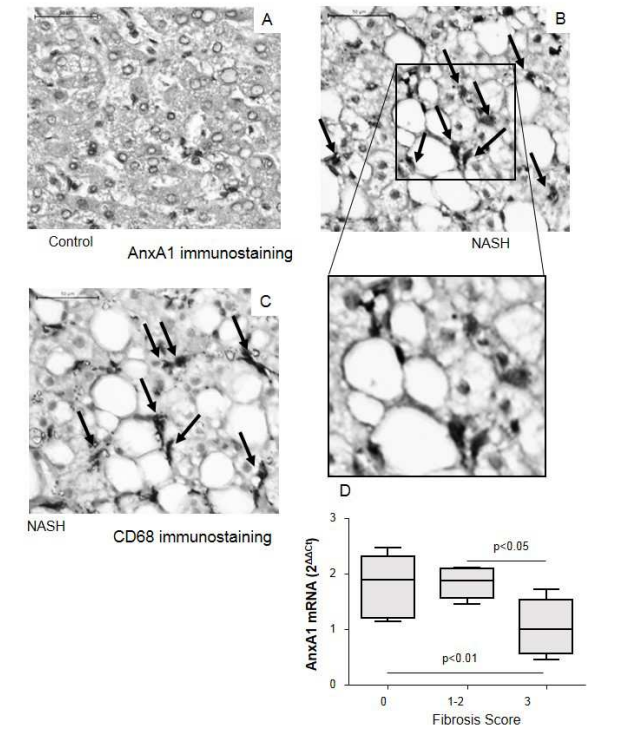

Figure 2

Figure 2. AnxA1 expression in human livers with or without NASH.

AnxA1 detection by immunohistochemistry in liver specimens from control individuals (A) and NASH patients (B). CD68 positive macrophages (from the same NASH patient; C) (magnification 40x). Control liver samples refer to surgical resections for hepatic metastasis of colon carcinoma. (D) AnxA1 mRNA was measured in the liver biopsies of 28 NASH patients using RT-PCR and normalized to that β-actin. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. The extent of liver fibrosis was scored according to Kleiner et al. [12].

AnxA1 deficiency stimulates hepatic inflammation in advanced NASH

To further make insights on the role of AnxA1 in the evolution of NASH, AnxA1 KO mice were administered the MCD diet. The livers of AnxA1 KO mice on a control diet displayed normal histological appearance apart from the sporadic presence of monocyte infiltration (Fig. 3). Upon feeding the MCD diet for either 4 and 8 weeks liver triglycerides and transaminase release in AnxA1 KO mice were comparable with those of WT

mice (Fig. 3). However, histology showed that already after 4 weeks on MCD diet, the semi-quantitative scores of lobular inflammation were higher in AnxA1 KO mice (2.2±0.4 vs. 1.4±0.5; p=0.01) and remained elevated by extending the treatment to 8 weeks (2.4±0.6 vs. 1.6±0.5; p=0.03). At this time point, NASH in AnxA1 KO mice was characterized by diffuse inflammatory foci containing mononucleated cells and by a marked up-regulation in liver and circulating levels of TNF-α (Fig. 3). Accordingly, week 8 MCD AnxA1 KO mice had higher mRNA for the chemokine CCL2 and its receptor CCR2 together with elevated number of hepatic macrophages when compared to paired WT animals (Fig. 4).

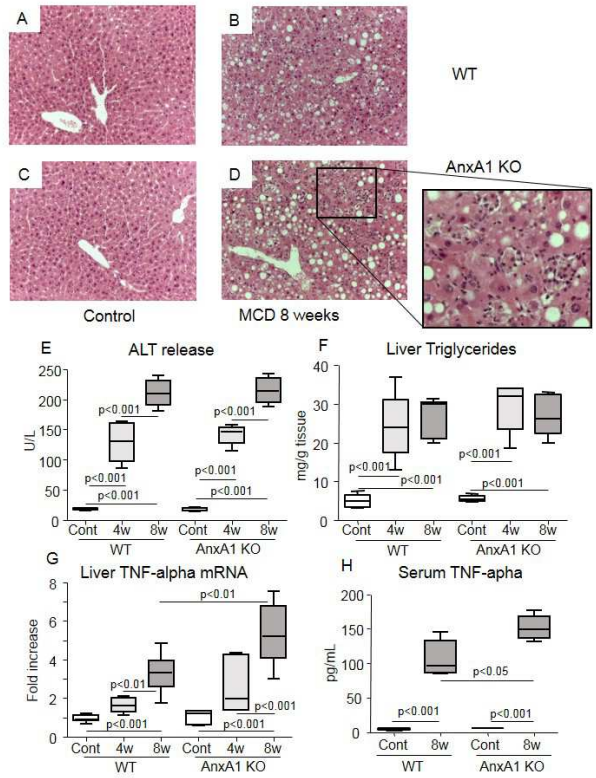

Figure 3. AnxA1 deficiency promotes steatohepatitis in mice with NASH.
Wild-type (WT) and AnxA1 KO C57BL/6 mice were fed the methionine-choline deficient (MCD) diet up to 8 weeks. (A-D) Liver histology was evaluated in hematoxilin/eosin stained sections from control or MCD-fed animals (magnification 20x). (E-G) NASH severity was assessed by circulating alanine aminotransferase (ALT) release, hepatic triglyceride content and liver TNF-α mRNA levels, measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene. (H) Circulating TNF-α levels were determined by ELISA. Values refer to 6-8 animals per group; boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

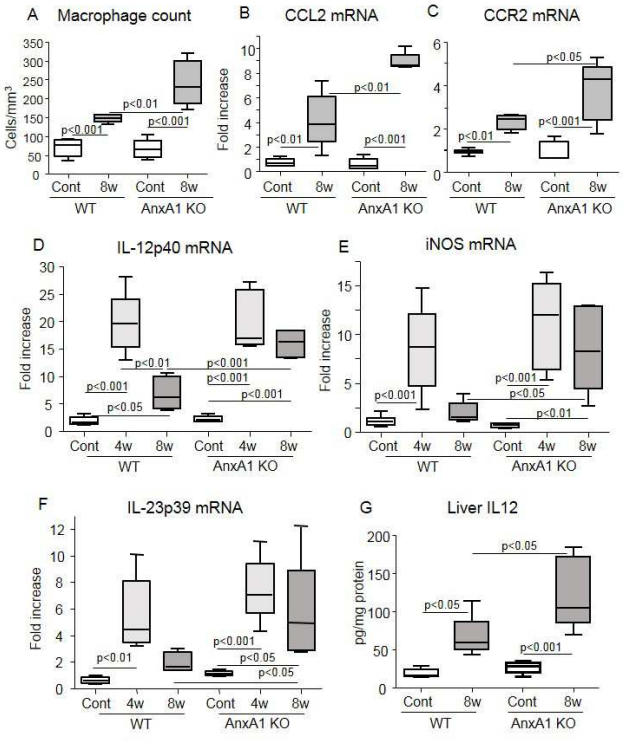

Figure 4. AnxA1 deficiency promotes liver macrophage recruitment and activation.
Wild-type (WT) and AnxA1 KO C57BL/6 mice were fed the methionine-choline deficient (MCD) diet up to 8 weeks. (A) Macrophage counts following immunostaining with anti-F4/80 antibody. (B-F) Liver mRNA levels for CCL2, CCR2 and the macrophage M1 activation markers IL-12p40, IL23p19 and iNOS as measured by RT-PCR. Data are expressed as fold increase over control values after normalization to the β-actin gene. (G) Liver IL-12 protein content as determined in the same animals. In all cases, values refer to 8-12 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Characterization of AnxA1 effect on hepatic macrophages functions

To further characterize the molecular and cellular events modulated by AnxA1, experiments with hepatic macrophages were conducted. In WT mice, NASH progression is characterized by a biphasic change in liver expression of macrophage M1 activation markers including inducible NO synthase (iNOS), IL-12p40, IL-23p19: these were elevated after 4 weeks on the MCD diet and declined thereafter (Fig. 4). In these animals, the individual AnxA1 mRNAs inversely correlated with those of iNOS (r=-0.62; p=0.01), IL-12p40 (r=-0.48; p=0.03) and IL-23p19 (r=-0.62; p=0.03), pointing to the possible contribution of endogenous AnxA1 in down-regulating macrophage M1 responses during disease progression. Supporting this view, AnxA1 KO mice receiving the MCD diet for 8 weeks did not show any decline in iNOS, IL-12p40 and IL-23p19 liver mRNAs: these markers were instead about 2-3 fold higher than those measured in WT animals (Fig. 4). On the same vein, hepatic IL-12 protein content was significantly enhanced in AnxA1$^{-/-}$ mice (Fig. 4).

Recent reports have linked the anti-inflammatory action of AnxA1 with a stimulation of IL-10 production through a p38 mitogen activated kinase (p38MAPK) signalling path, together with the induction of M2 polarization [16, 17]. Flow cytometry analysis of macrophages isolated from the livers of WT mice fed 4 weeks with the MCD diet showed that AnxA1-expressing F4/80^{+} cells had a higher IL-10 content than those AnxA1-negatives (Fig. 5). Furthermore, the incubation of isolated macrophages with recombinant AnxA1 halved the expression of iNOS and IL-12p40 without affecting that of the M2 markers arginase-1 and galactose-type C-type lectin 1 (MGL-1/CD301) (Fig. 5). In AnxA1-treated macrophages, the suppression of M1 activation was associated with a two-fold increase in IL-10 mRNA (Fig. 5); moreover, addition of the p38MAPK inhibitor SB203880 reverted both AnxA1-induced IL-10 stimulation and down-modulation of iNOS and IL-12p40 (Fig. 5). Interestingly, macrophage incubation with AnxA1 also significantly lowered CCL2 and CCR2 mRNA levels in a p38MAPK-dependent manner (Fig. 5), suggesting the capacity of AnxA1 to influence hepatic monocytic recruitment through CCL2 and CCR2 signalling.

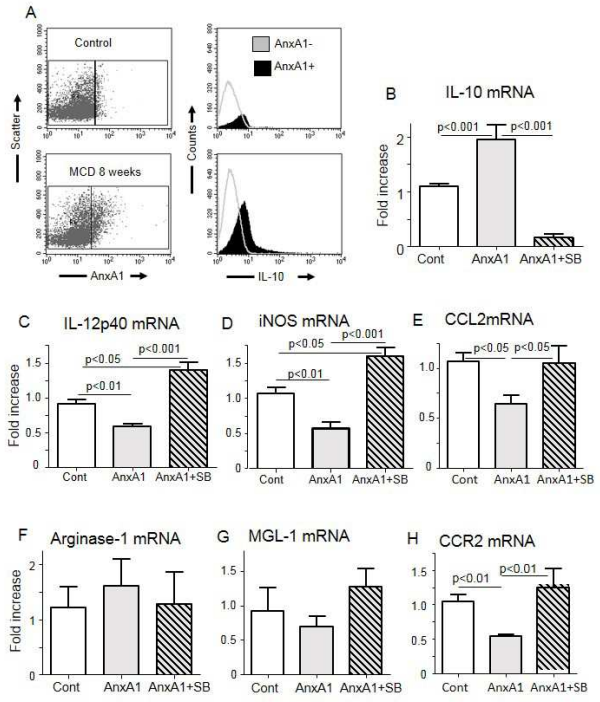

Figure 5. AnxA1 regulates macrophage functions through the stimulation of IL-10 production.

Hepatic macrophages were isolated from the livers of either control or 8 weeks MCD fed (MCD) mice. (A) F4/80 positive cells were analyzed by flow cytometry for the co-expression of AnxA1 and IL-10 in MCD-fed mice. (B) Macrophages isolated from livers of MCD-fed mice were incubated in vitro with or without recombinant AnxA1 (100 nM) and the p38MAPK inhibitor SB203880 (SB; 10 µM) and subsequently analysed for the expression of IL-10 mRNA (B). (C-H) RT-PCR analyses for other markers including M1 (iNOS and IL-12p40) and M2 activation markers (arginase-1 and MGL-1/CD301), as well as (E, H) CCL2 or CCR2. Values refer to 4-5 different cell preparations and are presented as mean ± S.D. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 5

AnxA1 deficiency stimulates the fibrogenic evolution of NASH

The data obtained in the patients with NAFLD/NASH indicate an inverse association between liver expression of AnxA1 and extension of hepatic fibrosis. By investigating the effects of AnxA1 deficiency on the fibrogenic evolution of experimental NASH we observed that the fibrosis markers pro-collagen-1α and α-smooth muscle actin (α-SMA) were higher in MCD-fed AnxA1 KO mice than in WT animals (Fig. 6). Hepatic collagen deposition, as evidenced by Sirius Red staining, and α-SMA-positive activated hepatic stellate cells (HSCs) (4.3±0.9 vs. 20.3±3.8 cells/hmf; p<0.005) were also more evident in AnxA1 KO mice with NASH, where they could be observed to surround cell foci containing mononucleated cells (Fig. 6). The stimulation of liver fibrosis in AnxA1 KO animals was unrelated to the regulation of hepatic TGF-β1 (Fig. 6), suggesting that additional factors may contribute to the pro-fibrogenic evolution of NASH.

The beta-galactoside-binding lectin galectin-3 may be implicated in the pathogenesis of NASH directing myofibroblast activation in fibrotic livers [18, 19]. In our experiments, liver galectin-3 expression was up-regulated in WT mice with NASH particularly following 8 weeks on the MCD diet (Fig. 7). At this time point, the hepatic content of galectin-3 in AnxA1 KO mice was two folds higher (Fig. 7). In line with these ex-vivo data, *in vitro* experiments showed that AnxA1-mediated signals effectively down-modulated galectin-3 expression in macrophages isolated from NASH livers (Fig. 7). Conversely, addition of AnxA1 to the same macrophage preparations did not affect TGF-β1 mRNA (not shown). At the immunohistochemistry level, galectin-3 production was particularly evident in the cell foci indicated above (Fig. 7). These galectin-3-positive foci were more frequent in AnxA1 KO livers (2.8±0.8 vs. 4.6±0.5 cell foci/hmf; p<0.03), where they were surrounded by abundant collagen fibres (Fig. 6).

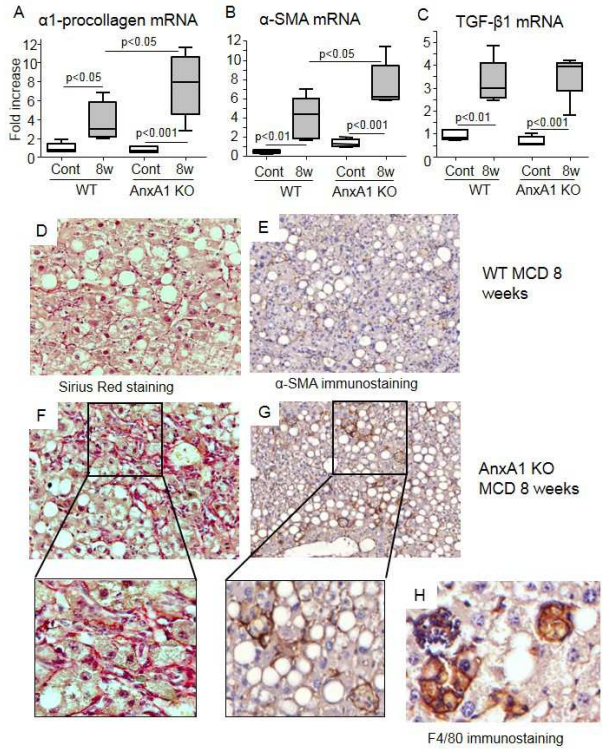

Figure 6

Figure 6. AnxA1 deficiency promotes hepatic fibrosis in mice with NASH.
Wild-type (WT) and AnxA1 KO C57BL/6 mice were fed the methionine-choline deficient (MCD) diet for 8 weeks. (A-C) Liver mRNA levels for pro-collagen-1α, α-SMA and TGF-β1 as measured by RT-PCR and are expressed as fold increase over control values after normalization to the β-actin gene. Values refer to 6-8 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons. (D, F) Collagen deposition as detected by Sirius Red staining in representative liver sections from 8-week MCD diet in WT and AnxA1 KO mice. (E, G) Activated hepatic stellated cells (HSCs) expressing α-smooth muscle actin (α-SMA) (magnification 40x and 20x). Enlargement show α-SMA-positive HSCs surrounded by collagen fibers forming cell foci with mononucleated. (H) These latter were stained by the macrophage marker F4/80 (magnification 40x).

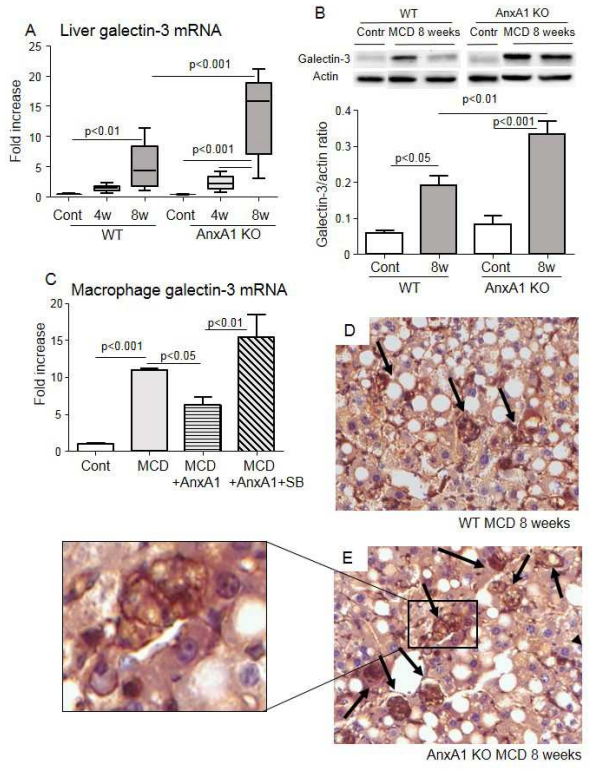

Figure 7

Figure 7. AnxA1 regulates galectin-3 production in the livers of mice with NASH.
Wild-type (WT) and AnxA1 KO C57BL/6 mice were fed the methionine-choline deficient (MCD) diet for 8 weeks. (A,B) Galectin-3 mRNA (boxes and whiskers) and protein levels (mean ± SD) were measured by RT-PCR and Western blot, respectively. Data are from 6-8 mice per group and statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons. (C) Macrophages isolated from livers of control (Cont) or 4 weeks MCD-fed (MCD) mice were incubated with or without recombinant AnxA1 (100 nM) and the p38MAPK inhibitor SB203880 (SB; 10 μM) for 16 hours, prior to quantification of galectin-3 mRNA. (D, E) Liver galectin-3 expression by immunohistochemistry in MCD-fed animals (magnification 40x). Enlargement shows cell foci containing galectin-3 macrophages and hepatic stellate cells.

Discussion

Growing evidences point to the importance of AnxA1 in the modulation of anti-inflammatory and pro-resolving responses in rodent models of acute inflammation [6, 7]. However, to our knowledge, no study has so far investigated the involvement of AnxA1 in the mechanisms leading to chronic liver disease. We now report that AnxA1 production is significantly up regulated in the livers of mice with chronic steatohepatitis induced by feeding with a MCD diet.

In experimental NASH, up-regulation of AnxA1 is particularly evident in the advanced phases of the disease and appears to specifically involve macrophages containing intra-cytoplasmic lipid droplets. The origin of these macrophages has not been characterized in detail though our preliminary data indicate that these cells express to low extent the monocyte markers Ly6C and CD11b [20], suggesting that they might derive from inflammatory macrophages which had undergone phenotypic changes after scavenging dying fat-laden hepatocytes. Interestingly, macrophages are also the predominant source of AnxA1 in the adipose tissue of obese mice [12], suggesting that during the metabolic syndrome AnxA1 up-regulation might be a common response in macrophages from different tissues. We are aware that NASH induced by the MCD diet does not reproduce some of the key features of the human disease such as obesity and insulin resistance. However, at variance from the high fat diet protocol, this model is suitable to study the inflammatory components of the disease as it causes extensive steatohepatitis that rapidly progresses to fibrosis [21].

In line with the homeostatic properties of AnxA1, we observed that AnxA1 deficiency promotes lobular inflammation in MCD-fed mice, particularly in the animals with more advanced NASH, as seen after 8 weeks of treatment. The mechanisms behind the inflammatory phenotype appear related to the direct action of AnxA1 on the macrophages. Indeed, in the livers of WT animals with advanced NASH, higher AnxA1 expression is associated with down-modulation of the macrophage M1 phenotype. Such an effect is absent in AnxA1 KO mice that experience instead elevated expression of iNOS, IL-12p40 and IL-23p19 gene products. Moreover, the *in vitro* addition of recombinant AnxA1 reduced M1 marker expression in macrophages isolated from NASH livers by more than 50%. Altogether, these results suggest that macrophage-derived AnxA1 represents an autocrine/paracrine loop that suppresses, at least in part, pro-inflammatory M1 responses. This new notion is consistent with data indicating a central role for AnxA1 in the down-modulation of TNF-α and IL-6 production by macrophages exposed to glucocorticoids [6, 22].

The homeostatic functions of AnxA1 are mediated by formyl peptide receptor-2/Lipoxin A_4 receptor (FPR2/ALX), a G protein–coupled receptor that is shared with others pro-resolving lipid mediators including lipoxin A_4 and resolvin D1 [23, 24] as well as with the pro-inflammatory protein serum amyloid A and cathelicidin LL-37 [25,26]. Recently, Cooray and co-workers unveiled an AnxA1 specific FRP2/ALX proresolving signal pathway involving p38 mitogen activated kinase (p38MAPK), MAPKAPK1/2 and heath shock protein 27 (Hps27), leading to generation of IL-10 [17]. In our experiments with isolated macrophages from NASH livers, we observed that down-modulation of iNOS and IL-12p40 is associated with a 2-fold rise in IL-10 expression and that the pharmacological inhibition of p38MAPK affects, in opposite ways, AnxA1-induced IL-10 stimulation and suppression of M1 polarization. This suggests that AnxA1 can down-regulate macrophage M1-responses through the action of IL-10.

In addition to modulation of the pro-inflammatory activity of macrophages, AnxA1 can regulate liver recruitment of monocytes by controlling the expression of the CCL2 and CCR2 pair. Indeed, CCR2 is one of the genes more extensively down regulated in human monocytes exposed to N-terminal AnxA1-derived peptide Ac1-25 [27]. However, differently from that reported by Lange and co-workers [27], in our hands the p38 inhibitor SB203880 effectively reverts the AnxA1 effect on CCR2 regulation in mice macrophages. So far, AnxA1 has been implicated in reducing granulocyte-tissue infiltration by blocking neutrophil–endothelial interactions and accelerating neutrophil apoptosis [6, 7]. Neutrophil infiltration is not relevant in the pathogenesis of NASH, where macrophages and lymphocytes are the predominant inflammatory cells [28]. Thus, our data point to the possibility that during chronic inflammation AnxA1 might also control the recruitment of monocytes. The capacity of AnxA1 to interfere with the CCL2/CCR2 axis might be particularly relevant in relation to the evolution of steatohepatitis as CCR2 deficiency, and CCR2 antagonism, ameliorate liver injury and fibrosis in experimental NASH [29, 30].

NASH is increasingly recognized as an important cause for liver fibrosis and about 15% of NASH patients progress to advanced fibrosis/cirrhosis [1]. However, the factors responsible for the large inter-individual variability in the development of fibrosis are still poorly characterized. Here, we report that i) AnxA1-expressing macrophages are evident in liver biopsies of patients suffering from NAFLD/NASH and ii) AnxA1 mRNA levels in these patients inversely correlate with the severity of fibrosis.

In line with these clinical data, experimental NASH in AnxA1 KO mice is characterized by increased liver fibrosis, suggesting that AnxA1 can prevent the fibrogenic evolution of NASH. This effect might involve modulation of galectin-3 expression. Galectin-3 is a member of the galectin family, a group of lectins that

participates in the regulation of cell adhesion, proliferation and survival as well as in the modulation of tissue inflammation and fibrosis [31]. In acutely injured livers, galectin-3 is mainly produced by macrophages and sustains M1 activation in models of acetaminophen- and concanavalin-A-induced hepatitis [32, 33]. Conversely, in rodent and human livers with fibrosis, galectin-3 is also expressed by activated α-SMA-positive hepatic stellate cells (HSCs) and regulates their pro-fibrogenic activity [19, 34]. Recent studies have implicated galectin-3 in the pathogenesis of NASH, although with controversial results, as galectin-3 deficient mice show either protection [18] or increased disease severity [35, 36] in relation to an impaired glucose metabolism and an increased susceptibility to obesity and systemic inflammation [37, 38]. In our hands, an increase in liver galectin-3 characterizes advanced NASH in MCD-fed mice and this was susceptible to AnxA1 application. Moreover, the worsening of fibrosis quantified in MCD-fed AnxA1 KO mice associates with a further up-regulation in galectin-3 levels. In the livers of these animals, collagen deposition is particularly evident around cell foci composed of galectin-3 expressing macrophages and HSCs further supporting a specific role of galectin-3 in stimulating pro-fibrogenetic cell-to-cell interactions. Consistently, galectin-3 secretion by macrophages has been implicated in the promotion of renal and vascular fibrosis [39, 40], while genetic deletion or inhibition of galectin-3 attenuates HSC activation and hepatic collagen deposition in CCl_4- or tioacetamide-treated mice [19, 41].

Collectively, these results make us to unveil a novel functional role for AnxA1 in NASH progression, a property effected through a control of hepatic inflammation and fibrogenesis, uneven modulation of galectin-3 and IL-10, leading to a reduced macrophage M1 response. It is plausible that strategies aiming at increasing hepatic AnxA1 expression, or the development of AnxA1 analogues [42], might have a potential for innovative therapeutic control on NASH evolution.

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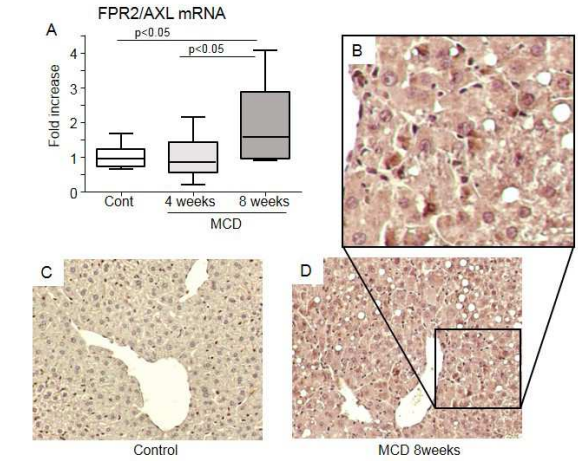

Supplementary Fig. 1

Supplementary Figure 1

Expression of AnxA1 receptor Fpr2 in the livers of mice with NASH induced by feeding a methionine-choline deficient (MCD) diet. Wild type C57BL/6 mice received the MCD diet up to 8 weeks. (A) Fpr2 mRNA was measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene (A). The values refer to 6-8 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. (B-D) Fpr2 liver localization by immunohistochemistry in control or MCD-fed mice.

Supplementary Table 1

Clinical and biochemical characterization of NAFLD/NASH patients investigated**.**

	Demographic Data
Patients Number (Male/Female)	28 (22/6)
Age (Years)	44.3 (29-66)
BMI	26.6 (17-31)
	Biochemical Data
HOMA-IR (n.v. <3)	5.18 (0.4-13.7)
AST (U/L– n.v. 5–40)	43.6 (15-198)
ALT (U/L n.v. 5–40)	77.0 (13-323)
γ-GT (U/L n.v. 5–45)	118.5 (12-792)
Fasting Glucose (mg/dL n.v. <100)	100.6 (78-245)
	Histological Data
Steatosis score	1.5 (1-3)
Inflammation score	0.7 (0-2)
Balloning score	1 (0-2)
Fibrosis score	1.4 (0-3)
NAS score	3.2 (1-5)

The values are expressed as median and inter-quartile range (IQR). For histological scores the range of variability is included.
BMI, body mass index; AST, alanine aminotransferase; ALT, aspartate aminotransferase; γ-GT, gamma-glutamyl transpeptidase; HOMA-IR, homeostatic model assessment-insulin resistance; ISI, insulin sensitivity index; n.v., normal values; NAS, NAFLD activity score.

Additional results

Role of NKT cells and osteopontin in sustaining inflammatory processes during NASH evolution

As previously outlined the down-modulation of macrophage M1 response occurring in mice with advanced NASH after eight weeks of feeding methionine and choline deficient (MCD) diet does not parallel with a decline in the liver production and circulating levels of TNF-α (Fig. 1), one of the key cytokines in the evolution of NASH (76). This indicates that other mechanisms might be responsible for sustaining inflammation in advanced NASH. The experiments reported in Paper 2 have shown that the progression of NASH in NF-kB1-null mice is much faster that in wild type animals as fibrosis is already evident after four weeks of treatment with MCD diet. In this experimental model, we have demonstrated an increased recruitment and activation of NKT cells likely due to an increased hepatic production of IL-15. Moreover, NASH in NF-kB1-deficient mice is characterized by a specific increase in the liver production of IFN-γ and osteopontin that, in turn, stimulate TNF-α synthesis. These observations suggest to investigate whether similar mechanisms might also characterize the disease evolution in wild-type animals.

Flow cytometry measurement of NKT cells (CD3+ NK1.1+) in the livers of C57BL/6 mice receiving the MCD diet shows that early NASH after four weeks of treatment is characterized by a decrease in the NKT pool, whereas in advanced NASH after eight weeks of diet the NKT cell population has grown to level significantly higher than in control mice (Fig. 4A, C). As we have observed in NF-kB1-null mice, the increase in the number of NKT cells in C57BL/6 mice fed with MCD diet for eight weeks is associated to an elevation in the liver production of IL-15 (Fig. 4D-E). In a recent study, Tang and colleagues have proposed the involvement of Tim3/galectin-9 in regulating NKT cells in liver with steatosis, as they observed that Galectin-9, a β-galactoside-binding lectin, can interact both with Tim-3-expressing Kupffer cells to induce secretion of IL-15, as well as with Tim-3-expressing NKT cells to promote their apoptosis, thus contributing to NKT depletion (77). However, this mechanism does not appear to account for the changes in NKT cells observed in our NASH model, since we have detected a reduction in liver mRNA expression of galectin-9, already after four weeks of MCD diet (Fig. 4F).

Furthermore, in these animals we have also found a significant up-regulation in the mRNA expression of CXCL16 (Fig. 4G), a chemokine expressed by macrophages and endothelial cells that by interacting with its receptor CXCR6 on NKT cell surface promotes cell migration, survival and

proliferation (78). In line with the effects on NKT cells, osteopontin (OPN) production is specifically increased in mice receiving the MCD diet for eight weeks (Fig. 5A-B). However, flow cytometry analysis of OPN-expressing cells in the livers with advanced NASH reveal that, in addition to NK1.1-positive cells, OPN production is evident in about 75% of F4/80-positive macrophages (Fig. 5C-D), indicating the hepatic macrophage pool as an important source of OPN in NASH. These data prompt to investigate whether macrophage OPN production is also implicated in worsening hepatic inflammation in AnxA1-null mice. As shown in Figure 5E, AnxA1$^{-/-}$ mice fed MCD diet for eight weeks have a higher liver mRNA expression of OPN than similarly treated wild type animals. Furthermore, AnxA1$^{-/-}$ animals, with the same treatment, confirm a possible anti-fibrogenic role of AnxA1 as it might control fibrosis and the change of macrophage phenotype through the modulation of OPN production.

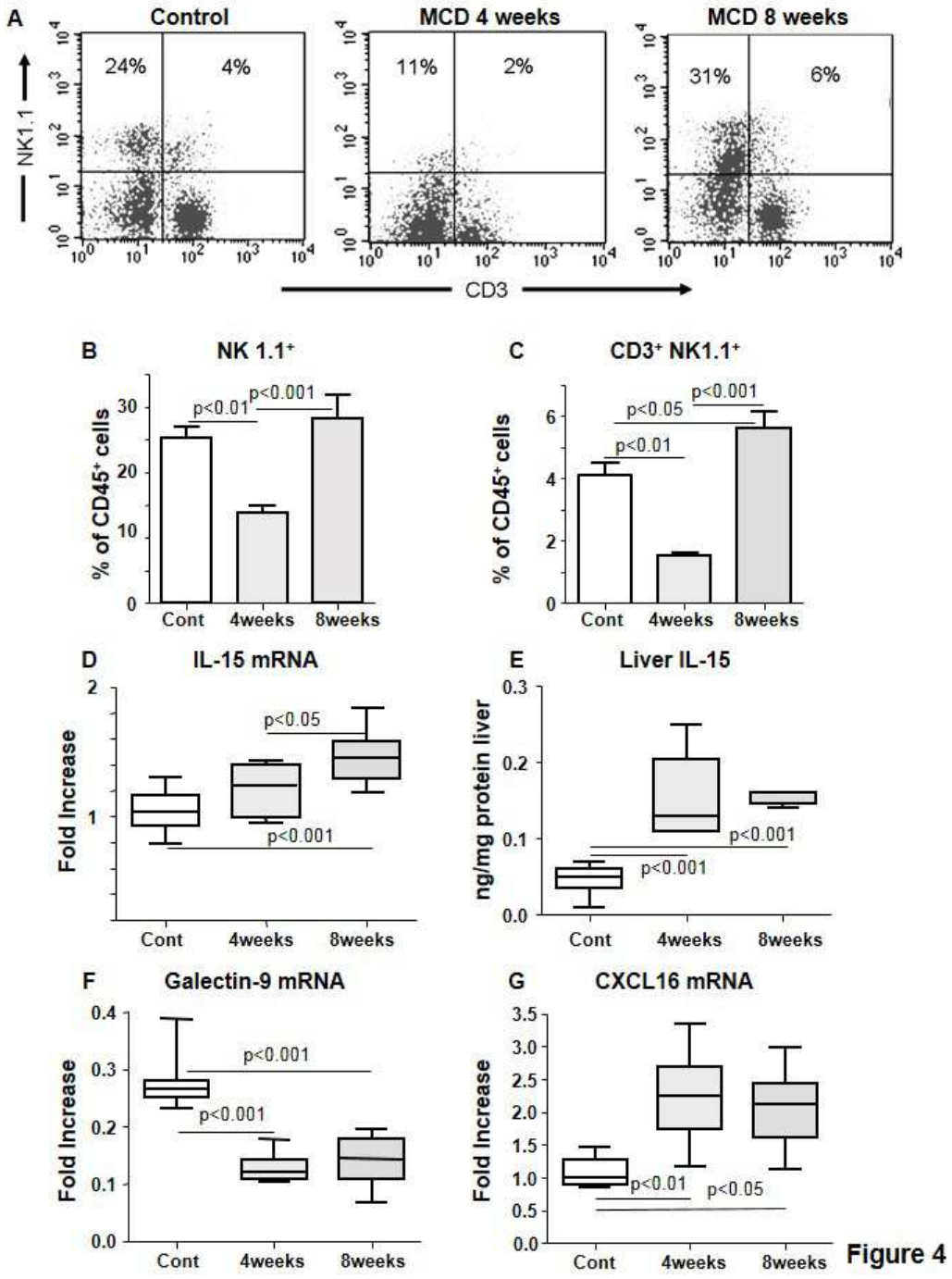

Figure 4

Figure 4: NASH evolution is associated with an increase in the number of NKT cells.
C57BL/6 mice were fed control or MCD diet up to eight weeks. Hepatic leucocytes were isolated from the livers of control and MCD-fed mice and analysed by flow cytometry for the expression of CD3 and NK1.1 (Panels A-C). Liver mRNA levels for IL-15, galectin-9 and CXCL16 were measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene (Panels D, F, G). The liver IL-15 content was determined by ELISA in the same animals (Panel E). The values refer to 6-8 animals in each group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values.

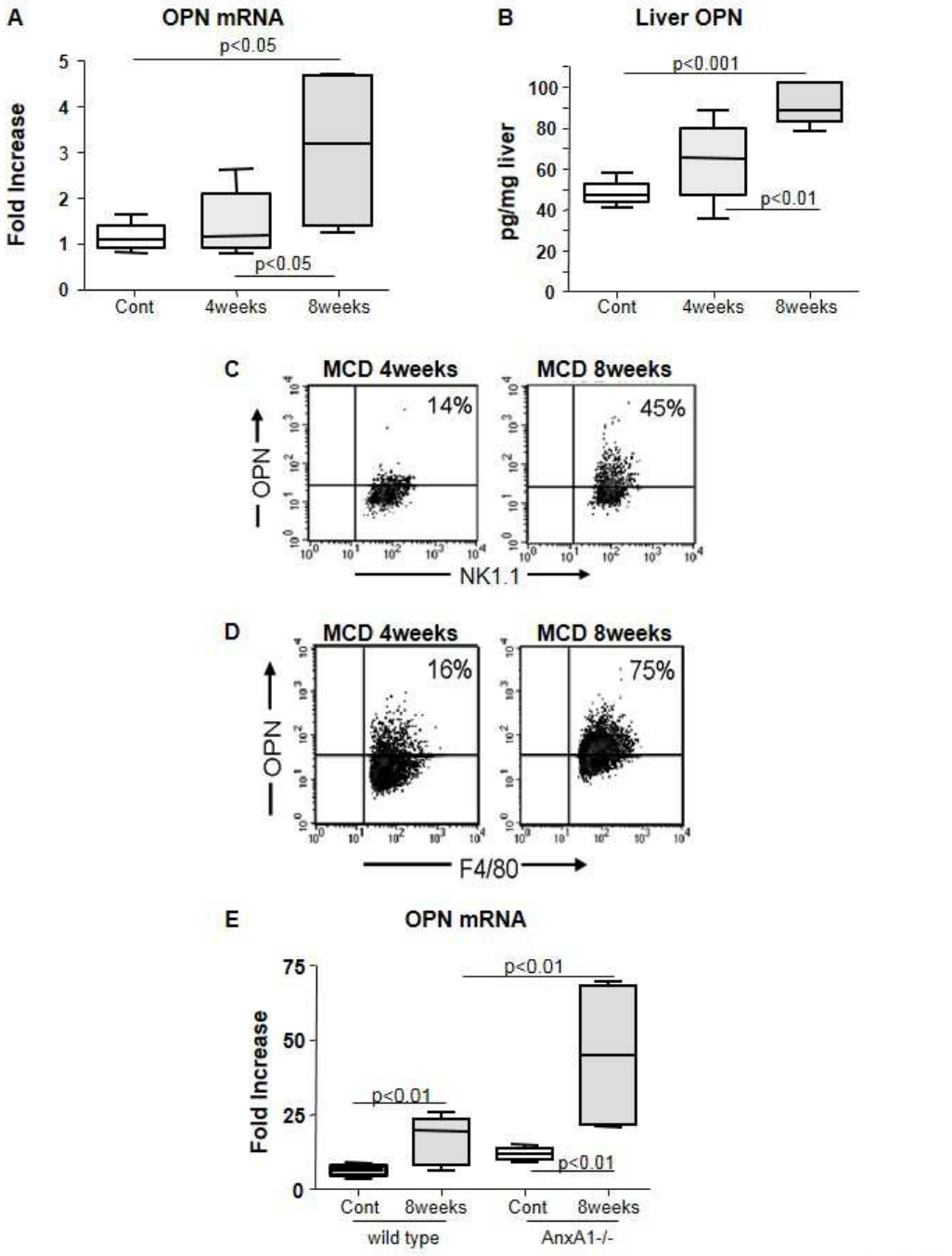

Figure 5

Figure 5: NASH evolution is associated with an increase in OPN production by NKT cells and macrophages. Wild type and AnxA1$^{-/-}$ C57BL/6 mice were fed control or MCD diet up to eight weeks. Liver osteopontin (OPN) was measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene (Panels A, E). The liver OPN content was determined by ELISA in the same animals (Panel B). The values refer to 6-8 animals in each group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Liver mononucleated cells were isolated from the livers of four and eight weeks MCD diet mice and the OPN expression by NK1.1+ and F4/80 hepatic macrophages was analysed by flow cytometry (Panels C-D).

General discussion

In the recent years, the worldwide growth of NAFLD/NASH incidence has stimulated a huge number of studies aimed to understand the mechanisms responsible for the pathogenesis of the disease in order to develop treatments able to block the evolution of liver damage. In fact, although NAFLD is a benign and indolent process, it can progress to NASH in approximately 15-20% of the patients. A still open question is why some patients with NAFLD develop NASH and others do not. It is known that in humans, genetic factors and gender differences influence the individual susceptibility to NASH (5).

So far, there is not a suitable experimental model able to reproduce all the aspects of human NASH. Rodent feeding a high fat diet causes obesity, insulin resistance and hepatic steatosis, but liver inflammation is usually modest and the animals do not have signs of fibrosis even prolonging the dietary treatment (73). On the other hand, the use of diet depleted of choline alone or by the combination of choline and methionine allows obtaining evident steatohepatitis evolving to fibrosis, but in the absence of obesity and insulin resistance (73). In our study, we relayed on the use of methionine and choline deficient (MCD) diet because this model is characterized by extensive steatohepatitis after four weeks of treatment that progresses to fibrosis by extending dietary regimen up to eight weeks (73). Nonetheless, even using this model there are differences between mice strains in susceptibility to experimental NASH (79, 80). Indeed, we have observed that after four weeks of MCD diet C57BL/6 mice develop more severe steatohepatitis as compared to Balb/c mice. We have taken advantage of these differences to investigate possible mechanisms involved in the inter-individual variability in the human disease. C57BL/6 and Balb/c mice are characterized by a different bias in cytokine production by CD4+ T-helper (Th) cells and macrophages. It is well established that C57BL/6 mice have a prominent Th-1 cytokine production (IL-2, TNF-α, IFN-γ) as opposed to a prevalence of Th-2 cytokine response (IL-4, IL-5 and IL-13) in Balb/c mice (81). Such a Th-1 bias has a major role in determining the increased susceptibility to acute inflammation of C57BL/6 mice. Conversely, a Th-2 bias is associated with an increased fibrosis in Balb/c mice (82). In experimental models of atherosclerosis, the Th1-slanted C57BL/6 mice develop more extensive plaques than Balb/C mice, supporting a proatherogenic role of Th1 response (83). This is confirmed by the blunting of Th1 cells that decreases atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) C57BL/6 mice treated with a high cholesterol diet (84). Although recent studies suggest several analogies between the inflammatory process of NASH and atherosclerosis (85), in our model, mice Th1/Th2 bias does not influence the severity of NASH as MCD diet alters neither the mRNA expression of IFN-γ and IL-4 nor that of Th1 and Th2 transcription factors T-bet and GATA-3. A

possible explanation might be that in our experiment the time required to induce steatohepatitis is too short (only four weeks) to recruit CD4+ T-cells. As mentioned before, during inflammation, macrophages have a predominant role and they can express different functional patterns of differentiation depending on the environmental signalling mediated by microbial products and cytokines. It is possible to divide macrophages in two subsets. The M1 polarized macrophages are activated through Toll-like receptors and INF-γ and exhibit enhanced killing of intracellular microorganisms, increased secretion of pro-inflammatory cytokines/chemokines (IL-1β, IL12, TNF-α, IL-12, CXCL9, CXCL10) and mediators (ROS, NO) and higher expression of co-stimulatory molecules. The M2 alternative activated macrophages are induced by IL-4 or IL-13 and express Arginase-1, mannose receptor and IL-4 receptor-α and promote anti-inflammatory, pro-fibrogenic, angiogenic and immuno-suppressive activities (86). Atherosclerosis and NASH share also similarities regarding the role of macrophages in the evolution of tissue injury. In atherosclerotic plaque, macrophages accumulate large amounts of lipids, transform into foam cells and drive atherogenesis. Lipid-loaded macrophages have also been observed in NASH, where hyperlipidemic mice have bloated foamy Kupffer cells upon high fat feeding. During the process of atherosclerotic lesions, macrophages acquire a classical M1 phenotype and produce inflammatory cytokines (87).

In our hands, macrophages isolated from the livers of MCD-treated C57BL/6 mice show M1 responses significantly higher than those obtained from similarly treated Balb/c mice and the M1 macrophage activation is responsible for the increased severity of NASH in C57BL/6 mice as compared to Balb/C animals. These observations are in line with reports showing that in rodents treated with choline-deficient or MCD diets Kupffer cells depletion by clodronate reduces liver TNF-α and IL-12 expression along with hepatic inflammation (39, 66). According to Tosello-Trampont and co-workers during the early phase of NASH, damaged hepatocytes express and release molecules that activate Kupffer cells which, in turn, produce IL-1α and TNF-α. These cytokines are able to activate in an autocrine manner Kupffer cells, which, in turn, produce chemokines, such as CCL2 and IP-10, that recruit circulating pro-inflammatory monocytes and amplify the inflammatory response (39). The relevance of these results to humans emerges from recent observations showing that obese subjects with NASH, but not those with NAFLD, have a specific increase in the liver expression of pro-inflammatory M1 chemokines/cytokines, such as CXCL1-9-10, CCL2, CCL5 and IL-1β (88). Collectively, these data indicate that the M1 activation of hepatic macrophages contributes to the increased susceptibility to the development of steatohepatitis in C57BL/6 mice, suggesting that the progression from NAFLD to NASH might be influenced by genetic and epigenetic factors able to modulate the macrophage response.

Among possible genetic factors influencing macrophage functions, we have investigated those involving the NF-kB transcription factor (NF-kB1) as a functional polymorphism of human NF-kB1 gene (-94ins/delATTG, rs28720239) that affects the protein production. This polymorphism has been associated with a higher prevalence of inflammatory and autoimmune diseases (89-91). NF-kB (Nuclear Factor kB) is a collective term that refer to homo- and hetero-dimeric transcription factors belonging to the Rel family that, in response to cell stimulation, migrate from the cytoplasm to the nucleus. All Rel polypeptides are characterized by an N-terminal RHD (Rel Homology Domain), which mediates DNA binding, nuclear localization and subunit dimerization. Mammals express five Rel proteins belonging to two classes. The first one comprises RelA, c-Rel and RelB, proteins that are synthesized as mature forms and do not require proteolytic cleavage. The second group is encoded by *Nfkb1* and *Nfkb2* genes: their products are first synthesized as large precursors, respectively p105 and p100, requiring proteolytic processing by proteasome that removes their C-terminal halves to produce the mature forms, p50 and p52 NF-kB proteins (92, 93). In unstimulated cells, NF-kB dimers are inactive, since they interact in the cytoplasm with inhibitory proteins termed IkBs (Inhibitors of NF-kB) (93). After stimulation with an agonist, IkBs are proteolitically degraded by the proteasome, releasing associated NF-kB proteins to translocate into the nucleus and modulate gene expression (94). NF-kB transcription factors play an important role in the regulation of immune and inflammatory responses. NF-kB induction is essential for the expression of immune response genes: pro-inflammatory cytokines (TNF-α, IL-1, IL-6), chemokines (MIP-1α, CCL5) and adhesion molecules (E-selectin, VCAM-1), which collectively regulate the recruitment of immune cells to sites of infections. Recent studies have been demonstrated that the NF-kB1 p50 subunit is involved in the regulation of macrophage response (92, 95). In particular, p50 homo-dimers have an important role both in the reduction of pro-inflammatory cytokines (TNF-α and IL-12) production by macrophages and stellate cells and in driving macrophage M2 alternative polarization, producing Arginase 1 and CCL17 and inhibiting iNOS (inducible NO synthetase) and TNF-α production (96, 97).

The physiological function of NF-kB1 in immunity has been investigated by NF-kB1-deficient (NF-kB1$^{-/-}$) mice, which produce neither p105 nor p50. The absence of p50 does not cause developmental abnormalities or lethality by itself. These mice have normal levels of B cells and normal ratios of κ and λ light chain usage (98). However, B cells isolated from these mice cannot be induced to differentiate in response to the mitogen LPS, and no NF-kB activation is observed. NF-kB1 deficient mice have dramatically greater susceptibility to bacterial infection but remarkably enhanced resistance to viral infection (93). Therefore, it appears that p50 subunit plays an important role in immune responses to acute inflammation but not in the overall development of the mouse. In

that context, it has been observed that NF-kB1$^{-/-}$ mice show exacerbated M1-driven inflammation and defective capacity to mount allergy and helminth-driven M2-polirized reactions (97).
By inducing NASH in NF-kB1$^{-/-}$ mice with the MCD diet, we have observed more severe steatohepatitis characterized by extensive lobular infiltration of mononucleated cells, frequent lipogranulomas, higher circulating TNF-α levels and increased hepatocyte apoptosis. Moreover, NASH in NF-kB1$^{-/-}$ mice associates with a more rapid progression of centrilobular fibrosis that is already present after only four weeks of treatment. However, analysis of the role of macrophages have surprisingly revealed any significant differences neither in the number of F4/80 positive cells nor in the mRNA expression of M1 markers, such as iNOS and IL-12p40, between NF-kB1$^{-/-}$ and wild type mice, indicating that NF-kB1 deficiency does not alter the macrophage response in MCD-fed mice. However, as discussed later, the data obtained in NF-kB1$^{-/-}$ animals has been useful to understand other aspects of inflammatory response in NASH.

In time course experiments we have observed that the progression of NASH is associated with a steadily increase in the number of F4/80 positive macrophages. In normal liver, the number of intrahepatic macrophages is maintained constant with a cell turnover that continuously repopulates the Kupffer cell population. There is a controversy about the origin of Kupffer cells and how their homeostasis is maintained over time. According to a more traditional view, tissue macrophages are not self-renewing and are replenished from bone marrow-derived monocytes (99, 100). In contrast, it has been argued that Kupffer cells are a self-renewing population and divide as mature cells or originate from local intrahepatic progenitors (101-103). Klein and colleagues have identified two distinct macrophage populations within the liver: a bone-marrow derived subset that is rapidly replaced after cell destruction by irradiation and a residual non-bone marrow-derived subset that is lost during cell isolation protocols. These two populations share the same morphology and phagocytic capabilities, but only the first one is engaged in inflammatory responses (104). During inflammatory responses, the migration of monocytes into tissues as well as their differentiation into macrophages or dendritic cells is largely determined by the inflammatory milieu, such as adhesion molecules, chemokines, pathogen-associated pattern recognition receptors (46). There are two major subsets of monocytes in peripheral blood: Ly6C^{high} (Gr1^{high}) monocytes that express CCR2, CD62L and CD64 and Ly6C^{low} (Gr1^{low}) monocytes that lack CCR2 and have higher levels of CD32. Both subsets express the receptor of fractalkine (CX_3CL1), CX_3CR1, but the second monocyte population characteristically expresses higher levels (105, 106).
There are some clear evidences suggesting that during the early phase of NASH the increase in hepatic macrophages involves the recruitment of circulating inflammatory Ly6C^{high} monocytes

through CCL2/CCR2-mediated signals (48, 107). In fact, Kirovski and colleagues have demonstrated that mice fed high fat diet develop hepatic steatosis without significant inflammation, but they have elevated CCL2 expression both in the liver and in the visceral adipose tissue. CCL2 serum levels are also elevated in patients with ultrasound-diagnosed NAFLD and correlate with the body-mass index and fasting glucose (108). Furthermore, CCR2 deficiency and the use of a specific CCR2 antagonist ameliorate liver injury and fibrosis in experimental NASH (48, 107). In our hands, the increase in hepatic macrophages occurring in the early phase of NASH is associated with an increase in mRNA expression of CCL2 and CCR2. However, CCL2/CCR2 signalling cannot explain the further expansion of macrophage pool detected in advanced NASH. Fractalkine (CX_3CL1) was originally defined as a chemoattractant for monocytes, but growing evidences indicate that its receptor is involved in controlling cell survival. In fact, in conditions of hepatic inflammation and fibrosis, CX_3CR1 represents an essential survival signal for monocyte-derived macrophages by activating anti-apoptotic bcl-2 expression (44). Monocytes and macrophages lacking CX_3CR1 undergo increased cell death following liver injury, which perpetuates inflammation, promotes prolonged inflammatory monocyte infiltration into the liver and enhances fibrosis (44). At present, the role of CX_3CL1 in NASH is still poorly investigated. However, our preliminary data indicate that CX_3CL1 increases in advanced NASH in parallel with the expansion of CX_3CR1-expressing macrophages. Thus, at this stage, factors controlling the differentiation and the survival of intrahepatic monocytes might have a predominant role in controlling macrophage expansion.

During the evolution of NASH, we have observed an apparent paradox involving hepatic macrophages. In fact, while in the early phase of the disease hepatic inflammation is characterized by an enhanced macrophage M1 activation, the expression of M1 markers progressively decreases in parallel with the disease evolution to fibrosis. These changes do not involve M2 polarization, as the expression of M2 markers is not affected. Thus, during the evolution of NASH we have observed both an increase in the number of F4/80 positive macrophages and a change in their phenotype and characteristics. Increasing evidences indicate that macrophages are the target of a variety of signals involved in terminating inflammatory responses and in the regulation of tissue healing (109). Many proteins and lipid mediators are involved during the process of resolution of inflammation: they down-modulate immune responses, control the clearance of tissue infiltrating leukocytes and promote functional changes in macrophages favouring healing processes (75). Among all the proteins involved in the termination of inflammatory response, we have focused our attention on AnnexinA1 (AnxA1), or lipocortin-1, a 37kDa anti-inflammatory protein originally

reported to be induced by glucocorticoids and inhibiting phospholipase activity, but it has subsequently been shown to regulate different cellular functions in a variety of cell types. It is the first element of the annexin family of calcium- and phospholipid-binding proteins, which consists of 13 members. Annexins are grouped according to similarities in the structure and in the sequence of the calcium-binding region. All annexins have a core constituted by four repeats of 60-70 amminoacids each, attached to an N-terminal region that gives specificity of action to each family member (110).

Several evidences suggest that AnxA1 mediates its effects through the interaction with FPRs (Formyl Peptide Receptors), a family of seven transmembrane domain, G protein-coupled receptors, consisting of three members (FPR1, FPR2 and FPR3). FPRs are expressed by several cell types including neutrophils, monocytes, macrophages, endothelial and epithelial cells. The G protein that couples to the FPRs belongs to the G_i family of hetero-trimeric G proteins and it induces the activation of phospholipase C (PLC) and the release of calcium from intracellular stores. Other signal pathways associated to FPRs involve the activation of PLA, PLD and members of the MAP kinase family.

The vast majority of AnxA1 effects are mediated by FPR2, which is also the receptor for the anti-inflammatory molecules lipoxin A_4 and for the pro-inflammatory mediators serum amyloid protein A (SAA) and cathelicidin LL-37 (111). By interacting with FPR2, AnxA1 can modulate anti-inflammatory and pro-resolving responses in rodent models of acute inflammation by down-regulating the production of pro-inflammatory mediators, such as eicosanoids, NO and IL-6, reducing neutrophil recruitment to inflammatory sites, promoting the clearance of apoptotic granulocytes and stimulating tissue repair (111, 112).

In the MCD model of NASH, hepatic AnxA1 increases during the evolution of the disease, becoming particularly evident in the advanced phases when is selectively expressed by macrophages with intra-cytoplasmic lipid droplets and inversely correlated with M1 markers. The origin of these macrophages has not been studied in detail, even if preliminary results indicate that they express at low extent the monocyte markers Ly6C and CD11b, suggesting that they can originate from inflammatory macrophages, which have changed their phenotype after having engulfed dying fat-laden hepatocytes.

To better understand the role of AnxA1 in the evolution of NASH we have used C57BL/6 AnxA1 deficient mice ($AnxA1^{-/-}$) (113). In general, it has been demonstrated that the absence of AnxA1 induces the exacerbation and the prolongation of inflammation. $AnxA1^{-/-}$ mice have higher inflammatory response as demonstrated by increased leukocyte transmigration, higher levels of inflammatory markers, increased neurological deficit in a stroke model and delayed repair in a

model of colitis (112, 114). In that condition, the administration of exogenous AnxA1 is able to rescue the phenotype in AnxA1$^{-/-}$ animals.

We have observed that NASH in AnxA1-deficient mice is characterized by an enhanced lobular inflammation that is particularly evident after eight weeks of treatment. The mechanism responsible for such exacerbation of inflammation is related to an enhanced M1 activation of macrophages. Indeed, the addition of recombinant AnxA1 on macrophages isolated from NASH livers decreases the expression of these M1 markers, without affecting M2 markers. Thus, these results suggest that the AnxA1 produced by macrophages might act in autocrine manner contributing in down-modulating pro-inflammatory M1 responses and in changing their phenotype. This mechanism of inflammatory regulation by AnxA1 in macrophages is in line with the study of Yang and colleagues in which they have demonstrated that AnxA1 exerts an inhibitory effects on LPS-induced macrophage IL-6 and TNF-α release and MAPK and NF-kB activation through the induction of GILZ (GC-induced leucine zipper) (115).

In a recent study using Resonance Energy Transfer (RET) technique, Cooray and colleagues have shown that AnxA1 induces the homodimerization of FPR2 and, subsequently, the activation of signalling pathway involving p38 mitogen activated kinase (p38MAPK), MAPKAPK1/2, the chaperon protein heat shock protein 27 (Hsp27) and the release of IL-10 (116). In our model, we have seen that in macrophages isolated from NASH livers the down-modulation of M1 activation markers is associated with an increase in IL-10 expression; moreover, the inhibition of p38MAPK through the chemical inhibitor SB203880 is able to revert both IL-10 stimulation and AnxA1-down-modulation of iNOS and IL12p40.

Interestingly, macrophage incubation with AnxA1 also lowers CCL2 and CCR2 mRNA expression in a p38MAPK-dependent manner, confirming the capacity of AnxA1 to contribute in regulating the liver recruitment of monocytes. Previously, Lange and co-workers have seen the anti-inflammatory activities of the N-terminal AnxA1-derived peptide Ac1-25 on the level of gene expression by using cDNA microarrays, systematically assessing gene regulation in response to Ac1-25 stimulation of human peripheral blood monocytes. Comparison of global gene expression profiles in combination with real-time PCR and flow cytometry has revealed that Ac1-25 is able to induce profound changes in the expression of genes involved in the inflammatory process; in this study, CCR2 has been reported to be one of the most extensively down-modulated gene (117). However, differently from that Lange and colleagues have reported in their study, in our model, the AnxA1 effect on CCR2 down-modulation in mice macrophages is p38MAPK-dependent: in fact, the p38 inhibitor SB203880 is able to block the down-regulation of CCR2. It is very important the involvement of AnxA1 in the signalling pathway mediated by CCL2 and CCR2, as previous works

have pointed the role of CCL2/CCR2 axis in the evolution of NASH: in fact, the pharmacological inhibition of both CCL2 and CCR2 diminishes liver macrophage infiltration, steatohepatitis and fibrosis (48, 107). Taken together these results suggest that AnxA1 might play a functional role in NAFLD/NASH progression by controlling hepatic inflammation through the down-regulation of M1 macrophage activation. As AnxA1 is also evident in liver patients with NASH and its expression inversely correlates with the severity of fibrosis, variability in AnxA1 expression might contribute to influence the evolution of the human disease.

In C57BL/6 mice fed with MCD diet up to eight weeks we have observed that, despite the down-modulation of M1 macrophage response, there is still appreciable liver damage and inflammation as confirmed by higher ALT release, triglyceride content serum TNF-α levels. This suggests that there are other mechanisms involved in maintaining inflammation in advanced NASH. By using flow cytometry, we have observed that the evolution of NASH is characterized by changes in hepatic distribution of NKT cells, as the early NASH is associated with a decrease in the number of NKT cells (CD3+ NK1.1+), while in the advanced NASH after eight weeks of diet the NKT pool grow to level higher than in control mice. An increase in hepatic NKT cells also characterized the rapid progression of NASH in NF-kB1$^{-/-}$ animals. These results are in line with previously works of other groups. For instance, liver NKT cells appear to be relatively depleted in *ob/ob* mice, a model of obesity-related pro-inflammatory cytokine excess, insulin resistance and mild NASH (65). The adoptive transfer of regulatory NKT cells into *ob/ob* animals results in a significant reduction of hepatic fat content, a shift from macro to microsteatosis and significant improvement in glucose homeostasis (118). Reductions in hepatic NKT cell numbers have also been reported to occur when wild type mice are fed high fat, high sucrose diets to induce obesity, insulin resistance and hepatic steatosis (64). Moreover, Kremer and colleagues have reported that hepatic NKT cells are decreased in hepatosteatosis, induced by choline-deficient diet, but are preserved in IL-12 deficient mice. Furthermore, while the administration of lipopolysaccharide leads to increase in hepatic IL-12 expression and the depletion of KCs by clodronate reduces hepatic IL-12 expression and restores NKT cells (66). Interestingly, in a recent work, Tang and colleagues, using a co-culture system of Kupffer cells and NKT lymphocytes, have demonstrated that pro-inflammatory Kupffer cells, expressing high levels of CD1d on cell surface and by presenting lipid antigen to activate NKT cells, led to NKT-cell activation-induced cell death (119). All these data support the concept that the relative depletion of hepatic NKT cells contributes to the metabolic and cytokine alterations involved in the pathogenesis of steatosis. However, a recent report by Syn and co-workers shows that there is an expansion of hepatic NKT cells in mice with more advanced NASH, supporting the

idea that the NKT pool might contribute to fibrogenesis in NASH (67). This is in line with human data where the number of intrahepatic CD3+CD56+ NKT cells in NAFLD patients positive correlates with the NAS score: these cells may enhance disease activity through cytokine production after the recognition of lipid antigens presented with CD1d in livers of NAFLD (68).
Although NKT cells are abundantly present in the liver and involved in hepatic inflammation, the molecular mechanisms involved in their recruitment and expansion in the advanced NASH remain elusive. We have observed that the expansion of NKT cell population in C57BL/6 mice treated for eight weeks with MCD diet is associated with an increase in the hepatic production of IL-15. IL-15 is a cytokine responsible for macrophage, T-cell, NK and NKT cell survival and maturation (120). In normal livers, IL-15 is constitutively produced by hepatocytes and has a key role in creating T-cell favourable microenvironment (121). Hepatic injury stimulates IL-15 formation by hepatocytes, hepatic progenitor cells and macrophages and this contributes in promoting innate and adaptive immune response as well as hepatic regeneration (122, 123). Besides IL-15, we have also observed a significant up-regulation in the mRNA expression of CXCL16 in C57BL/6 animals with advanced NASH. In murine liver, CXCL16 is a chemokine expressed by macrophages and endothelial cells that by interacting with its receptor CXCR6 on NKT cell surface promotes cell migration, survival and proliferation (77). In patients with chronic liver diseases, intra-hepatic CXCR6 and CXCL16 expression are up-regulated compared with controls (77). CXCR6 and CXCL16 up-regulation is also evident in experimental model liver diseases and macrophages have been found to be a major source of CXCL16, while CXCR6-deficient animals are protected against both NKT accumulation and hepatic inflammation (77). Thus, we propose that in advanced NASH the up-regulation of IL-15 and CXCL16, along with the lowering of IL-12, might lead to a more rapid increase in NKT number through a more efficient hepatic differentiation process and that, in turn, NKT cells might contribute to sustain hepatic inflammation. Interestingly, an increased hepatic expression of IL-15 is also evident in NF-kB1$^{-/-}$ animals after only two weeks of MCD diet in combination with an expansion of NKT cell pool. This suggest that the NF-kB1 deficiency may stimulate liver NKT lymphocytes by promoting IL-15 up-regulation.

At the moment, the mechanisms by which NKT cells can contribute to the lobular inflammation and the evolution of NASH to fibrosis are still incompletely characterized. The experiments performed using NF-kB1$^{-/-}$ mice have underlined a possible role of the cytokine osteopontin (OPN) as these mice, but not wild type animals, show a specific increase in liver OPN after four weeks of MCD diet. Furthermore, NF-kB1 deficiency is associated with OPN production by NKT cells. In a similar manner, we have also observed that liver OPN is up-regulated in wild type C57BL/6 mice fed with

MCD diet for eight weeks. Also in these conditions, hepatic NKT cells generate OPN. OPN is a multifunctional cytokine produced by both immune (activated T-cells, dendritic cells, macrophages) and parenchymal cells and plays important roles in inflammation and tissue healing (124). Moreover, OPN is classified as a Th1 cytokine and shown to be involved in monocyte/macrophage as well as dendritic cell migration and activation (125). OPN activates a wide variety of receptors including CD44 variants and integrins. Binding to these receptors induces signalling pathways such as MAPK kinase kinase1/JNK1/activator protein 1, IKK/IκB/NF-kB and phosphatidylinositol 3-kinase/Akt. Diao and colleagues have shown that in Concanavalin A-induced hepatitis, NKT cells secrete OPN, which augments NKT cell activation and triggers neutrophils infiltration and activation (126). Moreover, a humanized anti-osteopontin antibody significantly protects mice from Concanavalin A-induced liver injury in association with the reduction of transaminase activities and improvement of liver injury (127). The role of OPN in stimulating NASH is in line with previous observations where OPN-deficient mice are protected against steatohepatitis and fibrosis induced by MCD diet (128). Furthermore, in a recent work Syn and colleagues have shown that livers from NKT-deficient mice contains significantly less OPN and have less fibrosis than wild type mice after diet-induced NASH. Interestingly, human livers with NASH and advanced fibrosis contain more NKT cells and OPN than NASH livers with early fibrosis (60). Thus, NKT cell activation is associated with increased expression of OPN and this cytokine might be a useful biomarker of advanced fibrosis in NASH. Moreover, OPN is highly up-regulated in adipose tissue from obese patients and animal models of obesity. OPN-deficient mice are protected from high fat diet-induced adipose tissue inflammation and insulin resistance (129), indicating a functional involvement of OPN in obesity-induced insulin resistance. This increased insulin sensitivity is associated with reduced macrophage accumulation in adipose tissue, together with reduced expression of IL-6, TNF-α, iNOS and CCL2. Consistent with these results, neutralising antibody to OPN reduces macrophage numbers in the liver and adipose tissue in obese mice, associated with increased insulin sensitivity and an increased proportion of apoptotic macrophages (130). Moreover, in genetically obese *ob/ob* mice treated with D-galactosamine to induce liver injury, the neutralising antibody to OPN reduced liver injury in parallel with reduced macrophage recruitment and IL-12 and IL-18 production (131). Collectively, these results suggest that the up-regulated OPN expression might be an important mediator in the progression from steatohepatitis to fibrosis contributing to the promotion of inflammatory and immune responses.

Conclusions

From the results presented above, we can conclude that NAFLD progression from simple steatosis to steatohepatitis and to fibrosis is a complex process that involves multiple interactions between hepatocytes, inflammatory cells and hepatic stellate cells during the different phases of the disease evolution. In particular, we have demonstrated a role of both macrophages and NKT cells in the evolution of NAFLD/NASH. Macrophages progressively increase their number during NASH progression and change their phenotype and morphology, while NKT cells, after decreasing in the early phase of the disease, expand when fibrosis appears, leading the maintenance of inflammation also in advanced NASH. Feeding NF-kB1$^{-/-}$ mice with MCD diet, we have observed a more rapid progression of NASH in knockout animals as compared to wild type mice involving an increased recruitment and activation of NKT cells, pointing to possible importance of NF-kB1 polymorphisms as risk factor in the progression of human NASH. Moreover, we have analysed for the first time the role of AnnexinA1 in NASH progression showing that it play an important role in the evolution of the disease by controlling both hepatic inflammation and fibrogenesis. These results represent a good starting point to investigate whether genetic differences in the production of AnnexinA1 may account for the inter-individual variability in the evolution of NAFLD/NASH and to test AnnexinA1 analogues as possible novel treatments to control NASH evolution.

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