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CHARACTERIZATION OF INFLAMMATORY MECHANISMS INVOLVED IN THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS NASH.

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INTRODUCTION

NAFLD/NASH epidemiology.

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. 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 (1). 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 as single large droplet or as smaller, well-circumscribed droplets admixed with cytoplasmic contents (2, 3). Due to the growing diffusion of obesity, NAFLD is considered the most frequent hepatic lesion in western countries and its prevalence ranges from 3 to 15% in the general population, but reaches up to 70% among overweight individuals. Even if hepatic steatosis is often benign, about 15-20% of patients affected by NAFLD develop non-alcoholic steatohepatitis (NASH), characterized by hepatocellular damage and lobular inflammation that often evolves to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (4).

Mechanisms leading to hepatocyte injury in 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 parenchymal damage and inflammation typical of NASH are the factors determining 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 (5).

Several studies indicate that fat accumulation within hepatocytes is determined by insulin-resistance. Indeed, 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 (6). The latter, by flowing through 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) (1). Furthermore, NAFLD subjects exhibit changes in the adipokine pattern that 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. This imbalance promotes hepatic fat accumulation (7). Insulin resistance and 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. Furthermore, alterations in the mitochondrial electron transport chain are an important source of reactive oxygen species (ROS). In turn, ROS-dependent lipid peroxidation promotes a self-sustaining loop that leads to further mitochondrial damage and causes mitochondrial DNA (mtDNA) mutations (8).

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 (ER) stress and cell death, a phenomenon known as lipotoxicity (9, 10). Indeed, hepatocyte incapability to esterify such an excess of FFAs triggers endoplasmic reticulum stress and JNK1/2 activation (10, 11). 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 (11, 12). Thus, up to now, oxidative stress, ER stress, mitochondrial dysfunctions e JNK1/2 activation are considered important factors in the pathogenesis of liver injury during NASH.

Inflammatory mechanisms in the progression of NASH.

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. Pattern-recognition receptor, including Toll-like receptors (TLRs), contribute to the pro-inflammatory responses in fatty livers (13). 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-mediated production of TNF-α and IL-6 by hepatocytes that trigger Kupffer cells to secrete inflammatory mediators, which recruit to the liver other phagocytic cells (14).

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 (15). 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.

Macrophage responses represent an important factor in NASH evolution as they are the main source of pro-inflammatory and pro-fibrogenic mediators that stimulate liver infiltration by circulating monocytes. Resident liver macrophages, also known as Kupffer cells, represent about 20% of non-parenchymal cells in the liver. 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 (16). Their activation ranges between two separate polarization states: the “classically activated” pro-inflammatory M1 and the “alternatively activated” anti-inflammatory M2 states (17). 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. (16).

Under physiological condition, Kupffer cells display a prevalent M2 differentiation and some evidences suggest that a M2/M1 polarization shift might occur in the liver during the evolution from NAFLD to NASH. Indeed, inducing NASH in mice by feeding for ten days methionine and choline deficient (MCD) diet, Kupffer cells get activated releasing TNF-α and promote a

subsequent infiltration of pro-inflammatory $CD11b^{int}Ly6C^{hi}$ monocytes (18). 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 (19).
On top of resident macrophages, new macrophages can be recruited from circulating monocytes at the onset of NASH and infiltrate 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. In mice as in humans, circulating monocytes can be distinct in different subsets on the basis of 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; 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 $CD14^{++}CD16^{-}$ (20, 21). 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. 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 (21). Nonetheless, the actual functions of CX_3CR1^{high} monocytes during the evolution of hepatic injury is still poorly understood.
Recent studies have pointed out a possible involvement of hepatic dendritic cells. Liver DCs are a heterogeneous population of specialized bone marrow-derived cells responsible for antigen presentation to lymphocytes. DCs are sparsely distributed through the liver, and they are primarily found in the portal regions and occasionally in the parenchyma (22). In healthy livers, dendritic cells represent a small fraction of non-parenchymal cells and have a predominant tolerogenic phenotype (23), but a dramatic expansion occurs during chronic liver diseases in combination with a stimulation in their antigen presenting activity, the release of pro-inflammatory cytokines and an increase in their cellular lipid content (24). Unfortunately, a single surface marker that can be used to unequivocally identify DCs has yet to be determined, and distinguishing DCs from other cell types, such as monocytes and macrophages continues to be a challenge (24). In mice and humans DCs can be identified as $CD45^{+}$ cells that constitutively express high levels of major histocompatibility complex II (MHCII) and the use of additional markers, such as the high expression of CD11c, can be also useful in identifying DCs. Experiments using the MCD model of NASH have evidenced that hepatic DCs expand and mature in the early phases of the disease and acquire an immune-stimulating phenotype (25). Such activation likely relates to the activation of both humoral and cellular immune response in NASH (26). However, the specific features of NASH-associated DCs have not been characterized in details.

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 (6). 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 (27). In addition, 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 (27). Accordingly, recent work has recognized that in NASH the development of portal inflammation and fibrosis is a marker for progressive disease (6).

Aim of the study

Despite these information allow to understand some aspects of the evolution of the disease many questions remain open. What is less clear is how liver macrophages behave during the progression of NASH, particularly in relation to the development of fibrosis. Studies using different mice models of chronic liver injury have shown that infiltrating macrophages in injured livers derive from Ly6C/CD11b-expressing circulating monocytes and that these cells not only contribute to sustain inflammation, but also promote fibrosis by stimulating hepatic stellate cells (HSCs) activation and angiogenesis. However, the phenotype of macrophages responsible for the evolution of chronic liver diseases, including NASH, are still incompletely characterized.

During the first year of Doctorate, I focused my attention on studying morphological and functional changes occurring in liver infiltrating monocyte-derived macrophages during the progression of NASH taking advantage of a mouse model of the disease based on the feeding with a methionine-choline deficient (MCD) diet.

EXPERIMENTAL PROCEDURES.

Animal and Experimental protocol. Eight weeks old male C57BL/6 mice were purchased from Harlan-Nossan (Corezzana, Italy) and fed for 4 or 8 weeks with either methionine-choline deficient (MCD) or control diets (Laboratorio Dottori Piccioni, Gessate, Italy). In some experiments, 4 weeks MCD-fed mice received NaHS (1mg/kg body wt) daily for further 4 weeks while continuing on their deficient diet. The experimental protocols 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 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 as well as 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. Steatosis and lobular inflammation were scored blind according to Kleiner et al. (28) in hematoxilin/eosin stained sections. Necro-inflammatory foci and apoptotic cells were counted as reported in (29). Collagen deposition was evidenced by Sirius Red staining. Liver macrophages and activated hepatic stellate cells were evidenced in formalin-fixed sections using, respectively, anti-mouse F4-80 (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 and horse-radish peroxidase polymer kit (Biocare Medical, Concord, CA, USA).

mRNA extraction and Real time PCR. 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, CCL1, CCL2, CX_3CL1, iNOS, arginase-1, MGL-1, CCR2, CCR8, CX_3CR1, α1-procollagen, TGF-β1, α-SMA and beta-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.

Isolation and purification of liver macrophages. Liver macrophages were isolated from the livers of either controls or MCD-fed mice by collagenase perfusion according to Froh et al. (30) and purified

using biotinylated anti-F4-80 antibodies (eBiosciences, San Diego CA, USA) and streptoavidin-coated magnetic beads (Miltenyi Biotec, Germany). Cell purity, as estimated by flow cytometry following immunostaining for CD45 and F4-80, was above 85%. The cells were processed for mRNA extraction using ChargeSwitch® Total RNA Cell Kit (Invitrogen, Frederick, MD, USA).

Intrahepatic mononucleated cell isolation and flow cytometry analysis. Liver mononucleated cells were isolated from the livers of naive and MCD-fed mice and purified on a density gradient (Lympholyte®-M, Cedarlane Laboratoires Ltd. Burlington, Canada) as described in (31). Cells were then washed with Hank's medium and incubated 30 min with de-complemented mouse serum to block unspecific immunoglobulin binding. The cells were then stained with fluorochrome-conjugated antibodies for CD45, CD11b, Ly6C, CD11c, MHCII (eBiosciences, San Diego CA, USA), F4-80 (Invitrogen, Abingdon, UK) and CX_3CR1 (R&D System, Minneapolis, MN, USA) and analyzed with a FACScalibur (Becton Dikinson, Franklin Lakes, NJ, USA) flow cytometer. Intracellular staining for TNF-α and IL-12, was perfomed using specific fluorochrome-conjugated antibodies supplied by (eBiosciences, San Diego 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 functional and morphological changes in liver macrophages.

Feeding mice with a methionine-choline deficient (MCD) diet for either 4 or 8 weeks, resulted in a progressive worsening of steatohepatitis as evaluated by a time dependent increase in a liver triglyceride accumulation, transaminase release and hepatic inflammation. Although the hepatic mRNAs for pro-fibrogenic markers α-1 procollagen, α-smooth muscle actin (α-SMA) and TGF-β1 were significantly up-regulated already after 4 weeks of MCD diet, appreciable fibrosis, as evidenced by collagen staining with Sirius Red, and α-SMA-positive activated hepatic stellate cells (HSCs) were evident in more advanced disease after 8 weeks of treatment. In parallel with the progression of NASH, flow cytometry showed a time-dependent increase of F4-80 positive intra-hepatic macrophages (Fig. 1). However, while macrophage M1 activation markers inducible NO synthase (iNOS) and IL-12p40 sub-unit were up-regulated in the early phases of NASH (4 weeks of MCD diet), a significant decline in both iNOS and IL-12p40 expression was evident upon extending the treatment up to the eight week (Fig.2A, B). The same pattern was also evident by evaluating iNOS and IL-12p40 mRNAs in macrophages isolated from the livers of mice at different disease stages and confirmed by measuring circulating IL-12 levels (Fig. 2C, D, E).

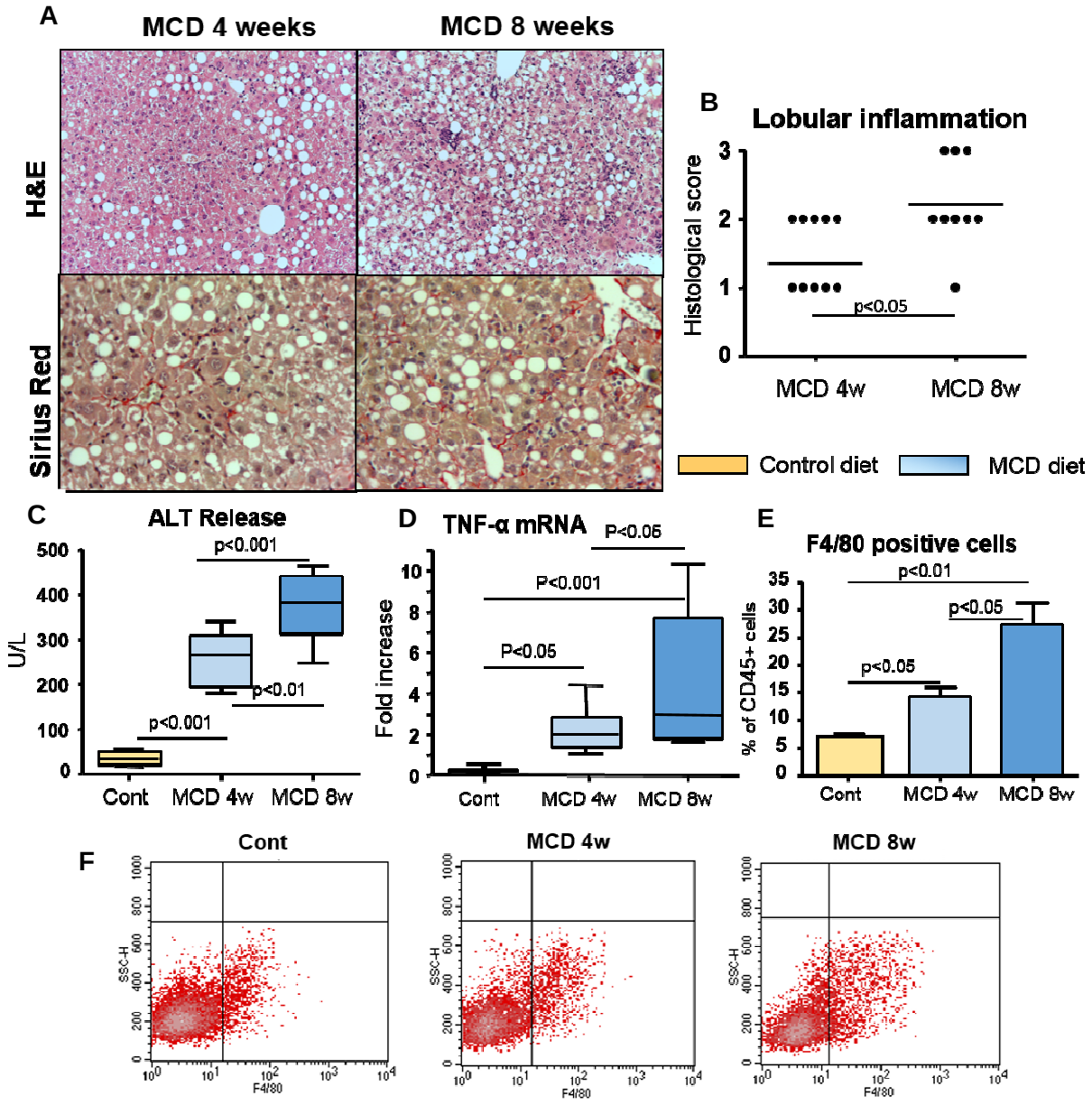

Figure 1. Time dependent variations of hepatic injury and inflammation during mice feeding with a methionine/choline deficient (MCD) diet.

Wild type C57BL/6 mice received the MCD diet for up to 8 weeks. (A, B) Liver histology was evaluated in hematoxilin/eosin (EE) stained sections by counting inflammatory foci as reported in (29) and in collagen Picro-Sirius Red (SR) stained sections from control or MCD-fed animals (magnification 200x). (C) Alanine aminotransferase (ALT) release was determined by enzymatic methods. (D) TNF-α mRNA was measured by Real Time PCR and expressed as fold increase over control values after normalization to the β-actin gene. The values refer to 5-6 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. (E, F) Intrahepatic F4-80 positive macrophages were evaluated by flow cytometry of liver mononucleated cells isolated at different time points. Data are from 4-6 animals per group.

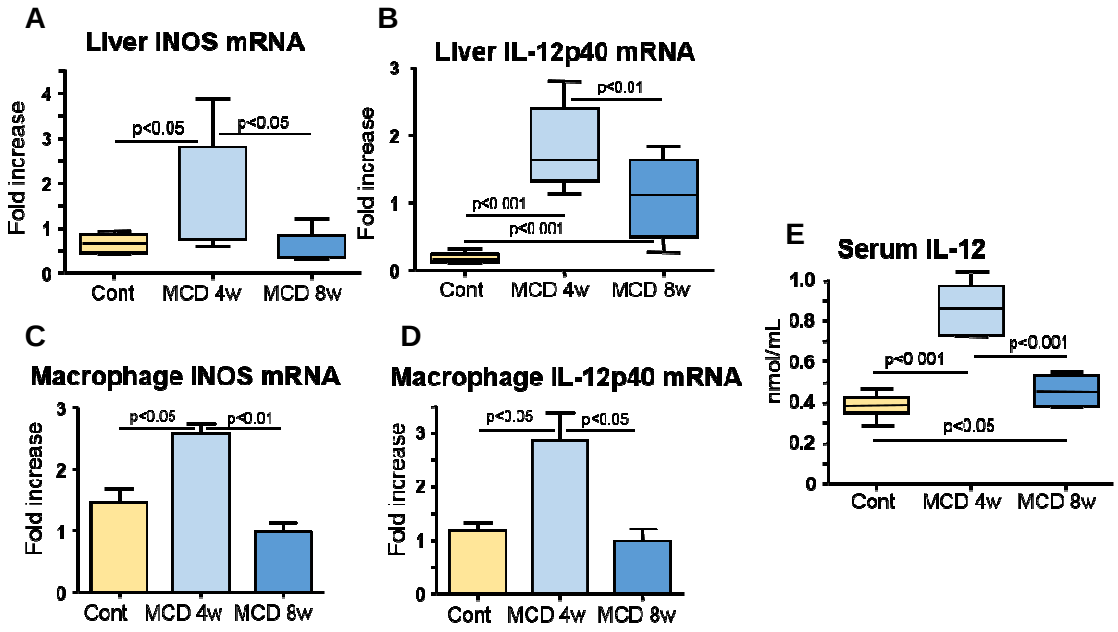

Figure 2. The evolution of NASH is associated with a down-modulation in the hepatic expression of macrophage M1 activation markers.

Mice were fed a methionine/choline deficient (MCD) over an 8-weeks time period. (A, B, C , D) The expression of the macrophage M1 activation markers inducible NO-synthase (iNOS) and IL-12p40 were evaluated by Real Time PCR in mRNA extracted from whole liver and isolated macrophages of either control or MCD-fed mice. (E) Circulating IL-12 protein levels were determined in the same animals. The Real Time PCR values are expressed as fold increase over control values after normalization to the β-actin gene. Data 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.

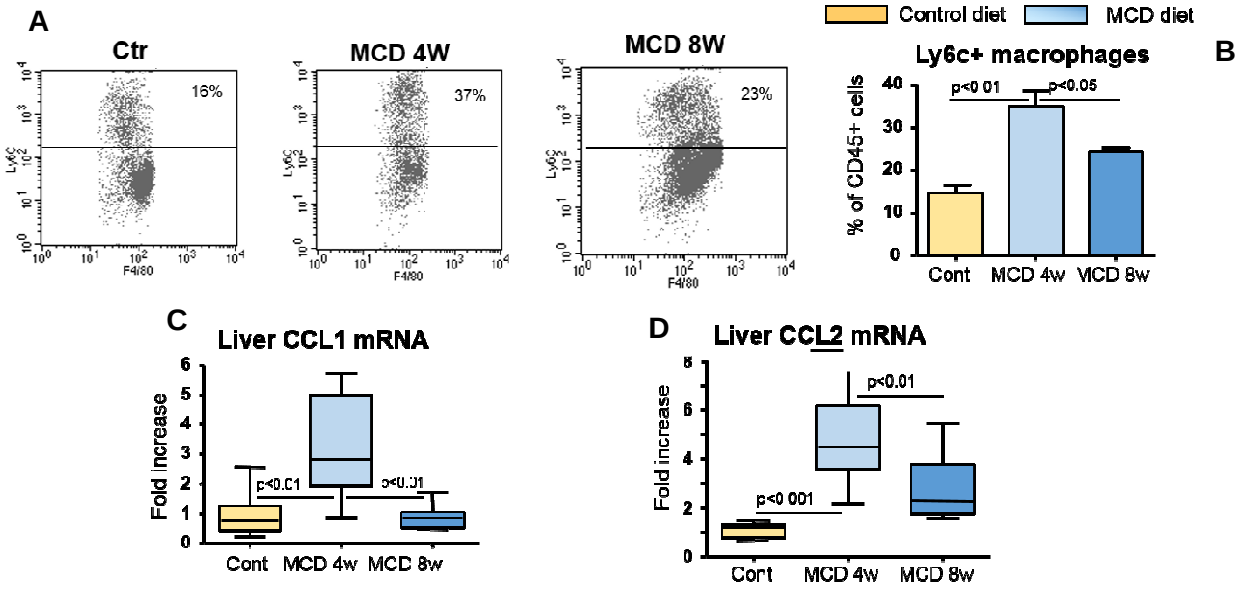

Figure 3. The evolution of NASH is associated with a down-modulation in the hepatic expression of chemokines recruiting Ly6C^{+}monocyte-derived macrophages.

Mice were fed a methionine/choline deficient (MCD) over an 8-weeks time period. (A, B) The relative prevalence of Ly6C^{hi}/F4-80^{+} subset was evaluated by flow cytometry of liver mononucleated cells isolated at different time points. Data are from 4-6 animals per group. (C, D) The expression of monocyte chemokines CCL1 and CCL2 were evaluated by Real Time PCR in mRNA extracted from whole liver. The values are expressed as fold increase over control values after normalization to the β-actin gene. Data 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.

The progression of NASH is associated with a decline of Ly6C^{high} macrophages.

Previous studies have reported that infiltrating macrophages in injured livers derive from Ly6C/CD11b-expressing blood monocytes. Accordingly, we observed that the peak of M1 activation was associated with the prevalence of Ly6C^{high}/CD11b^{+}/F4-80^{+} cells, but their frequency declined in advanced NASH in parallel with an increase in a sub-set of Ly6C^{low} macrophages (Fig. 3). In a similar manner, the liver expression of monocyte chemokines CCL1 and CCL2 as well as that of their respective receptors CCR8 and CCR2 (data not shown) peaked at 4 weeks and declined thereafter. Further characterization of Ly6C^{low} macrophages accumulating in advanced NASH showed that these cells were CD11b positive and largely CX3CR1 negative. Furthermore, they produced appreciably less IL-12 than those Ly6C^{high} (Fig. 4A). On the other hand, the macrophage expression of the M2 polarization markers arginase-1 and galactose-type C-type lectin-1 (MGL-1/CD301) was not appreciably affected during NASH progression (Fig. 4B, C), indicating that Ly6C^{low} macrophages might originate from a phenotypic switch of Ly6C^{high} cells and their prevalence might account for the decline of M1 polarized responses in advanced NASH.

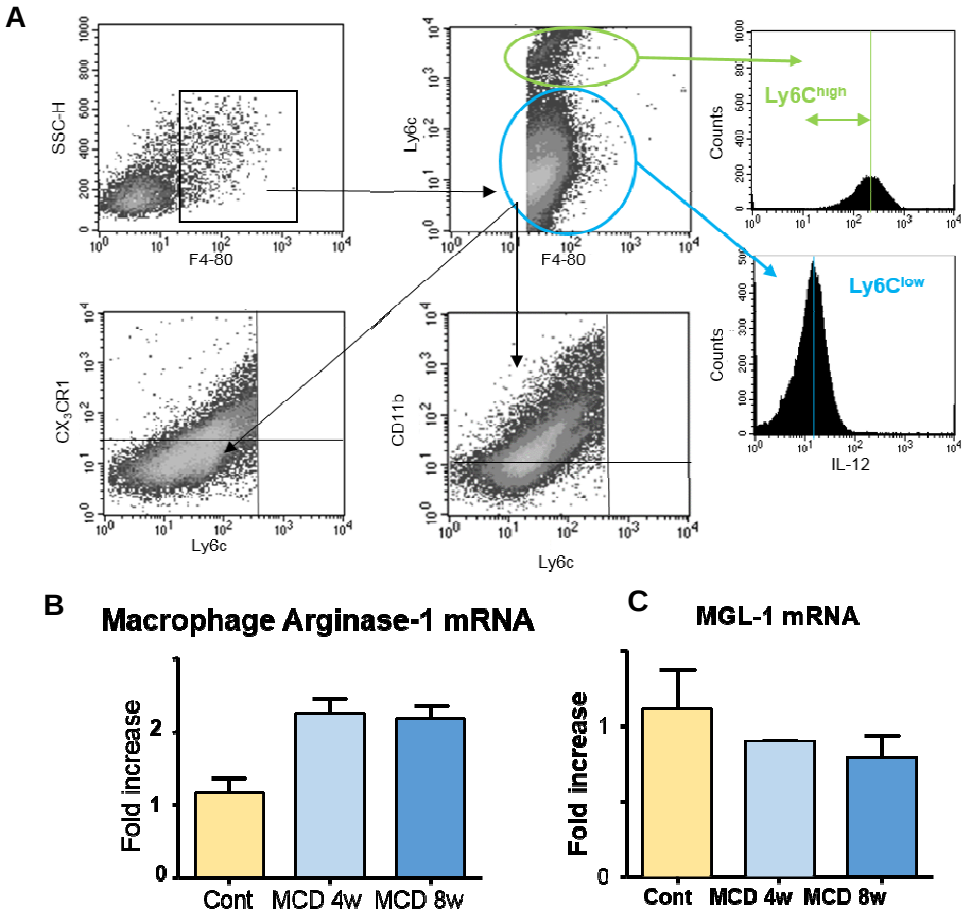

Figure 4. Changes in the hepatic recruitment of Ly6C^{high} inflammatory macrophages during the progression of NASH.

Mice were fed a methionine/choline deficient (MCD) over an 8-weeks time period. (A) Ly6C^{low} hepatic macrophages were characterized for the relative distribution of CD11b and CX_3CR1 and for the expression of IL-12. One experiment representative of three. (B, C) The expression of the M2 polarization markers galactose-type C-type lectin-1 (MGL-1) and arginase-1 were evaluated by Real Time PCR in mRNA extracted from whole liver. The values are expressed as fold increase over control values after normalization to the β-actin gene. Data 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.

An increase in CX_3CR1 expressing monocyte derived dendritic cells is associated with the progression of NASH.

The chemokine fractalkine (CX_3CL1) has recently been implicated in regulating macrophage survival and differentiation. In our hands, the hepatic mRNAs for CX_3CL1 and its receptor CX_3CR1 steadily increased with the progression of NASH (Fig. 5A, B). Interestingly, in mice with more advanced disease the decline of $Ly6C^{high}/CD11b^{+}$ inflammatory macrophages was combined with a tenfold expansion of $F4\text{-}80^{+}/CD11b^{+}$ cells expressing CX_3CR1 (Fig. 5D). Further analysis of these cells showed that they expressed the dendritic cell (DC) markers CD11c and MHCII (Fig. 5E, F).

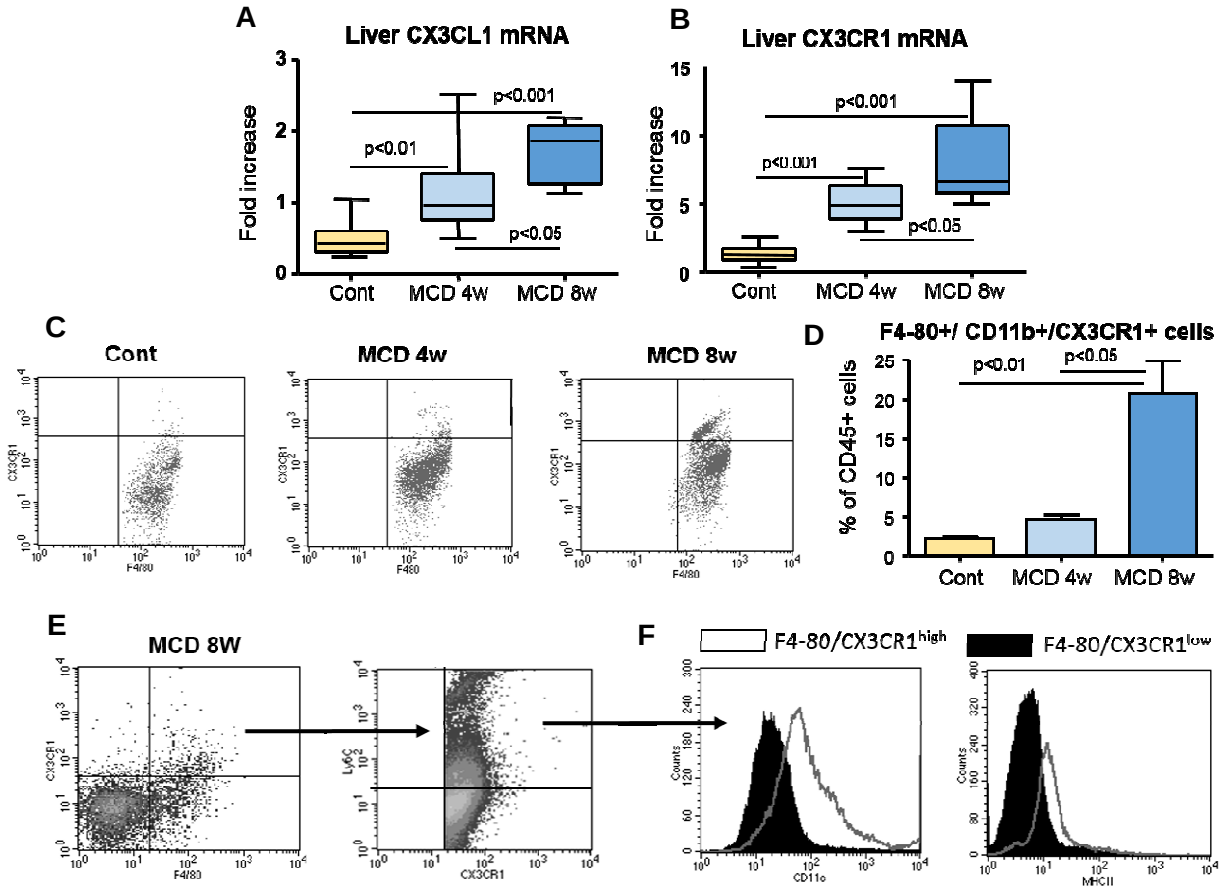

Figure 5. The progression of NASH is characterized by the increase of CX_3CR1-positive monocyte-derived cells.

Mice were fed a methionine/choline deficient (MCD) over an 8-weeks time period. (A, B) The hepatic expression of CX_3CL1 and CX_3CR1 was evaluated by Real Time PCR in mRNA extracted from the liver of control or MCD-fed mice. The values are expressed as fold increase over control values after normalization to the β-actin gene. Data 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. (C, D) The relative prevalence of $F4\text{-}80^{+}/CD11b^{+}/CX_3CR1^{+}$ cells was evaluated by flow cytometry of liver mononucleated cells isolated at different time points. Data are from 4-6 animals per group. (E, F) $F4\text{-}80^{+}/CX_3CR1^{hi}$ and $F4\text{-}80^{+}/CX_3CR1^{low}$ cells were characterized for the relative distribution of CD11b, Ly6C, CD11c and MHCII. One experiment representative of three.

Recently Henning and co-workers reported that the onset of NASH in MCD-fed mice is associated with an expansion in hepatic CD11c$^{high/int}$/MHCII^{+} dendritic cells (Fig. 6A). We confirmed these observations, but we also observed that the expansion of DCs in NASH involved a pool of CD11c$^{hiigh/int}$ cells, wich were positive for both MHCII and F4-80 (Fig. 6B). These cells were also by more than 85% CX$_3$CR1^{+}, while in control livers CD11c$^{high/int}$/MHCII^{+} dendritic cells were largerly F4-80 negative (Fig. 4). Altogether, these data suggested that the expansion of F4-80^{+}/CX$_3$CR1^{+} cells occurring in advanced NASH might involve a sub-set of cells with features of monocyte-derived inflammatory dendritic cells (moDCs). An elevated production of inflammatory mediators, including TNF-α, is a feature of moDCs (32,33). In line with this, we observed that F4-80^{+}/CX$_3$CR1^{+}/CD11c$^{high/int}$ cells had an enhanced production of TNF-α (Fig. 6C) and that CX$_3$CR1^{+}/ TNF-α^{+} cells accumulated in the liver of 8 weeks MCD-fed mice.

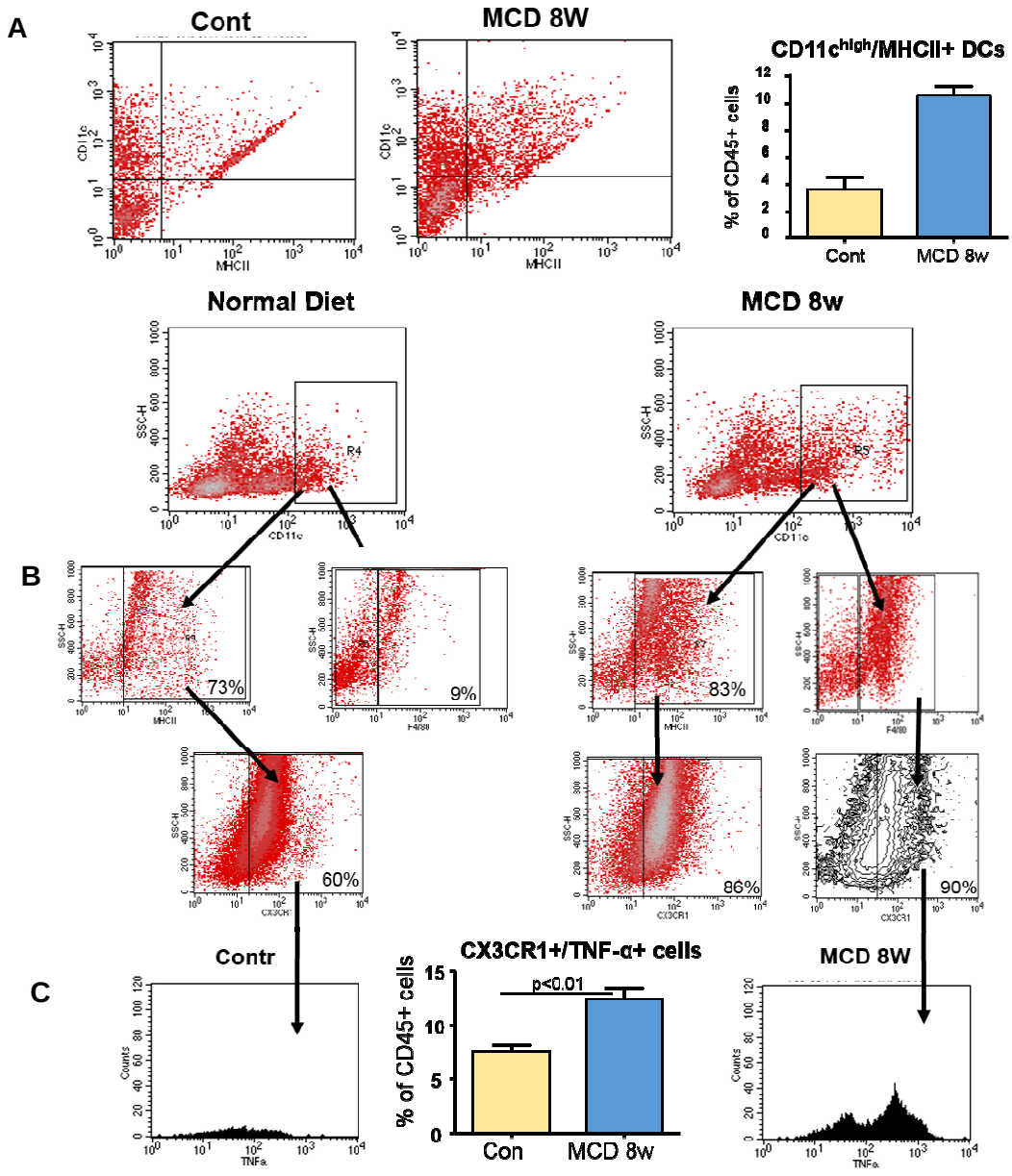

Figure 6. CX$_3$CR1-positive monocyte-derived cells associated with advanced NASH show features of monocyte-derived dendritic cells.

Mice were fed a methionine/choline deficient (MCD) over an 8-weeks time period. (A) The relative prevalence of CD11c $^{+}$/MHCII^{+} dendritic cells was evaluated by flow cytometry of liver mononucleated cells isolated from control and MCD-fed mice. Data are from 4 animals per group. (B) CD11c^{high}/MHCII^{+} hepatic dendritic cells were characterized for the relative distribution of F4-80 and CX$_3$CR1. One experiment representative of three. (C) The relative prevalence of TNF-α-producing F4-80 $^{+}$/MHCII^{+} cells was evaluated by flow cytometry of liver mononucleated cells isolated from control and MCD-fed mice. Data are from 4 animals per group.

DISCUSSION

The results presented above indicate that NASH evolution is characterized by complex changes in the differentiation pattern of the cells derived from monocytes infiltrating the liver. In particular, upon the disease evolution to fibrosis, hepatic macrophage responses are more diversified, as $Ly6C^{high}$ macrophages decline in parallel with the down-modulation of M1 activation markers, whereas a subset of monocyte-derived cells expressing CX_3CR1 and showing features of monocyte-derived dendritic cells become prevalent. These changes involve in one side the switch from $Ly6c^{hi}$ inflammatory macrophages into a $Ly6c^{lo}$ phenotype that characterizes the re-modulation of hepatic inflammation during the progression of NASH to fibrosis. These cells show an intermediate phenotype between that of inflammatory and "atypical" cells and might share similarities with $CD14^+/CD16^+$ macrophages observed during the progression of human chronic liver disease.
On the other side, NASH progression is associated with the monocyte differentiation to CX_3CR1-expressing inflammatory DCs. These cells not only can contribute to stimulate immune responses (26), but also directly sustain hepatic injury and inflammation through an elevated TNF-α production. These results are consistent with recent evidences indicating that under inflammatory conditions, infiltrating monocytes can differentiate into a special sub-set of inflammatory dendritic cells (moDCs), characterized by the co-expression of both dendritic and monocyte/macrophage surface markers and by high production of inflammatory mediators combined to an efficient antigen presenting activity (33).

Future perspectives.

In agreement with the involvement of fractalkine (CX_3CL1) in regulating macrophage survival and differentiation, with future experiments we plan to better characterize its role in regulating the macrophage $Ly6C^{high}$-$Ly6C^{low}$ switch. To this end, a recent study has shown that in macrophage cell lines stimulated with interferon-gamma or lipopolysaccharide, H_2S significantly inhibited CX3CR1 and CX3CL1 expression by modulating PPAR-gamma and NF-kB pathway. Based on these results, we want to investigate whether mice treatment with the H2S donor NaHS might influence macrophage differentiation to CX_3CR1^+ monocyte-derived DCs in MCD-induced NASH.
In this contest, we will evaluate the expression pattern of chemokines/cytokines, pro-inflammatory and pro-fibrogenic mediators, and the evolution of liver injury after the treatment.
Since two recent papers have reported that CX_3CR1 genetic deficiency exacerbates hepatic injury and fibrosis induced by chronic CCl_4 treatment and bile duct ligation, we don't plan to use CX_3CR1 knockout mice (34, 35). In addition, since we observed that about 60% of DCs in the livers of controls animals constitutively express CX_3CR1, is possible that genetic ablation of fractalkine receptor might affect a population of immune-regulatory hepatic DCs, enhancing damage-associated inflammation.
Nonetheless, further studies are required to better define the relative contribution of constitutive versus monocyte-derived CX_3CR1-expressing DCs during the evolution of chronic liver diseases. To this end, should be interesting to perform *in vitro* functional studies to assess the pro-inflammatory phenotype of the moDCs subset or to address further functional properties, evaluating the expression pattern of chemokines/cytokines produced by this cell population.

BIBLIOGRAPHY

1. Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Jarvinen H, Svegliati-Baroni G (2010) From the metabolic syndrome to NAFLD or vice versa? Dig. Liver Dis. 42, 320-330.
2. Kotronen A, Yki-Ja¨rvinen H, (2008) Fatty Liver: A Novel Component of the Metabolic Syndrome. Arterioscler Thromb Vasc Biol. 28, 27-38.
3. Brunt EM. (2010) Pathology of nonalcoholic fatty liver disease. Nat. Rev. Gastroenterol Hepatol. 7, 195-203 .
4. Farrel, G.C., Larter, C.Z. (2006). Nonalcoholic fatty liver disease: From steatosis to cirrhosis. Hepatol. 43, S99-S112.
5. Sanyal AJ. (2005) Mechanisms of disease: pathogenesis of nonalcoholic fatty liver disease. Nat. Clin. Pract. Gastroenterol. Hepatol. 2, 46-53.
6. Marra F, Gastaldelli A, Svegliati Baroni G, Tell G, Tiribelli C (2008) Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis. Trends Mol Med. 14(2), 72-81.
7. Tilg H, Moschen AR (2010) Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 53(5), 1836-46.
8. Reid AE. (2001) Nonalcoholic steatohepatitis. Gastroenterol. 121,711-723.
9. Neuschwander-Tetri BA. (2010) Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 52, 774-88.
10. Cazanave SC, Gores GJ (2010) Mechanisms and clinical implication of hepatocyte lipoapoptosis. Clin Lipidol. 5, 71-85.
11. Malhi H, Gores GJ. (2008) Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. Semin Liver Dis. 28, 360-9.
12. Czaja MJ. (2010) JNK regulation of hepatic manifestations of the metabolic syndrome. Trends Endocrinol Metab. 21, 707-13.
13. Broering R, Lu M, Schlaak JF. (2011) Role of Toll-like receptors in liver health and disease. Clin Sci (Lond) 121, 415-26.
14. Cortez-Pinto H, Caneiro de Moura M, Day CP. (2006) Non-alcoholic steatohepatitis: from cell biology to clinical practice. J. Hepatol. 44, 197-208.
15. Parker GA, Picut CA. (2005) Liver immunobiology. Toxicol Pathol. 33, 52-62
16. Mantovani A, Sica A, Locati M. (2007) New vista on macrophage differentiation and activation. Eur. J. Immunol. 37, 14-6.
17. Sica A, Mantovani A. (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 22, 787-95.
18. Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn YS. (2012) Kupffer cells trigger nonalcoholic steatohepatitis development I diet-induced mousemodel through tumor necrosis factor-α production. J Biol Chem. 287, 40161-72.
19. Bieghs V, Wounters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, Lutjohann D, Febbraio M, Moore KJ, van Bilsen M, Hofker MH, Shiri-Sverdlov R. (2010)

20. Zimmermann HW, Trautwein C, Tacke F. (2012) Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. Front Physiol.
21. Tacke F, Alvarez D, Kaplan Tj, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen, Lira SA, Habenicht AJ, Randolph GJ. (2007) Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest. 117, 185-94.
22. Bosma BM, Metselaar HJ, Mancham S, Boor PP, Kusters JG, Kazemier G, Tilanus HW, Kuipers EJ, Kwekkeboom J. Characterization of human liver dendritic cells in liver grafts and perfusates. Liver Transpl. 12(3): 384-93.
23. Crispe IN. (2014) Immune tolerance in liver disease. Hepatol. doi: 10.1002/hep.27254. [Epub ahead of print].
24. Rahman AH, Aloman C. (2013). Dendritic cells and liver fibrosis. Biochim. Biophys. Acta. 1832: 998-1004.
25. Henning JR, Graffeo CS, Rehman A, Fallon NC, Zambirinis CP, Ochi A, et al. (2013) Dendritic cells limit fibroinflammatory injury in nonalcoholic steatohepatitis in mice. Hepatol. 58:589-602.
26. Sutti S, Jindal A, Locatelli I, Vacchiano M, Gigliotti L, Bozzola C, et al. (2014) Adaptive immune responses triggered by oxidative stress contribute to hepatic inflammation in NASH. Hepatol. 59:886-897.
27. Friedman SL. (2008) Mechanisms of hepatic fibrogenesis. Gastroenterology. 134, 1655-69
28. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, et al. (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatol. 41:1313-1321.
29. Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, Bozzola C, Albano E.(2012) Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin Sci (Lond) 122:545-553.
30. Froh M, Konno A, Thurman RG. (2002) Isolation of Kupffer cells. Currents Protocols in Toxicology 14.4.1-14.4.12.
31. Crispe NI. (1997) Isolation of mouse intrahepatic lymphocytes. In: Current Protocols in Immunology. 3.21.1-321.8.
32. Connolly MK, Bedrosian AS, Mallen-St Clair J, Mitchell AP, Ibrahim J, Stroud A, et al. (2009) In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha. J Clin Invest. 119:3213-3225
33. Domininguez PM, Ardavin C. (2010) Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. Immunol Rev. 234:90-104.
34. Aoyama T, Inokuchi S, Brenner DA, Seki E. (2010) CX3CL1-CX3CR1 interaction prevents carbon tetrachloride-induced liver inflammation and fibrosis in mice. Hepatol. 52:1390-1400.
35. Karlmark KR, Zimmermann HW, Roderburg C, Gassler N,Wasmuth HE, Luedde T, et al.(2010) The fractalkine receptor CX_3CR1 protects against liver fibrosis by controlling differentiation and survival of infiltrating hepatic monocytes. Hepatol. 52:1769-1782.

LESSONS

- “Gene Therapy” – Pros. Antonia Follenzi – 19 June 2014
- "The C-value paradox, junk DNA and ENCODE" – Dr. Diego Cotella – 30 June 2014
- “ Gene Therapy Applications” – Prof. Antonia Follenzi – 14 July 2014

SEMINARS

1. “Epigenetic modification that control stem cells differentiation” - Prof. Salvatore Oliviero – 19 February 2014 - Department of Life sciences and system Biology, University of Turin & HuGeF.
2. “Role of Phosphoinositides-3-kinase C2-alpha, a class II PI 3-kinase, in development and cancer” - Prof. Emilio Hirsch – 19 March 2014 – Department of Molecular & Cellular Biology and Molecular Genetic, University of Turin.
3. ‘‘Atmospheric pressure plasma sources ad processes for biomedical and surface treatment applications’’ – Prof. Vittorio Colombo and Dr Matteo Gherardi – 5 May 2014.
4. “Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari a biomarker” - Miltenyi Biotec – 12 May 2014.
5. “Assessment of cervical cancer control in Rwanda and Bhutan” – Dr. Iacopo Baussano– 9 June 2014 - University of Eastern Piedmont, IARC Lyon.
6. “Ribosome alteration in cancer: effect or cause?” – Prof. Fabrizio Loreni - 11 June 2014 – Department of Biology, Tor Vergata University, Rome.
7. “Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli” - Prof. Gianni Cesareni - 12 June 2014 - Department of Biology, Tor Vergata University.
8. “Disarming mutant p53 in cancer” - Prof. Gianni Del Sal – 26 June 2014 - Department of Life Science, University of Trieste, LNCIB.
9. “Has nature done the experiment for us? Evolutionary insights into infection susceptibility and autoimmunity” Prof. Manuela Sironi - 27 June 2014 - University of Milan, Principal Investigator at Bioinformatics Laboratory – IRCCS “E.Medea – Association La Nostra Famiglia”, Bosisio Parini.
10. “The potential of small regulatory RNAs for the treatment of ovarian cancer” - Prof. John F. McDonald – 16 July 2014 - Georgia Institute of Technology, Atlanta, USA.
11. “A functional link between ARX and KDM5Cgenes linked to neurophenotypes defines acrucial epigenetic disease path” – Dr. Maria Giuseppina Miano – 21 July 2014 - Institute of Genetics and Biophysics ABT CNR – Napoli.
12. Cycle of seminars “The Borgese Sessions”- Prof. Steve Ellis – 8-22 September 2014.
13. “Clonal evolution and clinical relevance of subclonal mutations in chronic lymphocytic leukemia” – Dr. Ciardullo Carmela – 1 October 2014 - Laboratory of Hematology, University of Eastern Piedmont.

CONGRESSES

- **The International Liver Congress 2014 – London – 9-13 April 2014**

"ROLE OF CX3CR1-EXPRESSING MACROPHAGES IN THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS (NASH)."
Salvatore Sutti, Irene Locatelli, **Stefania Bruzzì**, Aastha Jindal, Marco Vacchiano, Cristina Bozzola, Emanuele Albano.

- **6th European Club of Liver Cell Biology – Castelfranco Veneto – 11-13 September 2014**

"CHANGES IN MACROPHAGE DIFFERENTIATION PATTERN DURING THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS NASH."
Salvatore Sutti, **Stefania Bruzzì**, Irene Locatelli, Aastha Jindal, Marco Vacchiano, Cristina Bozzola, Emanuele Albano.

- **United European Gastroenterology Week 2014 – Wien – 19-22 October 2014 (Travel Grant)**

"MORPHOLOGICAL AND FUNCTIONAL CHANGES OF LIVER MACROPHAGES DURING THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS (NASH)."
Stefania Bruzzì, Salvatore Sutti, Aastha Jindal, Irene Locatelli, Marco Vacchiano, Cristina Bozzola, Emanuele Albano.

Oral Presentations.

- **6th European Club of Liver Cell Biology – 11-13 September 2014 – Castelfranco Veneto.**

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Salvatore Sutti, **Stefania Bruzzì**, Irene Locatelli, Aastha Jindal, Marco Vacchiano, Cristina Bozzola, Emanuele Albano.

Abstracts.

- **The International Liver Congress 2014 – London – 9-13 April 2014**

"ROLE OF CX3CR1-EXPRESSING MACROPHAGES IN THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS (NASH)."
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