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ANNUAL REPORT

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

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FIRST YEAR RESULTS' SUMMARY

During the first year of the PhD program in Science and Medical Biotechnologies, I demonstrated 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 (1), 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 (2).

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 (3). 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 (4, 5). 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 (6).

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 (7).
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 (8). 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) (3). 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 (9). Insulin resistance, 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 (10).
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 (11, 12). Indeed, hepatocyte incapability to esterify such an excess of FFAs triggers endoplasmic reticulum stress and JNK1/2 activation (12, 13). 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 (13, 14).

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 (15). 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 (16).
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 (17). Available evidence suggests that adaptive immune responses are prevalent in NASH and mainly involves macrophages.

Macrophage responses represent an important factor in NASH evolution. 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 lipopolysaccharides, 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 (18). Their activation ranges between two separate polarization states: the "classically activated" pro-inflammatory M1 and the "alternatively activated" anti-inflammatory M2 states (19). Pro-inflammatory mediators (TLR ligands and IFN-γ) induce M1 polarized macrophages while 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. Under physiological conditions, 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 (46).
Studies using different mice models of chronic liver injury have shown that circulating monocytes are the precursors of infiltrating macrophages in injured livers, that further contribute to drive lobular inflammation and fibrosis (20, 21). In mice as in humans, circulating monocytes can be distinguished in different subsets on the basis of antigens and receptors exposed on the cell surface. Inflammatory monocytes are characterized as $Ly6C^{high}$ ($Gr1^{high}$)/ $CCR2^{high}$/$CX3CR1^{-}$ in mice and $CD14^{+}$/$CD16^{-}$ in humans and migrate to tissues in the early phase of the response to injury producing pro-inflammatory mediators (22, 23). A second population defined as $Ly6C^{-}$($Gr1^{-}$)/ $CCR2^{-}$/ $CX3CR1^{+}$ in mice and $CD14^{-}$/$CD16^{+}$ in humans has less characterized functions and appears to contribute in promoting tissue healing (22, 24). Accordingly, Kupffer cell depletion or interference with monocyte recruitment prevent hepatic injury and inflammation in experimental models of NASH (25, 26). However, the phenotype of the monocyte-derived cells responsible for the evolution of chronic liver diseases, including NASH, are still incompletely characterized.

Growing evidence indicates that infiltrating monocytes can also differentiate into dendritic cells (2). 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 (31). In healthy livers, dendritic cells represent a small fraction of non-parenchymal cells and can be divided into two main functional classes as classical (cDCs) and plasmacytoid (pDCs), this latter representing the majority of hepatic DCs (32). DCs in healthy livers display a predominant immature tolerogenic phenotype characterized by a low capacity to endocytose antigens and to stimulate T-lymphocytes combined with a high production of interleukin-10 (IL-10) (33, 34). However, recent studies have evidenced that hepatic DCs greatly expand following chronic liver injury in combination with a stimulation in their antigen presenting activity and the release of pro-inflammatory cytokines (35, 36). In these conditions infiltrating monocytes can also differentiate into monocyte-derived inflammatory dendritic cells (IDCs) that co-express both dendritic and monocyte/macrophage surface markers and produce large amounts of inflammatory mediators (2, 37). These cells co-express both DC and monocyte/macrophage surface markers and show a high production of inflammatory mediators combined with an efficient antigen-presenting activity (2). Interestingly, the pro-inflammatory and immunostimulating functions of hepatic DCs appear associated with a cell sub-set with high lipid content (38).
Nevertheless, a detailed characterization of hepatic DCs implication in the mechanisms leading to chronic liver injury is hampered by their still poor phenotypic characterization as well as by the dual suppressive or activating actions that these cells can have on immune and inflammatory responses within the liver (32). 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 (35). Such activation likely relates to the activation of both humoral and cellular immune response in NASH (1). However, the specific features of NASH-associated DCs have not been characterized in details.

Aims of the study

Aim I

During the first year of Doctorate, I demonstrated that NASH progression is associated with monocyte differentiation to CX_3CR1-expressing inflammatory DCs. These cells not only can contribute to stimulate immune responses (1), but also directly sustain hepatic injury and inflammation through an elevated TNF-α production (39). Based on these results, the research activity of the second year was addressed to explore this topic to better understand the role of this DC sub-set in the evolution of the pathology.

Aim II

Recent studies have pointed out that hepatic macrophages in human NASH, but not in patients with simple steatosis often cluster around lipid droplets derived from dead hepatocytes forming crown-like aggregates similar to those present in the inflamed visceral adipose tissue of obese patients (27, 28, 29). These macrophages appear enlarged and contain lipid vesicles and cholesterol crystals resembling foam cells of atherosclerotic plaques (29). Interestingly, clusters of foamy macrophages are also evident in several mice models of experimental NASH in association with lobular inflammation and hepatic fibrosis (29, 30).
As growing evidence indicates that lipid accumulation in macrophages of either adipose tissue or liver promotes pro-inflammatory responses and primes these cells to lymphocytes recruitment (40, 41), the aim of this study was to investigate the phenotype and the possible role of foamy macrophages in modulating lobular inflammation during the evolution of steatohepatitis to fibrosis.

Experimental model

To these aims, we used an experimental model of steatohepatitis induced by feeding mice with a methionine-choline deficient (MCD) diet that, in spite it is different in its pathogenesis by human NASH, allowed us to follow hepatic chronic inflammation up to the development of overt fibrosis (42).

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 Gesan Production s.r.l. (Campobello di Mazara, Italy) and Sigma Diagnostics (Milano, Italy), respectively. Circulating TNF-α 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. (43) in hematoxilin/eosin stained sections. Necro-inflammatory foci and apoptotic cells were counted as reported in (1). 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), α-smooth muscle actin (α-SMA), and CD68 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). F4/80 or α-SMA-positive cells were counted in ten diferent microscopic fields (magnification x20). AnxA1 producing cells were detected using specific antibodies from Zymed Laboratories-Invitrogen (Carlsbad, CA, USA). Hepatic collagen deposition was evidenced by Picro-Sirius Red staining. Immunofluorescence double staining were performed in frozen mice liver sections using fluorescein-labelled annexin V (Roche Diagnostics, Penzberg, Germany) and Texas Red-labelled goat anti-rat IgG antibodies (Sigma, Milan, Italy).

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-1β, IL-12p40, CD11b, CX_3CL1, CX_3CR1, CCL2, CCR2, iNOS, arginase-1, MGL-1, 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.

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 (44). 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 following prior gating for CD45 and the absence of cell aggregates. Intracellular staining for TNF-α and IL-12, was perfomed using specific fluorochrome-conjugated antibodies supplied by (eBiosciences, San Diego CA, USA). Intracellular staining for TNF-α, IL-12, and IL-10 was perfomed using specific fluorochrome-conjugated antibodies supplied by (eBiosciences, San Diego CA, USA). 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).

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. (45) and purified using biotinylated anti-F4/80 antibodies (eBiosciences, San Diego CA, USA) and streptavidin-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).

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 I

DC expansion during the evolution of steatohepatitis mainly involve a pool of CX_3CR1^+ inflammatory moDCs.

As mentioned before, NASH progression is associated with an increase in CX_3CR1-positive monocyte-derived DCs producing TNFα (Fig. 1). To better characterize the DC sub-sets involved during the evolution of the pathology, we analyzed the plasmocytoid and lymphocytoid pools. Interestingly, we observed that $CD11c^+/MHCII^+/B220^+$ DCs and $CD11c^+/MHCII^+/CD8^+$ were significantly decreased (Fig. 1), suggesting that DC expansion occurring during the evolution of steatohepatitis mainly involved CX_3CR1^{high} inflammatory moDCs (2).

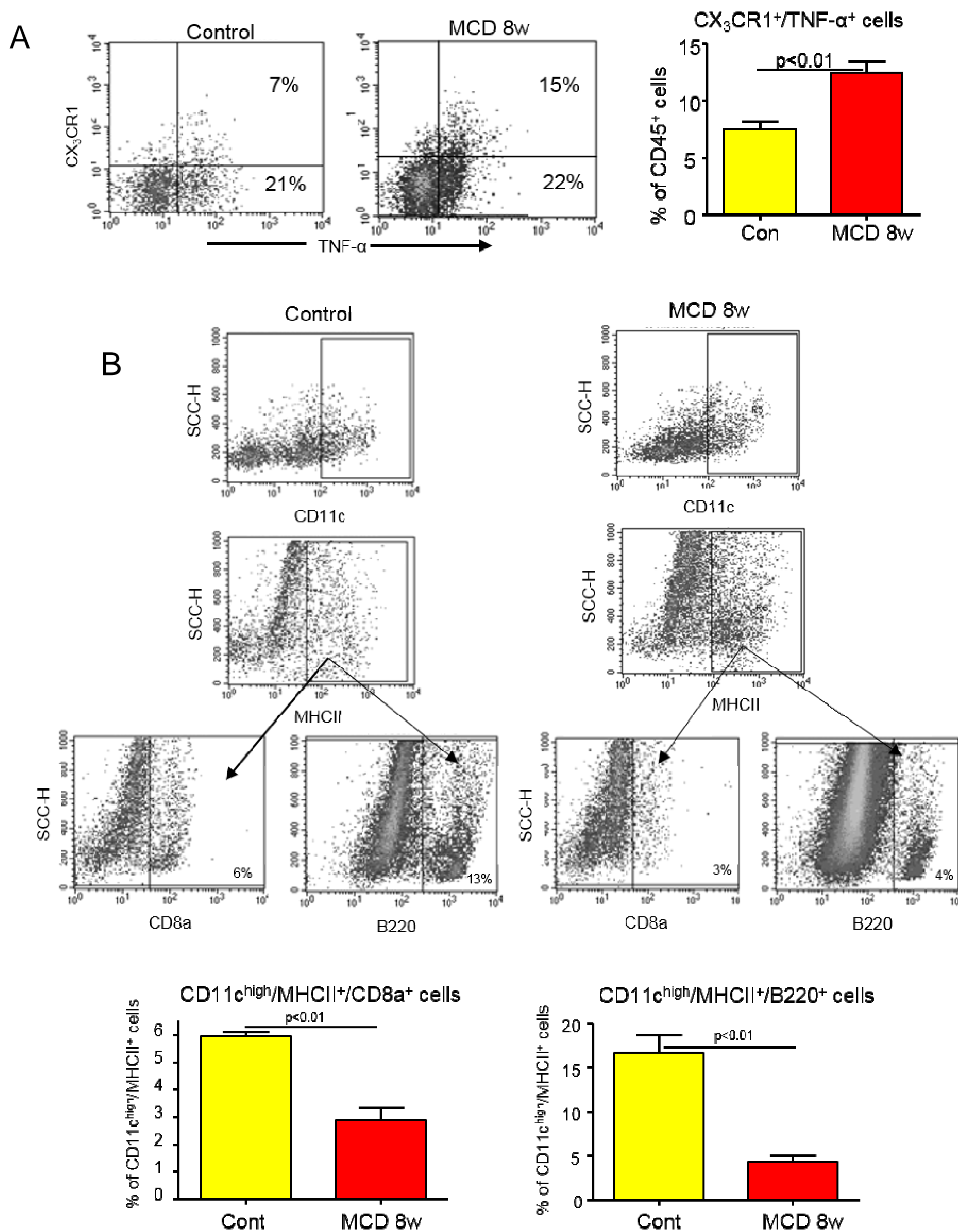

Fig. 1: DC expansion during the evolution of NASH involve a pool of TNFα-producing $CX3CR1^+$ moDCs. Mice fed on a control or an MCD diet for 8 weeks. (**A**) The proportion of TNFα-producing $CX3CR1^+$ cells was evaluated by flow cytometry in control and NASH livers. (**B**) $CD11c^{high}/MHCII^+$ hepatic DCs were characterized for the expression, respectively, of the plasmocytoid and lymphocytoid DC markers CD8a and B220 in either control or NASH livers. Quantitative data were from four to five animals per group.

The increase of TNF-α levels parallels with a decrease in M1 macrophage activation markers.

M1 activation of hepatic macrophages has been shown to be an important factor in driving inflammation in NASH through the production of TNF-α and other pro-inflammatory mediators (46, 40). However, advanced steatohepatitis is characterized by a lowering of M1 activation markers as compared with early NASH (Fig. 2). The intracellular TNF-α content of Ly6C^{high}/CD11b^{+}/F4/80^{+} macrophages also peaked in early NASH and subsequently decreased in more advanced disease (Fig. 3). Yet, a steady elevation in both the hepatic mRNA and serum levels of TNF-α was evident during NASH progression (Fig. 3) and the individual levels of circulating TNF-α positively correlated with transaminase release (r = 0,82; p = 0,035).

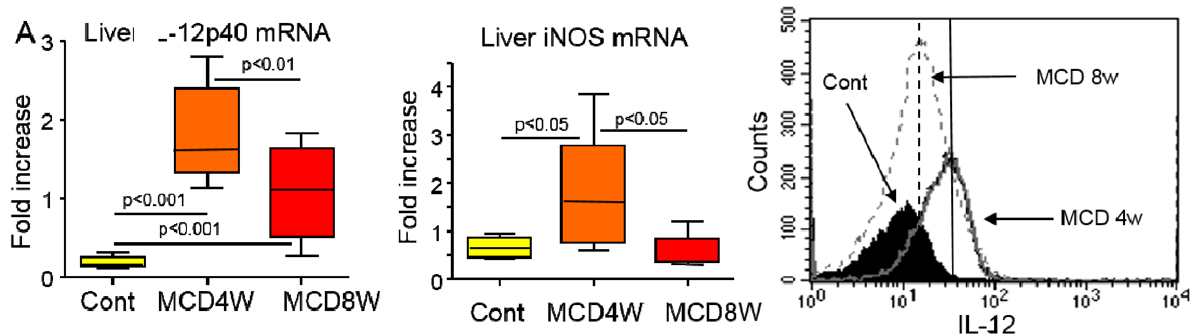

Fig.2: Advanced steatohepatitis is characterized by a lowering of M1 activation markers. Mice were fed with either control or methionine-choline deficient (MCD) diet over a 4 or 8-week time period. (**A**) The hepatic expression of inducible NO-synthase (iNOS) and IL-12p40 was evaluated by RT-PCR. The RT-PCR values are expressed as fold increase over control values after normalization to the β-actin gene. The data are from 5-6 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. (**B**) Intrahepatic F4/80^{+}/Ly6C^{+} macrophages were analyzed by flow cytometry for intracellular IL-12 expression. The data refer to 3-4 animals per group.

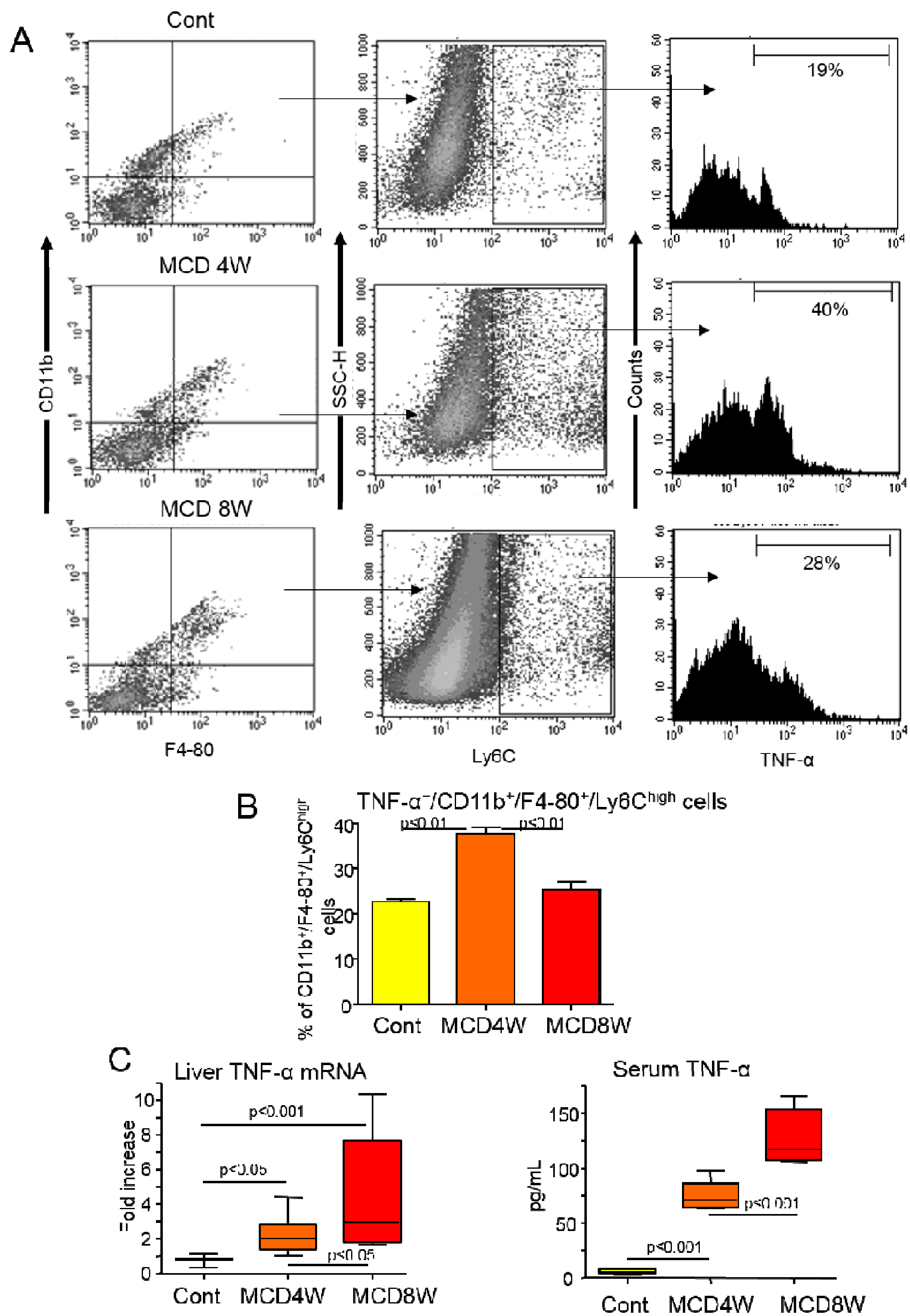

Fig.3: NASH progression is characterized by the increase of TNF-α levels. Mice were fed a methionine/choline deficient (MCD) over a 4 or 8-weeks time period. (**A-B**) F4/80^{+}/Ly6C^{+} cells was analyzed by flow cytometry for intracellular TNF-α expression. The percentages refer to 4 animals per group. (C) Hepatic mRNA expression and circulating levels of TNF-α were evaluated in control and MCD-fed mice. Boxes include the values within the 25th and 75th percentile, whereas the horizontal bars represent the medians. The extremities of the vertical bars (10th - 90th percentile) comprise 80% of the values. Results are from six to eight animals per group.

Effect of treatment with NaHS on CX_3CR1-positive moDCs associated with the progression of steatohepatitis.

Previous studies have shown that the genetic and pharmacological interference with CX_3CR1 ameliorates the evolution of atherosclerotic plaques (36, 2). In this context. Hydrogen sulfide (H_2S) has been reported to improve atherosclerosis by preventing the up-regulation of CX_3CL1/CX_3CR1 in monocyte/macrophages exposed to pro-inflammatory stimuli (2). As CX_3CR1-expressing DCs are already present in healthy livers (Fig. 4), in subsequent experiments we sought to investigate whether the treatment of mice with the H_2S donor sodium sulfide (NaHS) might selectively influence the development of CX_3CR1^+ moDCs in MCD-induced steatohepatitis. Preliminary analysis showed that chronic administration of NaHS (1mg/kg of body weight) did not influence transaminase release and hepatic inflammation markers in control mice (results not shown). In subsequent experiments, mice fed for 4 weeks on the MCD diet received daily NaHS while continuing on the diet up to the eight week. In these animals, we observed that NaHS ameliorated CX_3CL1 and CX_3CR1 mRNA up-regulation (Fig. 4), without interfering with that of CCL2, CCR2 or CD11b (Fig. 4). NaHS supplementation did not modify the hepatic pools of inflammatory macrophages and of DCs , but halved CX_3CR1 expression in F4/80$^+$ or CD11c^{high} cells (Fig. 5). In particular, NaHS treatment selectively reduced the fraction of CX_3CR1^{high}/F4/80$^+$/CD11c^{high} moDCs (Fig. 5).

Furthermore, NaHS decreased intracellular TNF-α levels as well as the fraction of TNFα-producing cells (Fig. 6). In line with this, hepatic TNF-α mRNA and circulating TNF-α levels were lowered in NaHS-supplemented mice (Fig. 6). NaHs treatment did not appreciably influenced the histopathological scores of steatosis (2.3 ± 0.8 compared with 1.8 ± 0.4; $p = 0.1$) and lobular inflammation (1.7 ± 0.6 compared with 1.6 ± 0.5; $p = 0.8$). However, it significantly reduced the number of necrotic foci and apoptotic cells (Fig. 6), and also prevented further elevation of transaminase release in the animals maintained on the MCD diet (Fig. 6), indicating that the sustained production of TNF-α by CX_3CR1^+ moDCs contributed to hepatocellular injury in advanced NASH. Although TNFα-producing DCs have also been implicated in promoting hepatic fibrosis (37), in our hands, treatment of mice with NaHS did not appreciably affected α1-procollagen, α-SMA and TGF-β mRNAs as well as collagen staining with Picro-Sirius Red (Fig. 7).

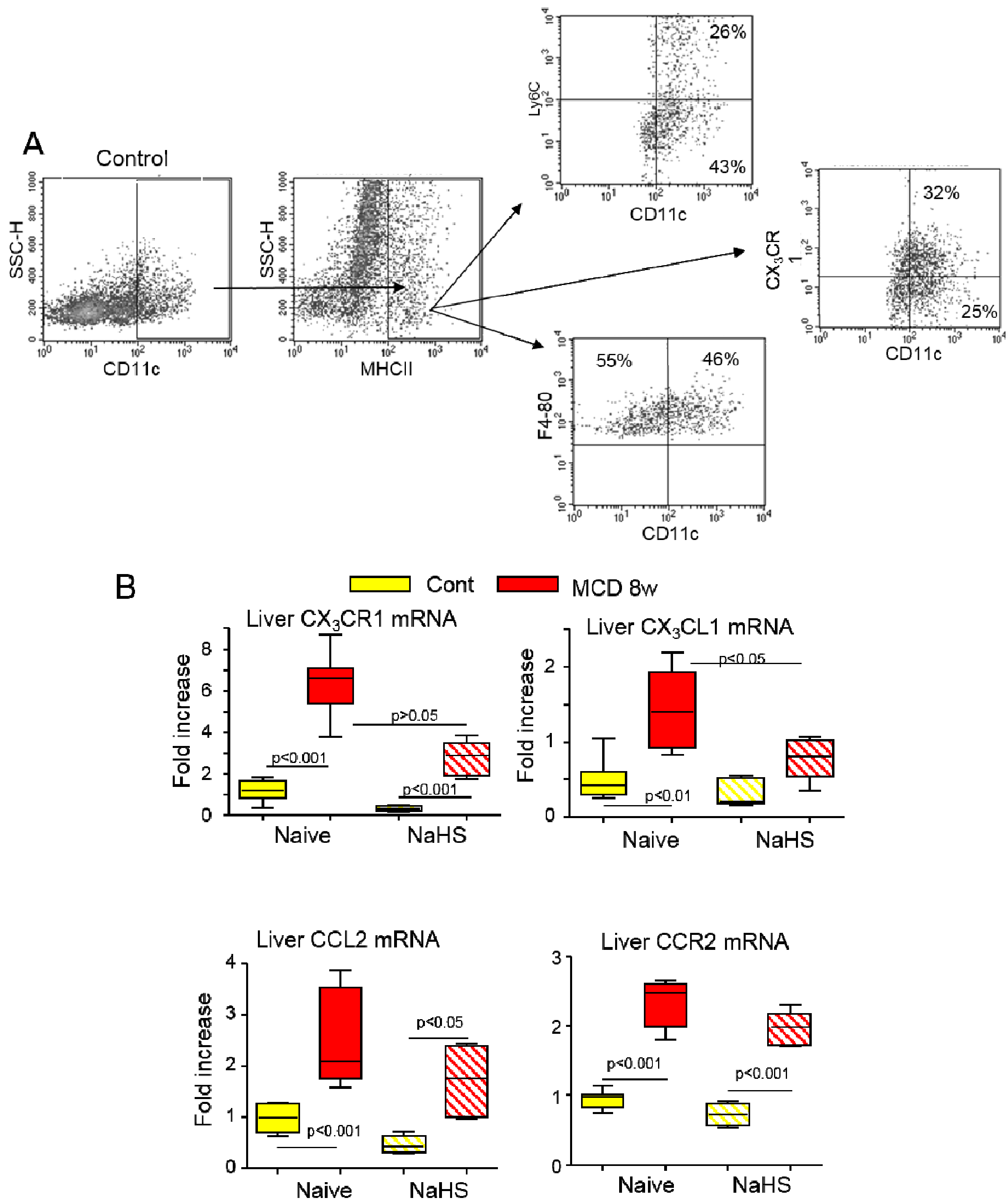

Fig. 4: Effects of hydrogen sulfide on CX_3CR1^+ DCs in NASH. Mice were fed with a control or an MCD diet and liver $CD45^+$ mononucleated cells were analyzed by flow cytometry. NaHS (1 mg/kg) was administered to MCD-fed mice starting from the fourth week of diet. (**A**) $CD11c^{high}/MHCII^+$ hepatic DCs were characterized for the expression of inflammatory monocyte markers F4/80 and Ly6C and of that of CX_3CR1. The percentages indicate the proportion of cells gated as $CD11c^{high}/MHCII^+$, with quantitative evaluation from 4 animals per group. (**B**) The hepatic expression of CX_3CL1, CX_3CR1, CCL" and CCR2 was evaluated by reverse transcription-PCR in mRNA extracted from control of MCD-fed mice woth or without NaHS supplementation. Results are expressed as fold increases over control (Cont) values after normalization to the β-actin gene and are from 6 to 9 animals per group; boxes include the values within the 25th and 75th percentile, whereas the horizontal bars represent the median. The extremities of the vertical bars (10th - 90th percentile) comprise 80% of the values.

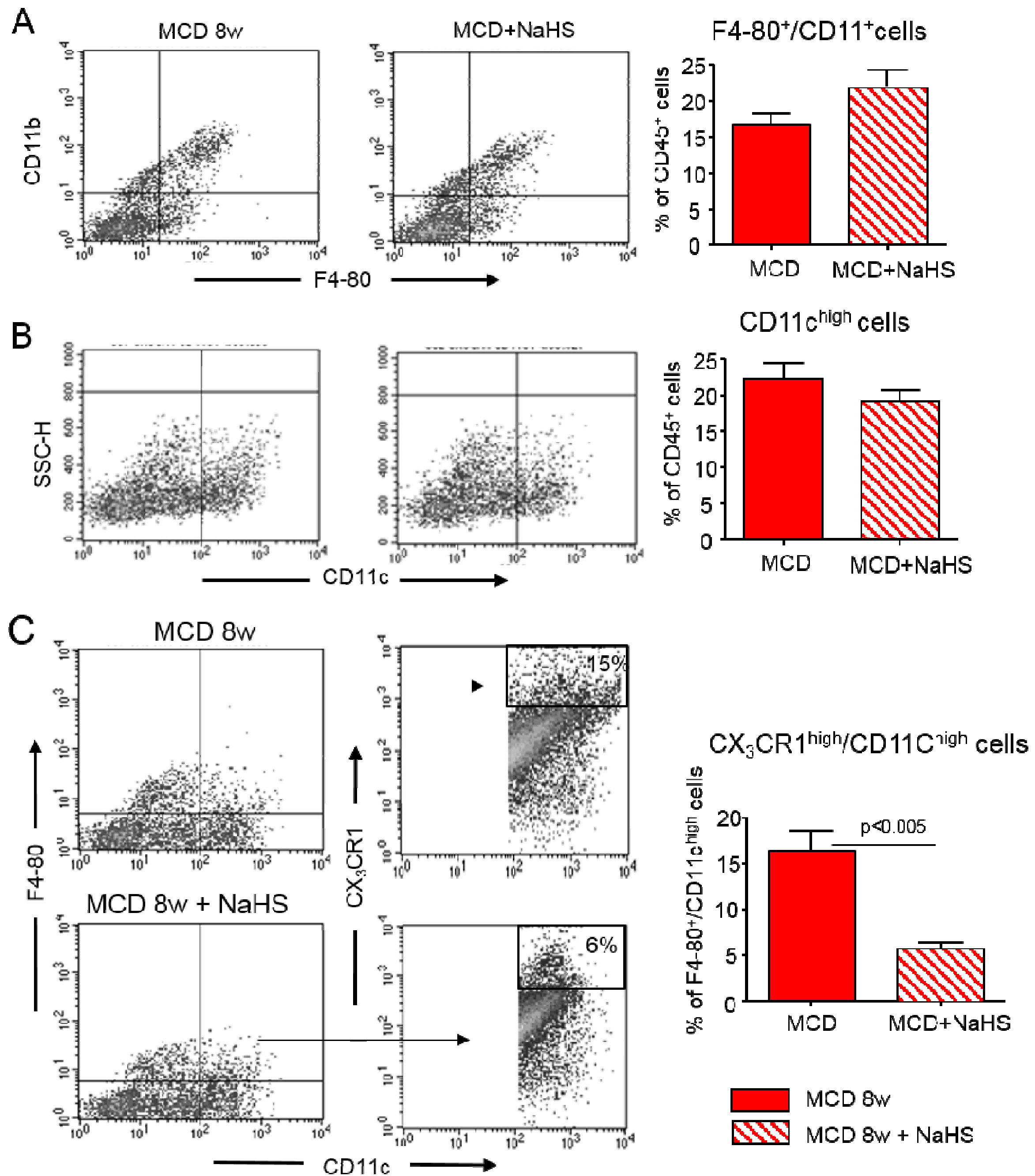

Fig. 5: Treatment of mice with the H_2S donor NaHS reduces hepatic CX_3CL1 expression and CX_3CR1-positive monocyte derived DCs associated with the progression of NASH. Mice were fed on an MCD diet for 8 weeks NaHS (1 mg/kg) was administered to MCD-fed mice starting from the fourth week of diet. (**A-B**) Intrahepatic mononucleated cells were analyzed by flow cytometry for the expression of the macrophage marker F4/80 and the DC marker CD11c. The results are from 3-4 animals per group. (**C**) Changes in the distribution of CX_3CR1^{high} moDCs following NaHS supplementation of MCD-fed mice. The percentages indicate the proportion of cells gated as F4/80^{+}/CD11c^{high}. Results are from three or four animals per group.

Fig. 6: Treatment of mice with the H_2S donor NaHS reduces TNF-α production and improves hepatic injury during the progression of steatohepatitis. Mice were fed with MCD-diet for 8 weeks. NaHS (1 mg/kg) was administered to MCD-fed mice starting from the fourth week of diet.(**A**) TNF-α expression and the relative prevalence of liver F4/80^{+}/ TNF-α^{+} cells was evaluated by flow cytometry. Results are from three or four animals per group. Isotypic controls are shown as broken lines. (**B**) Hepatic TNF-α mRNA and circulating TNF-α levels were evaluated in MCD-fed mice with or without NaHS supplementation. Liver TNF-α mRNA levels were measured by reverse transcription-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. Results are from 6 to 9 animals per group; boxes include the values within the 25th and 75th percentile, whereas the horizontal bars represent the median. The extremities of the vertical bars (10th - 90th percentile) comprise 80% of the values. (**C-D**) Liver histology was evaluated in hematoxylin/eosin-stained sections from control or MCD-fed animals (Magnification 200x). Necro-inflammatory foci and apoptotic cells were counted as described in (2). € Liver damage was assessed by circulating alanine aminotransferase (ALT) release.

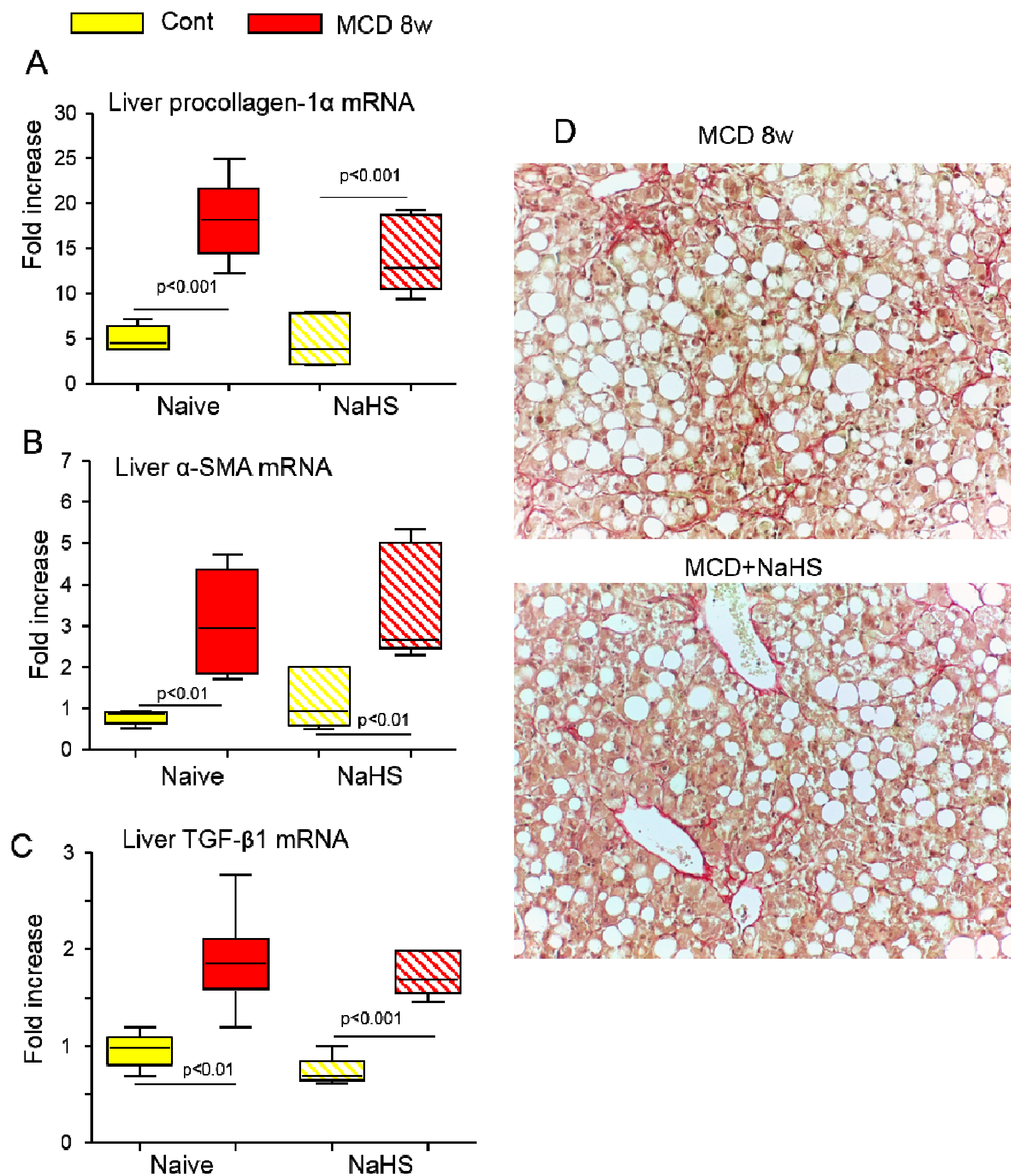

Fig. 7: NaHS treatment don't prevent hepatic fibrosis during the progression of NASH. (**A**) Liver procollagen-1α, α-SMA and TGF-β1 mRNA levels were measured by reverse transcription-PCR and expressed as fold increase over control values after normalization to the β-actin gene. Boxes include the values within the 25th and 75th percentile, whereas the horizontal bars represent the median. The extremities of the vertical bars (10th - 90th percentile) comprise 80% of the values. (**B**) Hepatic collagen deposition was evidenced by Picro-Sirius Red staining.

Discussion

The results presented above indicate that DC expansion occurring during the progression of experimental NASH involves, among different DC subcellular populations, a subset of CX_3CR1-positive monocyte-derived DCs producing TNF-α. Interestingly, the increase of TNF-α levels paralleled with the decrease of M1 macrophage activation markers.
H_2S is increasingly recognized as an endogenous mediator exerting anti-inflammatory and cytoprotective activity in several tissues including the gastrointestinal tract (47, 48). In our hands, NaHS does not have a generalized anti-inflammatory action, but specifically interferes with the up-regulation of CX_3CL1/CX_3CR1 dyad associated with the progression of steatohepatitis. Furthermore, NaHS selectively blocks the development of CX_3CR1^{high} moDCs, indicating that CX_3CL1/CX_3CR1 signaling might have an important role in the differentiation of $Ly6C^{high}$ inflammatory monocytes to moDCs. NaHS treatment also prevents the exacerbation of liver injury indicating that CX_3CR1^{+} moDCs can contribute to steatohepatitis by sustaining hepatic TNF-α production.
In conclusion, our results also show that interference with CX_3CR1 up-regulation prevents the differentiation of moDCs, pointing to CX_3CR1 as a possible target for the therapy of NASH.

Future perspectives.

As NaHS has not a specific anti-inflammatory effect on the CX_3CL1/CX_3CR1 dyad we would like to selectively block the expression of CX_3CL1/CX_3CR1 using more specific methods.
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 exclude to use CX_3CR1 knockout mice (36, 43). 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. To solve this problem, we plan to use an amino terminus–modified CX_3CL1 endowed with CX_3CR1 antagonist activity, able to reduce both CX_3CR1-dependent migration and adhesion (49), in order to determine if CX_3CR1 blockade could prevent the exacerbation of liver injury in our experimental model of steatohepatitis.

Results II

Experimental NASH is characterized by the presence of lipid-laden macrophages.

Steatohepatitis in mice receiving the methionine-choline deficient (MCD) diet was characterized by a time dependent worsening of liver histology, triglyceride accumulation and transaminase release that led to appreciable fibrosis after 8 weeks of treatment (Fig 1.). In these animals immunohistochemistry for the monocyte/macrophage marker F4/80 evidenced that the livers of MCD-fed mice showed the diffuse presence of small clusters of enlarged and vacuolated macrophages that were particularly evident after 8 weeks of treatment (Fig. 1). Double staining of frozen sections with anti-F4/80 antibodies and the lipid dye Oil Red O confirmed that the cytoplasmic vacuoles contained lipid droplets (Fig. 1). Furthermore a fraction of the cytoplasmic vacuoles in F4/80^{+} cells were also stained with the apoptotic cell marker Annexin V (Fig. 1), suggesting the phagocytosis of apoptotic bodies originating from dying fat-laden hepatocytes.

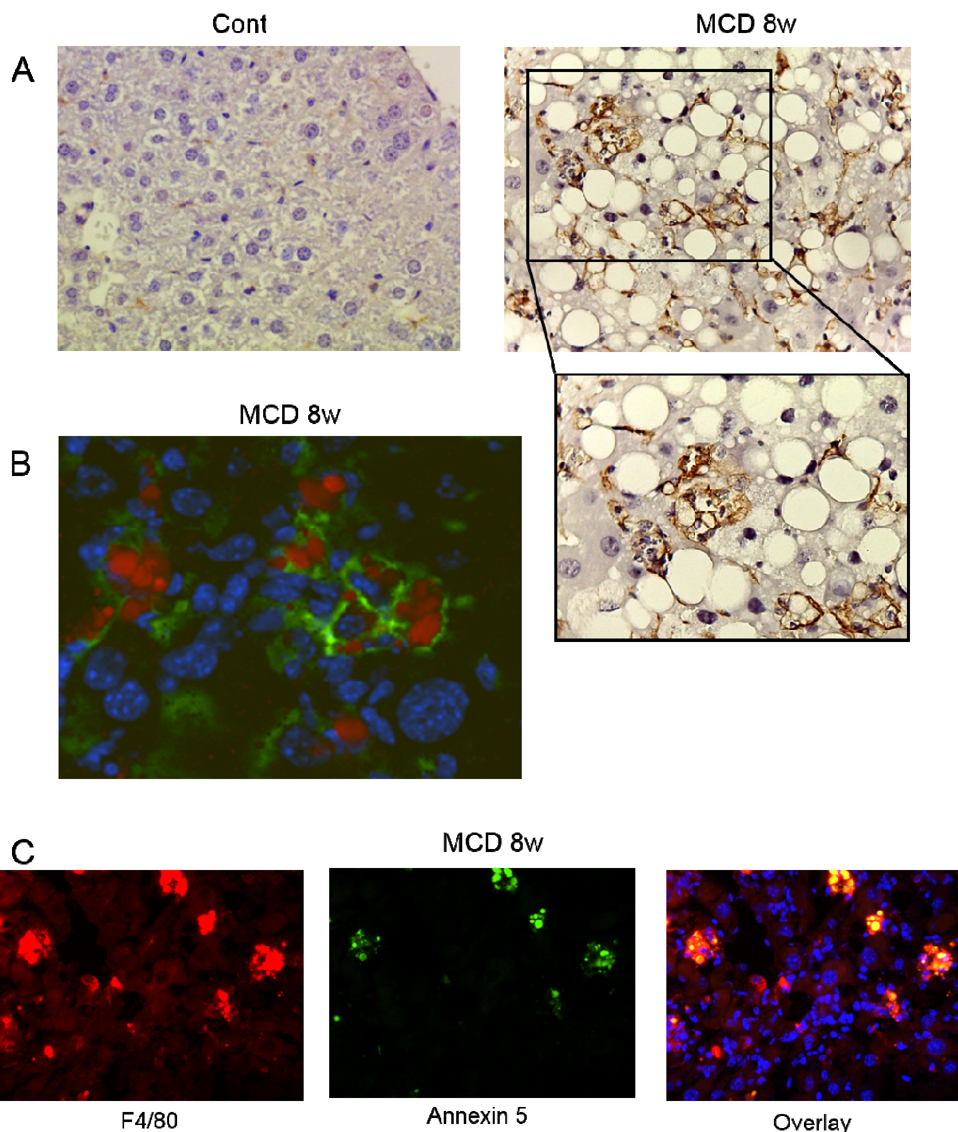

Fig. 1: Morphological changes in liver macrophages during the evolution of steatohepatitis. Mice were fed either with control or methionine-cholone deficient (MCD) diet over an 8-week time period. (**A**) Hepatic macrophages were evidenced by immunohistochemical staining with anti-F4/80 antibodies (magnification 40x). (**B**) Double staining of frozen liver sections eith the lipid dye Oil Red O (ORO) and anti-F4/80 antibody (green immunofluorescence; magnification 40x). (**C**) Co-localization of macrophages stained with Texas Red anti-F4/80 antibodies (red) and fluorescein-labeled annexin V (green) in frozen sections from NASH livers. Cell nuclei were counter-stained with DAPI. Images are representative of 3-4 distinct samples.

In line with these findings, flow cytometry analysis of hepatic mononuclear cells from controls or MCD-fed mice evidenced a steadily increase in F4/80-positive cells during the progression of NASH (Fig. 2). In parallel, we observed that among F4/80$^+$ cells the fraction of enlarged cells, as evidenced by a high forward scatter (FSC-H) parameter, also significantly increase in the liver of animals with more advanced disease (Fig. 2). Further characterization of high volume macrophages associated with NASH revealed that these cells had an enhanced expression of leucocyte activation markers CD11b (CD18b) and CD11c (CD18) as well as of Class II Major Histocompatibility Complex (MHCII) (Fig. 2). Furthermore, enlarged F4/80$^+$ cells associated with NASH were prevalently Ly6C^{high}, in line with an origin from circulating inflammatory monocytes (Fig. 2).

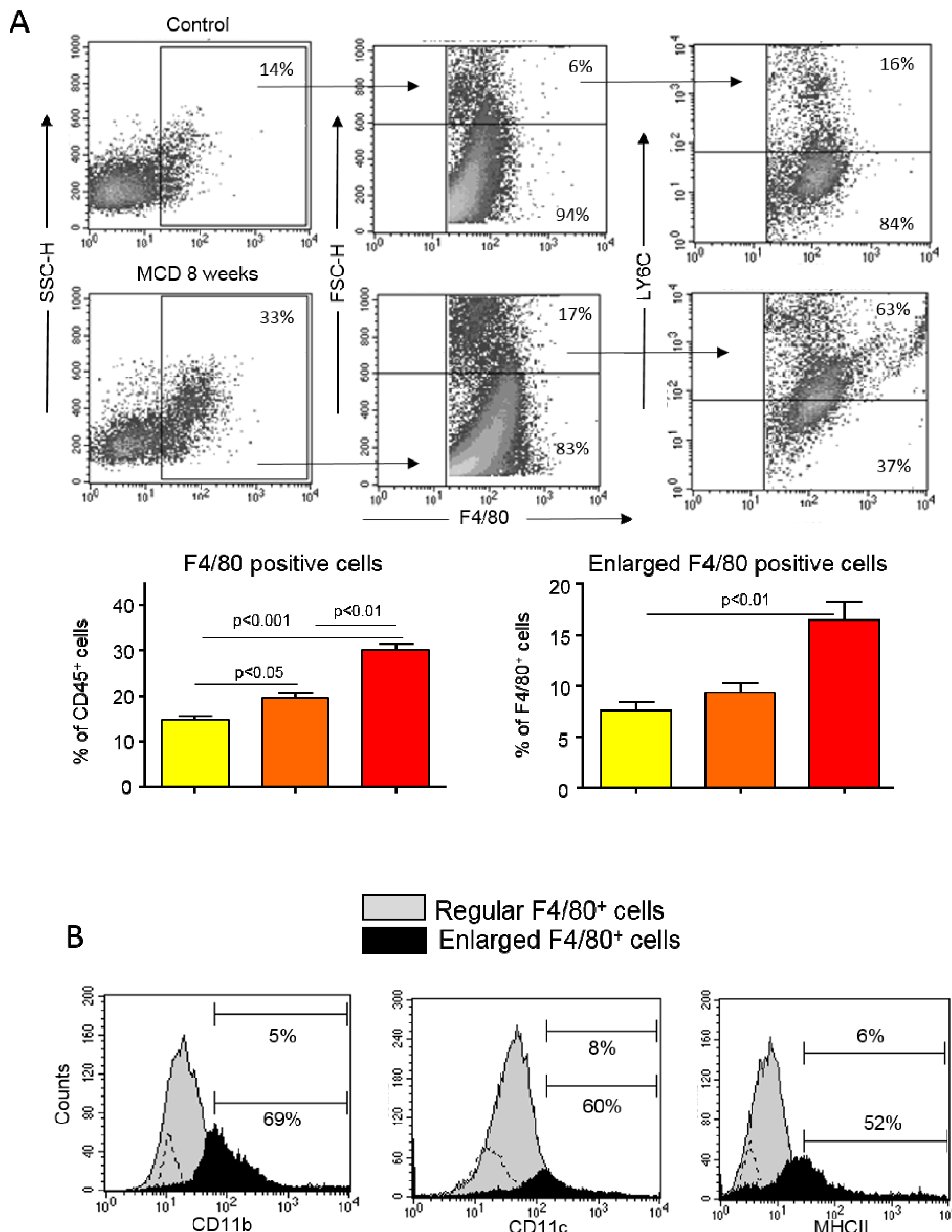

Fig. 2: Flow cytometry analysis of hepatic macrophages during the evolution of steatohepatitis. CD45$^+$ mononucleated cells were isolated from the livers of mice either with control or methionine-choline deficient (MCD) diet over an 8-week time period. (**A**) F4/80$^+$ macrophages were analyzed for cell volume (FSC-H) and the monocyte marker Ly6C distribution. The percentages refer to the number of cells gated as F4/80$^+$. The data were from 3-4 animals per group. (**B**) Expression of leucocyte activation markers CD11b, CD11c and Class II Major Histocompatibility Complex (MHCII) among regular or enlarged F4/80$^+$ cells. Dotted lines refer to isotypic controls. On experiment representative of three.

The accumulation of enlarged fat-laden macrophages during the progression of NASH is associated with changes in the hepatic inflammatory pattern.

Previous studies have shown that lipid-laden macrophages in human NASH had pro-inflammatory features and stained positive for myeloperoxidase and TNF-α (27). We observed that enlarged F4/80^{+} cells not only express more TNF-α but had also a higher production of interleukin-12 (IL-12), a marker of M1 activation (Fig. 3).

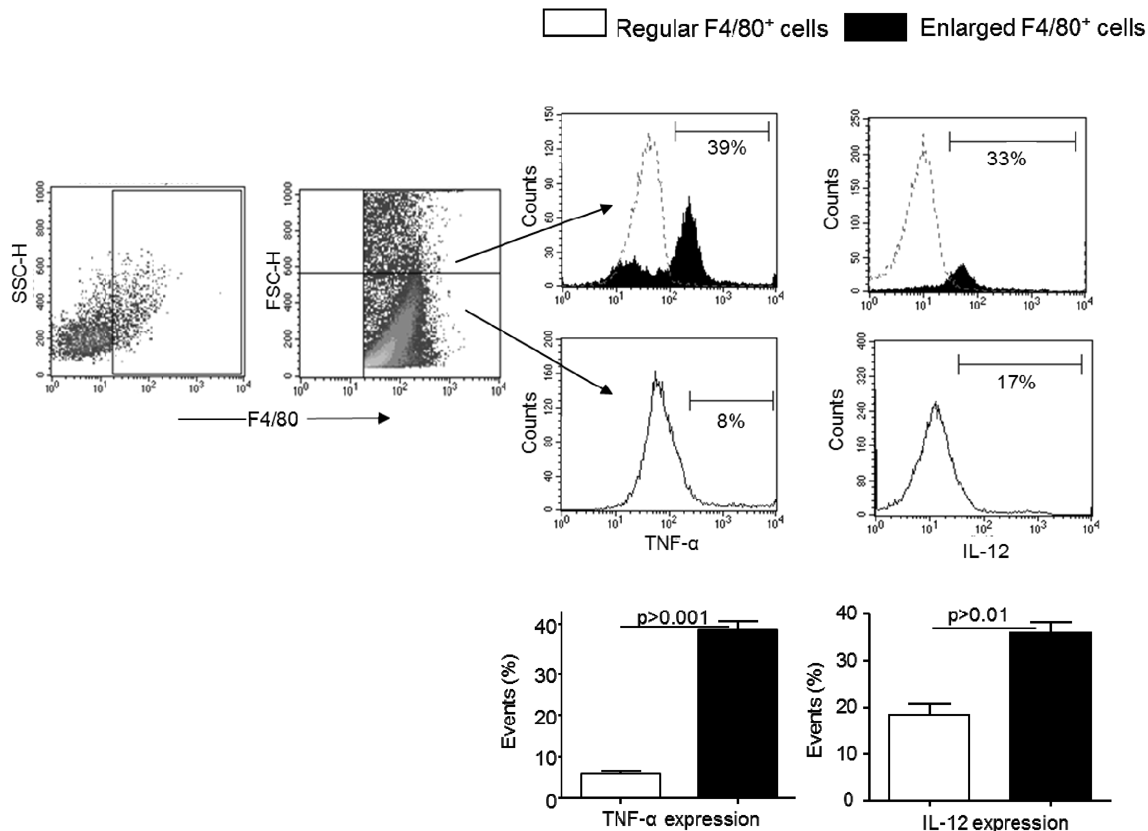

Fig. 3: Enlarged macrophages associated with the advanced phases of steatohepatitis shows an increased production of TNF-α.
CD45^{+} mononucleated cells were isolated from the livers of mice fed methionine-choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. F4/80^{+} macrophages were analyzed for the production of TNF-α and IL-12 among regular or enlarged cells. The percent values refers to the number of cells gated as F4/80^{+}. The dotted lines refer to the distribution of TNF-α^{+} and IL-12^{+} cells in the controls. The data were from 3-4 animals per group.

In spite of these pro-inflammatory features, the accumulation of enlarged fat-laden macrophages during the progression of experimental NASH was associated with changes in the hepatic inflammatory pattern. In fact, the expression of macrophage M1 activation markers such as inducible NO synthase (iNOS), IL-12p40 sub-unit and CXCL10 peaked in mice receiving the MCD diet for 4 weeks and decline thereafter (Fig. 4). In line with these finding, macrophages isolated from the liver of MCD-fed mice at different stages of the disease showed that iNOS and IL-12p40 mRNA levels were significantly lower in the cells obtained from mice with advanced NASH as compared to those in the early phases of the disease (Fig. 5). The same pattern was also confirmed by evaluating IL-12 in F4/80^{+} macrophages by flow cytometry or by measuring circulating IL-12 levels (Fig. 5). Interestingly the lowering of IL-12 expression mainly involved the macrophage sub-set with regular size (Fig. 5). On the other hand, macrophage expression of the M2 polarization markers arginase-1 and galactose C-type lectin-1 (MGL-1/CD301) was not affected in advanced NASH (Fig. 6). It is noteworthy that the up-regulation in macrophage arginase-1 that characterized steatohepatitis prevalently involved enlarged cells (Fig. 6).

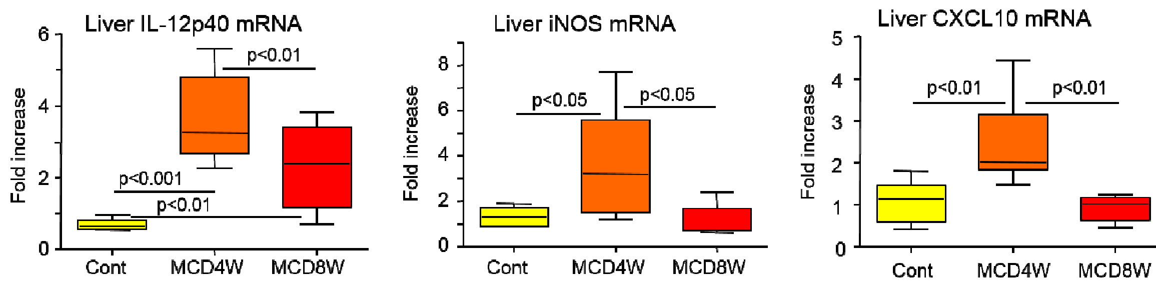

Fig. 4: The evolution of NASH is associated with a differential modulation of liver inflammatory markers. Mice were fed methionine-choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. The hepatic expression of inducible NO-synthase (iNOS) and IL-12p40 and CXCL10 was evaluated by RT-PCR. The RT-PCR values are expressed as fold increase over control values after normalization to the β-actin gene. The data are from 5-6 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.

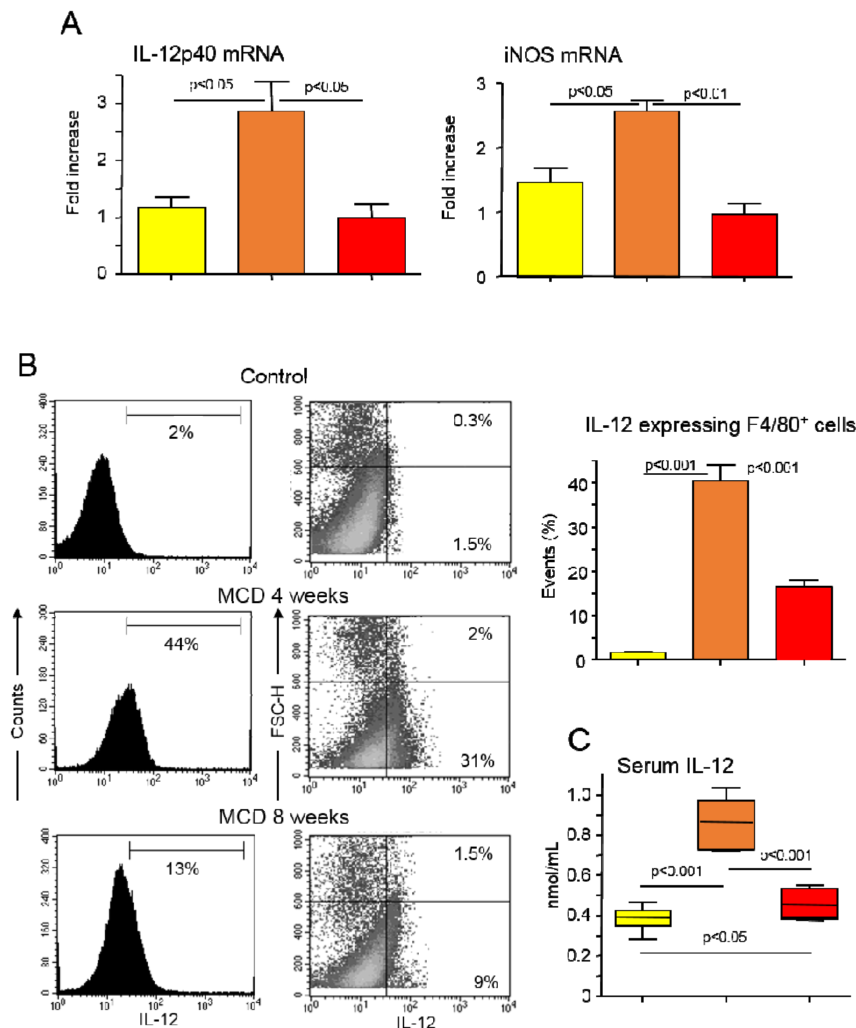

Fig. 5: The evolution of steatohepatitis is associated with a down-modulation in the M1 activation of liver macrophages. Mice were fed with either a control or a methionine-choline deficient (MCD) diet over an 8-week time period. (**A**) Isolated intrahepatic macrophages were isolated using magnetic beads coated with anti-F4/80 antibodies and evaluated for the expression of M1 activation markers inducible NO-synthase (iNOS) and IL-12p40 by RT-PCR. The values are expressed as fold increase over control values after normalization for the β-actin gene. The data are from 4 animals per group. (**B**) Intrahepatic F4/80^{+} macrophages were analyzed by flow cytometry for intracellular IL-12 expression and IL-12 distribution in relation to cell volume (FSC-H). The values refers to the percentage of cells gated as F4/80^{+}and represent 3-4 animals per group. (**C**) Circulating IL-12 were determined either in control and MCD-fed mice by immunoenzymatic assay. The data are from 5-6 animals per group; boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the median. The extremities of the vertical bars (10th- 90th percentile) comprise the eighty percent of the values.

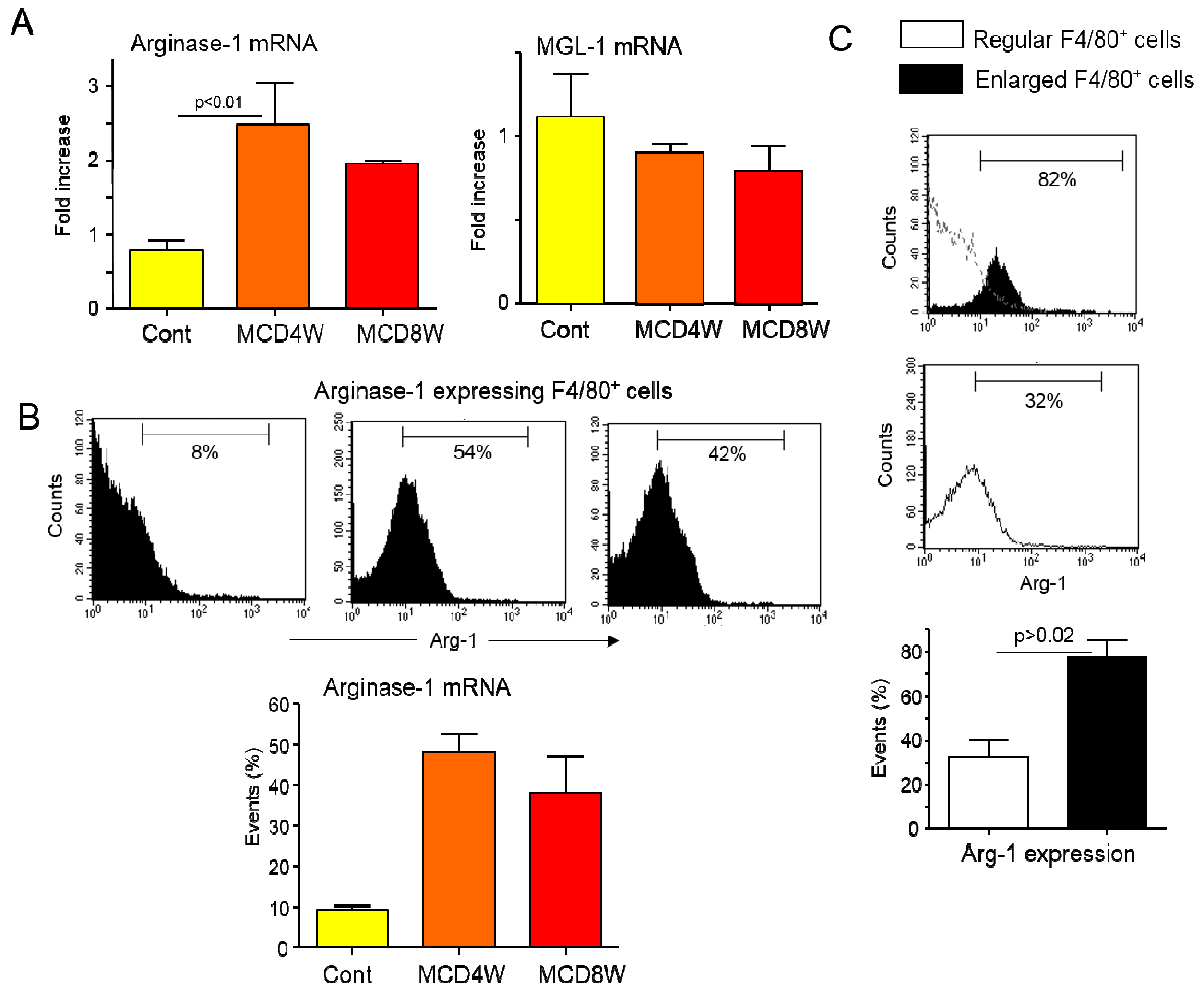

Fig. 6: The evolution of NASH does not affect macrophage expression of M2 activation markers.
Mice were fed methionine-choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. (Panel A) The hepatic expression of M2 polarization markers arginase-1 and galactose-type C-type lectin-1 (MGL-1/CD301) was evaluated by RT-PCR. The values are expressed as fold increase over control values after normalization to the β-actin gene. The data are from 5-6 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. (Panel B) Flow cytometry evaluation of the intracellular arginase-1 (Arg-1) in F4/80$^+$ intrahepatic macrophages. The values refers to the percent of cells gated as F4/80$^+$ and represent 3-4 animals per group. (Panel C) F4/80$^+$ macrophages were analyzed for arginase-1 production among regular or enlarged cells. The percent values refers to the number of cells gated as F4/80$^+$. The dotted lines refer to the distribution of Arg-1$^+$ cells in the controls. The data were from 3-4 animals per group.

Lipid-laden macrophages have anti-inflammatory capabilities.

To get more inside in the mechanisms leading to the decline of M1 responses we measured macrophage production of anti-inflammatory proteins such as interleukin-10 (IL-10) and annexin A1 (AnxA1) that have been previously implicated in modulating hepatic inflammation in NASH (50, 51, 52). Flow cytometry showed that the fraction of cells producing IL-10 and AnxA1 increased among F4/80^{+} hepatic macrophages cells isolated from 8 weeks MCD-fed mice (Fig. 7). Interestingly, the expression of both these anti-inflammatory mediators was 3-7 folds higher in the enlarged F4/80^{+} cell sub-set (Fig. 7). This suggested that AnxA1 and IL-10 released by enlarged lipid-laden macrophages can down-regulate M1-polarized responses.

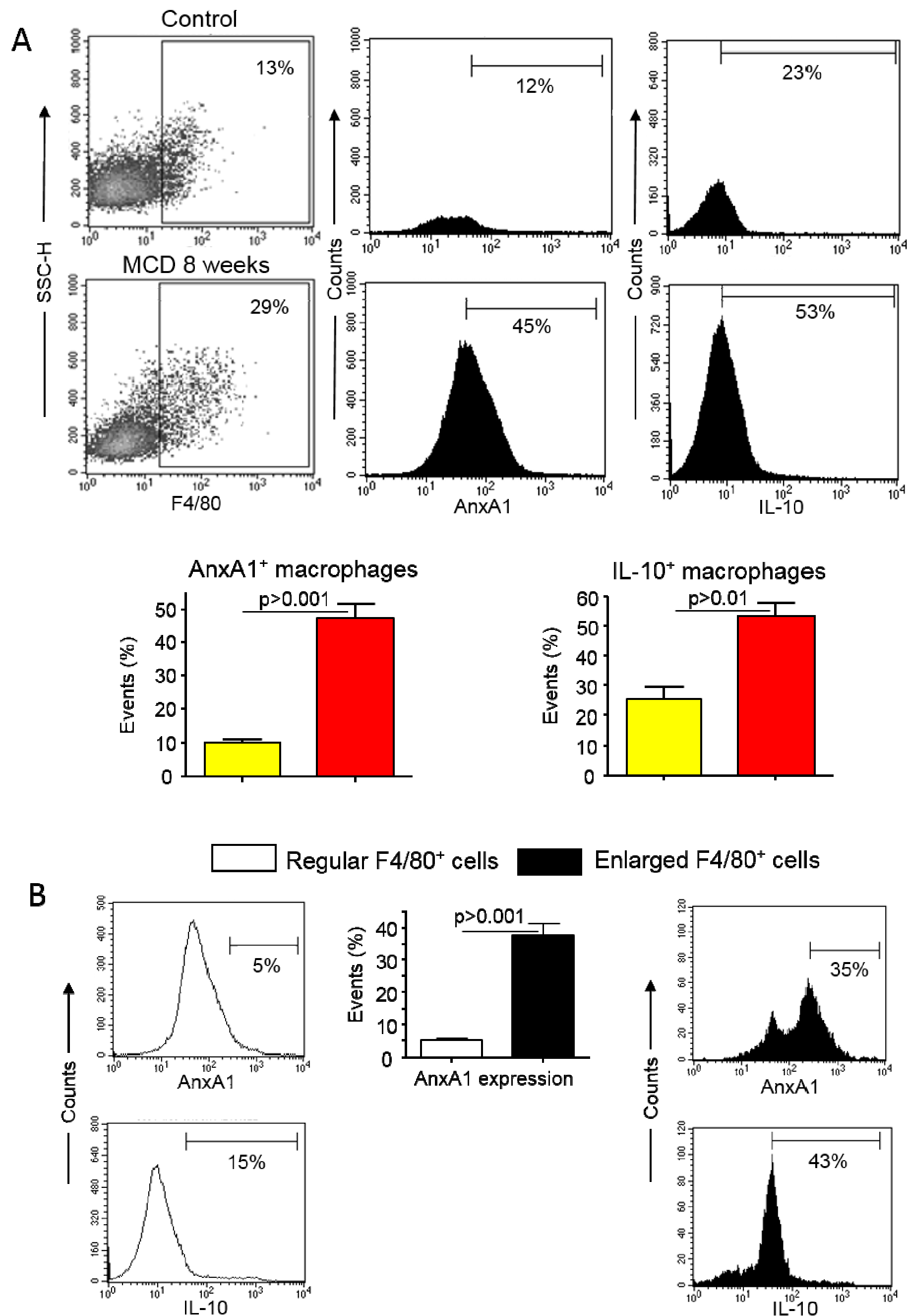

Fig. 7: Enlarged macrophages associated with the advanced phases of steatohepatitis show increased production of anti-inflammatory mediators interleukin-10 (IL-10) and annexinA1 (AnxA1).

CD45^{+} mononucleated cells were isolated from the livers of mice fed either with control or methionine-choline deficient (MCD) diet over an 8-weeks time period. (**A**) F4/80^{+} positive macrophages were analyzed for the production of IL-10 and AnxA1 among regular or enlarged F4/80^{+} cells. The percentages refer to the number of cells gated as F4/80^{+}. The data were from 3-4 animals per group.

According to previous studies (27, 28, 29, 30), enlarged vacuolated macrophages with morphology comparable to those detected in the livers of MCD-fed mice were also detected by CD68 immunostaining in liver biopsies from NASH patients (Fig. 8). These cells were also selectively stained with anti-AnxA1 antibodies (Fig. 8), confirming that also in human NASH lipid-laden macrophages contributed to AnxA1 production.

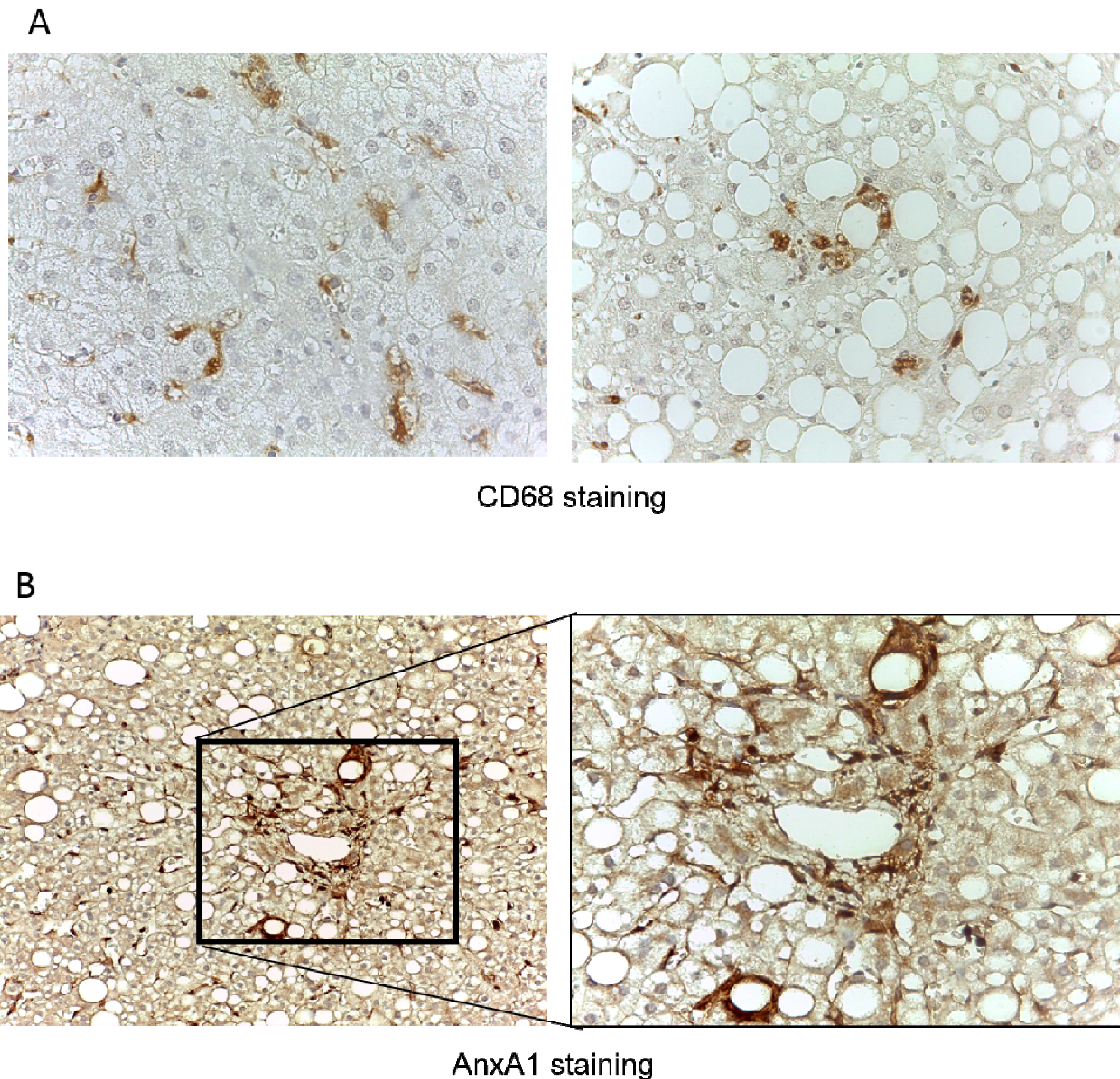

Fig. 8: Immunohistochemical detection of enlarged-foamy macrophages in human NASH. Formalin-fixed sections of liver biopsies from NASH patients were immunostained with human anti-CD68 (**A**) or anti-AnxA1 (**B**) antibodies in combination with horse-radish peroxidase polymer kit (magnification 20x).

Discussion

Immunohistochemical studies in human liver biopsies have shown that fat-laden macrophages in NASH express leucocyte activation makers CD11b and CD11c along with TNF-α and myeloperoxidase suggesting pro-inflammatory capabilities (27, 30). Our present data show that beside an increased expression of CD11b and CD11c, enlarged macrophages associated with NASH are prevalently $Ly6C^{high}$, supporting an origin from circulating $Ly6C^{high}/CCR2^{+}$ monocytes. However, despite showing a pro-inflammatory phenotype, these same cells display an increased production of the anti-inflammatory mediators annexin A1 (AnxA1) and IL-10 along with a high expression of arginase-1. Such a mixed phenotype is consistent with that observed by Zigmond and co-workers (53) in $Ly6C^{high}$ monocyte-derived macrophages, which infiltrates the liver immediately after acute injury. Interestingly, AnxA1 is also selectively expressed by enlarged vacuolated $CD68^{+}$ macrophages in liver biopsies from NASH patients. Moreover, we observed that annexin V stains intracellular lipid vesicles in $F4/80^{+}$ cells, suggesting that the phagocytosis of apoptotic bodies derived from dead fat-laden hepatocytes might contribute to AnxA1 up-regulation. As a result of AnxA1 and IL-10 up-regulation in enlarged lipid-laden macrophages we observed a down-modulation of liver M1-polarized responses that mainly involve the macrophages sub-set with regular size, suggesting that AnxA1 and IL-10 act in an autocrine/paracrine loop affecting pro-inflammatory responses by hepatic macrophages.
In conclusion, our data indicate that, despite their pro-inflammatory phenotype, fat-laden macrophages accumulating during the progression of NASH produce anti-inflammatory mediators suggesting their contribution in the down-modulation of hepatic inflammation associated with the development of fibrosis.

Future perspectives.

Growing evidence points to the importance of AnxA1 in the modulation of anti-inflammatory and pro-resolving responses in rodent models of acute inflammation (54, 55). Based on these observations, one of the next steps aims to increase hepatic expression of AnxA1, or to develop AnxA1 analogs, to understand if they might have a potential for the therapeutic control of NASH evolution. To this aim, we will first administer recombinant-AnxA1 to MCD-fed mice and we will analyze how the treatment is able to influence the progression of the disease and the expression of pro/anti-inflammatory mediators.
Nonetheless, further studies are required to better characterize phenotype and functions of these foamy-like macrophages in human NASH.

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LESSONS

- "Tissue engineering: the state of the art" – 14th November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont.
- "Regenerative Medicine" – 21st November 2014 – Prof. Maria Prat - Department of Health Sciences, University of Eastern Piedmont.
- "Ribosomopathies" – 25th May 2015 – Prof. Steve Ellis – Medical School, University of Louisville (Kentucky)
- "Basis of scientific research" – 10th June 2015 – Prof. Nicoletta Filigheddu – Università del Piemonte Orientale (Italy)

COURSES

- "Le Marie Sklodowska-Curie (MSCA) in Horizon 2020" – 25th June 2015 – Angelo D'Agostino.

SEMINARS

1. "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders" – 06 November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA).
2. "Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention" – 17 November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.
3. "La scoperta del bosone di Higgs" – 25 November 2014 - Dott. Roberta Arcidiacono - DiSCAFF, University of Eastern Piedmont - Dott. Marta Ruspa - Department of Health Sciences, University of Eastern Piedmont.
4. "Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori" – 27 November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women's Health, Paris.
5. "Humoral responses to HCV infection and clinical outcomes" – 28 November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK).
6. "Microglia microvescicles: messengers from the diseased brain" – 17 December 2014 - Dott. Roberto Furlan, San Raffaele University, Milan.
7. "Anticancer strategy Targeting cancer cell metabolism in ovarian cancer" – 19 January 2015 - Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University.
8. "Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states" – 20 January 2015 - Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive "L. Spallanzani" IRCCS, Rome.

9. “Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis” – 21 January 2015 - Prof. Valeria Poli - Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin.

10. “Myeloid cells as therapeutic target in cancer” – 27 January 2015 - Prof. Antonio Sica - DiSCAFF, UPO, Novara.

11. “Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells” – 11 March 2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.

12. “Signal control in iNKT cell development and function” – 09 April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).

13. “Actin-based mechanisms in the control of gene expression and cell fate” – 21st April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).

14. “An integrated approach to the diagnosis and treatment of ovarian cancer” – 7th May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Biosciience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).

15. “Conflicting interests and scientific communication” – 14th May 2015 – Prof. Kathleen Ruff – RightOnCanada Founder, Senior Advisor to the Rideau Institute (Ottawa, Canada).

16. “Recent developments in (cutaneous) Human Polyomavirus research” – 5th June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).

17. “High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach” – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).

18. “Le cellule staminali nel danno renale acuto e nel trapianto di rene” – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).

19. “Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)” , “Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)” – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).

20. Miniworkshop on “Biotechnology for Dermatology” – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France.

CONGRESSES

- **The International Liver Congress 2015 – Wien – 22-26 April 2014**
  CX_3CR1-EXPRESSING INFLAMMATORY DENDRITIC CELLS CONTRIBUTE TO THE PROGRESSION OF NONALCOHOLIC STEATOHEPATITIS (NASH) IN MICE. **Stefania Bruzzì**, Salvatore Sutti, Aastha Jindal, Irene Locatelli, Marco Vacchiano, Cristina Bozzola, Emanuele Albano. Dept. of Health Sciences and Interdisciplinary Research Centre for Autoimmune Diseases, University "Amedeo Avogadro" of East Piedmont, Novara, Italy.

Abstract

- **The International Liver Congress 2015 – Wien – 22-26 April 2014**
  FAT-LADEN MACROPHAGES MODULATE LOBULAR INFLAMMATION IN NONALCOHOLIC STEATOHEPATITIS (NASH).
  Salvatore Sutti, **Stefania Bruzzì**, Aastha Jindal, Irene Locatelli, Marco Vacchiano, Cristina Bozzola, Emanuele Albano. Dept. of Health Sciences and Interdisciplinary Research Centre for Autoimmune Diseases, University "Amedeo Avogadro" of East Piedmont, Novara, Italy.
- **SIPMET "Meeting Our Young Scientists" – Alba (CN) – 11-12 September 2015**
  CX_3CR1-EXPRESSING INFLAMMATORY DENDRITIC CELLS ARE INVOLVED IN THE PROGRESSION OF CHRONIC LIVER INJURY
  Salvatore Sutti, **Stefania Bruzzì**, Aastha Jindal, Irene Locatelli, Cristina Bozzola, Emanuele Albano. Dept. of Health Sciences and Interdisciplinary Research Centre for Autoimmune Diseases, University "Amedeo Avogadro" of East Piedmont, Novara, Italy.

Publications.

- **Is there a role for adaptive immunity in nonalcoholic steatohepatitis?**
  Sutti S, Jindal A**, Bruzzì S**, Locatelli I, Bozzola C, Albano E.
  World J Hepatol. 2015 Jul 8;7(13):1725-9. doi: 10.4254/wjh.v7.i13.1725.

- **Fat-laden macrophages modulate lobular inflammation in nonalcoholic steatohepatitis** (NASH).
  Jindal A, **Bruzzì S**, Sutti S, Locatelli I, Bozzola C, Paternostro C, Parola M, Albano E.
  Exp Mol Pathol. 2015 Aug; 99(1):155-62. doi: 10.1016/j.yexmp.2015.06.015. Epub 2015 Jun 22.

- **CX3CR1-expressing inflammatory dendritic cells contribute to the progression of** steatohepatitis.
  Sutti S, Locatelli I, **Bruzzì S**, Jindal A, Vacchiano M, Bozzola C, Albano E.
  Clin Sci (Lond). 2015 Nov 1; 129(9):797-808. doi: 10.1042/CS20150053. Epub 2015 Jun 25.