CX_3CR1-EXPRESSING INFLAMMATORY DENDRITIC CELLS CONTRIBUTE TO THE PROGRESSION OF STEATOHEPATITIS

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Running title: Inflammatory dendritic cells in steatohepatitis.

Keywords: Nonalcoholic fatty liver disease, macrophages, dendritic cells, liver fibrosis, chronic inflammation, CX_3CR1.

Words: 2806
Figure 6
Tables 0
References: 40

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Abbreviations:

α-SMA; α-Smooth Muscle Actin

DCs; Dendritic Cells

moDCs; monocyte-derived Dentritic Cells

MCD; Methionine-Choline Deficient diet

NAFLD; NonAlcoholic Fatty Liver Disease

NASH; NonAlcoholic SteatoHepatitis

Abstract

Liver monocytes play a major role in the development of non-alcoholic steatohepatitis (NASH). In inflamed tissues, monocytes can differentiate in both macrophages and dendritic cells. In this study, we have investigated the role of monocyte-derived inflammatory dendritic cells (moDCs) in experimental steatohepatitis induced in C57BL/6 mice by feeding a methionine-choline deficient (MCD) diet.

The evolution of steatohepatitis was characterized by an increase in hepatic $CD45^+$/ $CD11b^+$ myeloid cells displaying the monocyte/macrophage marker F4-80^+. In the early phases, (4 weeks treatment) $Ly6C^{high}$/$CD11b^+$/F4-80^+ inflammatory macrophages predominated. However, their frequency did not further grow with the disease progression (8 weeks treatment), when a fourfold expansion of $CD11b^+$/F4-80^+ cells featuring and the fractalkine receptor (CX_3CR1) was evident. These CX_3CR1^+ cells were also characterized by the combined expression of inflammatory monocyte (Ly6C, CD11b), and dendritic cell (CD11c, MHCII) markers as well as by a sustained TNF-α production, suggesting monocyte differentiation to inflammatory moDCs. The expansion of TNF-α-producing CX_3CR1^+ moDCs was associated with an elevation in hepatic and circulating TNF-α level and by the worsening of parenchymal injury. Hydrogen sulfide (H_2S) has been shown to interfere with CX_3CR1 up-regulation in monocyte-derived cells exposed to pro-inflammatory stimuli. Treating 4 weeks MCD-fed mice with the H_2S donor NaHS while continuing on the same diet prevented the accumulation of TNF-α-producing CX_3CR1^+-moDCs without interfering with hepatic macrophage functions. Furthermore, NaHS reduced hepatic and circulating TNF-α levels and ameliorated transaminase release and parenchymal injury.

Altogether, these results show that inflammatory CX_3CR1^+-moDCs contributed in sustaining inflammation and liver injury during steatohepatitis progression.

Introduction

The development of lobular inflammation and parenchymal injury represents the key feature in the transition from non-alcoholic fatty liver disease (NAFLD) to steatohepatitis (NASH) and is clinically relevant because inflammatory mechanisms are the driving forces for the disease evolution to fibrosis/cirrhosis [1]. Circulating free fatty acids, oxidative damages, endoplasmic reticulum stress and adipokine unbalances have been proposed to trigger hepatic inflammation by stimulating Kupffer cell activation [2,3]. Indeed, at the onset of NASH Kupffer cells significantly contribute to the production of pro-inflammatory cyto/chemochines, which, in turn, stimulate the liver infiltration by circulating monocytes [4,5]. These latter rapidly differentiates to M1 polarized macrophages [6] and, by interacting with activated CD4 helper T-lymphocytes and NKT cells, drive lobular inflammation [7,8]. Accordingly, Kupffer cell depletion or interference with monocyte recruitment through CCL2/CCR2 signaling prevent hepatic injury and inflammation in experimental models of NASH [4,9,10]. Furthermore, the extent of macrophage M1 responses appears to modulate NASH severity among different mice strains [6].

Recent studies have revealed the existence of at least two distinct monocyte subsets. Inflammatory monocytes are characterized as $Ly6C^{high}/CCR2^{+}/CX3CR1^{-}$ in mice and $CD14^{+}/CD16^{-}$ in humans and migrate to tissues in early phase of the response to injury producing pro-inflammatory mediators [11,12]. A second population defined as $Ly6C^{-}/CCR2^{-}/CX3CR1^{+}$ in mice and $CD14^{-}/CD16^{+}$ in humans has less characterized functions and appears involved in tissue healing [11,13]. Studies using different mice models of chronic liver injury have shown that $Ly6C^{high}$/CD11b-expressing circulating monocytes are the precursors infiltrating macrophages in injured livers [14,15]. However, the phenotype of the monocyte-derived cells responsible for the evolution of chronic liver diseases, including NASH, are still incompletely characterized [16].

Growing evidence indicates that under inflammatory conditions monocytes can also differentiate into a special sub-set of dendritic cells, called monocyte-derived inflammatory dendritic cells (moDCs) [17]. These cells co-express both dendritic and monocyte/macrophage surface markers and show a high production of inflammatory mediators combined to an efficient antigen presenting activity [17]. As a recent report has associated the development of NASH with an expansion in hepatic dendritic cells (DCs)[18], in this study we have investigated the possible contribution of moDCs to the progression of experimental steatohepatitis. To this aim, we used steatohepatitis induced in mice by feeding a methionine-choline deficient (MCD) diet that, in spite differing in its pathogenesis from the human NASH, it allows to follow hepatic chronic inflammation up to the development of overt fibrosis [19].

Material and Methods

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.

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 were evaluated by commercial ELISA kits supplied by Peprotech (Milano, Italy).

Histology and immunohistochemistry. Steatosis and lobular inflammation were scored blind according to Kleiner et al. [20] in hematoxilin/eosin stained liver sections. Hepatocyte apoptosis was detected by terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) as reported in [7]. Collagen deposition was evidenced by Picro-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 horse-radish peroxidase polymer kit (Biocare Medical, Concord, CA, USA). F4-80- or α-SMA-positive cells were counted in ten different microscopic fields (magnification 20x).

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, CD11b, iNOS, CX_3CL1, 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 [21]. Cells were 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) or 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 performed using a specific fluorochrome-conjugated antibody (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.

Feeding mice with a methionine-choline deficient (MCD) diet for up to 8 weeks resulted a progressive worsening of steatohepatitis as evaluated by a time dependent increase in liver triglyceride accumulation, transaminase release and hepatic inflammation (Supplementary Fig. 1). Although the hepatic mRNAs for α-1 procollagen was significantly up-regulated already after 4 weeks on the MCD diet, appreciable fibrosis, as evidenced by collagen staining with Picro-Sirius Red and α-SMA-positive activated hepatic stellate cells (HSCs), was evident in more advanced disease after 8 weeks of treatment (Supplementary Fig. 1). In parallel with the progression of NASH, flow cytometry showed a time-dependent expansion of intra-hepatic mononuclear cells expressing the mice monocyte/macrophage marker F4-80/CD11b^{+} (Fig. 1). Consistently, F4-80-positive cells in liver sections increased from 71.8±23 cell/mm^{2} in controls up to 146.8±10.2 cells/mm^{2} ($p<0.01$) in 8 weeks MCD-fed mice (Supplementary Fig. 2). Previous studies have reported that in injured livers inflammatory macrophages derive from Ly6C^{+}/CD11b^{+} blood monocytes [16]. Accordingly, we observed that in the early phases of steatohepatitis (4 weeks on the MCD diet) Ly6C^{high}/CD11b^{+}/F4-80^{+} macrophages were prevalent. However, their frequency did not further increase in advanced disease (Fig. 1) and this paralleled with a decline in the mRNAs of inflammatory M1 activation markers inducible NO synthase (iNOs) and IL-12p40 (Supplementary Fig. 1). At this stage, an elevation in the liver expression of both fractalkine (CX_3CL1) and the fractalkine receptor CX_3CR1 become evident (Supplementary Fig. 2). CX_3CR1 up-regulation largely involved monocyte-derived F4-80^{+} cells (Supplementary Fig. 2) and led to a fourfold expansion of a pool of F4-80^{+}/CD11b^{+}/CX_3CR1^{high} cells (Fig. 1). Further characterization of these cells showed that they co-expressed CD11b and Ly6C and were largely Ly6C$^{high/int}$ (Fig. 2), suggesting their origin from liver infiltrating Ly6C^{high} inflammatory monocytes rather than from the Ly6C^{-}/CCR2^{-}/CX3CR1^{+} monocyte sub-set. Moreover, these cells showed an increased expression of the dendritic cell markers CD11c and major histocompatibility class II (MHCII) complex (Fig. 2).

In line with previous observations implicating dendritic cell (DC) in NASH [18], we observed that the progression of steatohepatitis was characterized by an appreciable increase in the fraction of hepatic CD11c^{high}/MHCII^{+} DCs (Fig. 2). Such an expansion involved a pool of cells that were F4-80^{high} and Ly6C$^{high/int}$ and expressed CX_3CR1 (Fig. 3), Interestingly, CX_3CR1^{high} cells were particularly evident within the pool CD11c^{high}/F4-80^{+} DCs (Fig. 3). On the other hand, CD11c^{+}/MHCII^{+}/B220^{+} plasmocytoid DCs and CD11c^{+}/MHCII^{+}/CD8a^{+} lymphocytoid DCs were significantly decreased (Supplementary Fig. 3). Altogether, these data suggested that DC expansion occurring during the progression of steatohepatitis involved CX_3CR1^{high} monocyte-derived inflammatory DC (moDCs) [17].

M1 activation of hepatic macrophages has been shown to be an important factor in driving hepatic inflammation in NASH through the production of TNF-α and other pro-inflammatory mediators [4,6]. However, as mentioned above advanced steatohepatitis was characterized by a lowering of M1 activation markers as compared to the early NASH (Supplementary Fig. 1). The intracellular TNF-α content of Ly6C^{high}/CD11b^{+}/F4-80^{+}

macrophages also peaked in early NASH and subsequently decreased in more advanced disease (Supplementary Fig. 4). Yet a steadily elevation in both the hepatic mRNA and serum levels of TNF-α was evident during NASH progression (Fig. 4) and the individual levels of circulating TNF-α positively correlated with transaminase release (r=0.82; p=0.035). An elevated production of inflammatory mediators, including TNF-α, is a feature of moDCs [17,26]. In line with this, we observed that $CD11c^{high}/F4\text{-}80^{+}$ moDCs had an enhanced expression of TNF-α which specifically involved cells that were CX_3CR1^{+} (Fig. 4). Furthermore, the prevalence of $CX_3CR1^{+}/TNF\text{-}\alpha^{+}$ cells increased in the livers of 8 weeks MCD-fed mice (Fig. 4), suggesting that CX_3CR1^{+} moDCs might sustain hepatic TNF-α production during the progression of steatohepatitis.

Recent studies have shown that genetic and pharmacological interference with CX_3CR1 ameliorates the evolution of atherosclerotic plaques [22-24]. 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 [24]. As CX_3CR1-expressing DCs are already present in healthy livers (Fig. 3), in subsequent experiments we sought to investigate whether mice treatment with the H_2S donor NaHS might selectively influence the development of CX_3CR1^{+} moDCs in MCD-induced steatohepatitis. Preliminary analysis showed that chronic administration of NaHS (1mg/kg body wt) did not influence transaminase release and hepatic inflammation markers in control mice (not shown). In subsequent experiments, mice fed for 4 weeks with the MCD diet received daily NaHS while continuing on the diet up to the eighth week. In these animals, we observed that NaHS ameliorated CX_3CL1 and CX_3CR1 mRNA up-regulation (Fig. 5), without interfering with those of CCL2, CCR2 or CD11b (Supplementary Fig. 5). NaHS supplementation did not modify the hepatic pools of inflammatory macrophages and of DCs (Supplementary Fig. 5), but halved CX_3CR1 expression in $F4\text{-}80^{+}$ or $CD11c^{high}$ cells (Fig. 5). In particular, NaHS-treatment selectively reduced the fraction of $CX_3CR1^{high}/F4\text{-}80^{+}/CD11c^{high}$ moDCs (Fig. 5). Furthermore, NaHS decreased intracellular TNF-α levels as well as the fraction of TNF-α-producing cells (Fig. 6). In line with that, hepatic TNF-α mRNA and circulating TNF-α were lowered in NaHS-supplemented mice (Fig. 6). NaHS treatment did not appreciably influence the histopathological scores of steatosis (2.3±0.8 vs 1.8±0.4; p=0.1) and lobular inflammation (1.7±0.6 vs 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 TNF-α-producing DCs have also been implicated in promoting hepatic fibrosis [25], in our hands, mice treatment with NaHS did not appreciably affected pro-collagen-1α, α-SMA and TGF-β1 mRNAs as well as collagen staining with Picro-Sirius Red (Supplementary Fig. 6).

Discussion

Liver dendritic cells (DCs) are a heterogeneous population of specialized bone marrow-derived cells mainly involved in antigen presentation to lymphocytes [26]. In healthy livers, DCs represent a small fraction of non-parenchymal cells and have a predominant tolerogenic phenotype [27], but a dramatic DC expansion occurs in chronic liver disease in combination with a stimulation in their antigen presenting activity and the release of pro-inflammatory cytokines [28]. Although most of dendritic cells derive from common bone marrow precursors, growing evidence indicates that under inflammatory conditions infiltrating monocytes can differentiate into monocyte-derived inflammatory dendritic cells (moDCs) that co-express both dendritic and monocyte/macrophage surface markers and produce large amounts of inflammatory mediators [17,29].

Experiments in a mice model of NASH have evidenced that hepatic DCs expand and mature in the early phases of the disease and acquire the capacity to specifically stimulate $CD4^+$ T-cells [18]. Such an activation is likely related to the stimulation of both humoral and cellular immune responses that also characterizes the evolution of NASH [7]. However, the specific features of NASH-associated DCs have not been investigated in detail. Our present data add on the involvement of DCs in NASH by showing that, with the progression of steatohepatitis, DC expansion involves a sub-set of cells featuring monocyte markers (F4-80 and Ly6C) along with CX_3CR1 expression and TNF-α production. On these bases, we propose that during the evolution of NASH a sub-set of $Ly6C^{high}$ monocytes might acquire CX_3CR1 and differentiate to TNF-α-producing moDCs. Supporting this view, Barlic and co-workers have observed that during the development of atherosclerosis oxidized lipids stimulate human monocytes to switch from CCR2 to CX_3CR1 expression [30]. Furthermore, recent studies have shown that $Ly6C^{high}$ monocytes differentiate to $Ly6C^+/CD11b^+/CX_3CR1^+$ moDCs in the intestinal *lamina propria.* This process is greatly enhanced during gut inflammation and CX_3CR1^+ moDCs exacerbate colitis by secreting TNF-α [31,32].

Hydrogen sulphide (H_2S) is increasingly recognized as an endogenous mediator exerting anti-inflammatory and cytoprotective activity in several tissue including the gastrointestinal tract [33,34]. Zhang and co-workers [24] have recently shown that in either RAW 246.7 cells or mice peritoneal macrophages H_2S selectively antagonizes CX_3CR1 expression induced by LPS or interferon-γ by signaling through the transcription factor NFkB and peroxisome proliferator activated receptor-γ (PPAR-γ). They also demonstrate that, by interfering with the CX_3CL1/CX_3CR1 dyad, mice supplementation with the H_2S donor NaHS reduces the development of atherosclerotic plaques [24]. Mice feed with a high fat diet show a reduced hepatic H_2S production [35], while H_2S supplementation ameliorates oxidative stress and hepatic inflammation in mice with MCD-induced NASH [36]. In our hands, NaHS has not 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 TNF-α-producing 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 further elevation of transaminase release in the animals maintained on the MCD diet, indicating that CX_3CR1^+ moDCs can

contribute to steatohepatitis by sustaining hepatic TNF-α production. This is in line with the observation that TNF-α-producing DCs sustain hepatic inflammation in mice treated with the hepatotoxic agent tioacetamide [25].
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 [37,38]. In particular, Karlmark and co-workers [38] have shown that CX_3CR1 is required for the survival and the differentiation of liver infiltrating macrophages and can limit their M1 polarization. On the same vein, unspecific hepatic DC destruction exacerbates acetaminophen hepatotoxicity [39] and unexpectedly worsens the evolution of experimental NASH [18]. At present, there is no explanation for the discrepancies between these findings and our present results. We have observed that in the livers of control animals about 30% of DCs constitutively express CX_3CR1. Liver DCs are known to have a predominant immune-suppressive activity [27] and CX_3CR1^+ DCs also share these properties [40]. Thus, it is possible that genetic CX_3CR1 deficiency or hepatic DC ablation might enhance damage-associated inflammation by affecting a population of tolerogenic DCs and that this latter effect may overcame the protection given by preventing moDC differentiation.

We are well aware that steatohepatitis induced by the MCD diet does not reproduce important features of the human NASH such as obesity and insulin resistance [19]. However, the difficulties to differentiate the role of moDCS in both clinical and experimental settings justify the use of this model as, differently from other experimental protocols, it causes extensive steatohepatitis. 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.

In conclusion, the data presented indicate that the evolution of steatohepatitis involves the emergence of CX_3CR1-expressing moDCs that can sustain hepatic inflammation in advanced disease through TNF-α production. Our results also show that interference with CX_3CR1 up-regulation prevents the differentiation of moDCs, pointing CX_3CR1 as a possible target for the therapy of NASH.

Clinical Perspectives

In healthy livers, dendritic cells (DCs) represent a small fraction of non-parenchymal cells and have a predominant tolerogenic phenotype. Recent evidence indicates that NASH is associated with liver DC expansion and activation. However, the features of DCs involved in NASH progression have hot been characterized in detail.

We have observed that DC expansions during the evolution of steatohepatitis involves a sub-set of cells with features of monocyte-derived inflammatory dendritic cells (moDCs) and expressing the fractalkine receptor CX_3CR1. MoDCs sustain hepatic inflammation in the advanced phases of steatohepatitis by producing TNF-α**.** Interfering with CX_3CR1 expression, with the H_2S donors NaHS prevents moDC differentiation and ameliorates parenchymal injury.

These results indicate that preventing the differentiation of CX_3CR1-expressing moDCs can have application in the therapy of NASH.

Author contribution

SS and IL designed the study and performed the experiments; AJ, SB and MV performed the experiments; CB evaluated pathology; EA contributed to the study design and supervised the study and the manuscript preparation.

Acknowledgements

This work has been supported by a grant from the Fondazione Cariplo, Milan, Italy [Grant N°2011-0470]. The authors have no competing interests on the matter concerning the present manuscript.

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Legends to the Figures

Figure 1. Changes in the hepatic recruitment of Ly6C^{high} and CX3CR1+ monocyte-derived cells during the progression of steatohepatitis.
Mice were fed a control or a methionine-choline deficient (MCD) diet over an 8-week time period. The time changes of intrahepatic CD11b$^+$/F4-80$^+$ monocyte/macrophages the relative prevalence of Ly6C^{high}/CD11b$^+$/F4-80$^+$ and CX_3CR1^+/CD11b$^+$/F4-80$^+$ cell subsets were evaluated by flow cytometry of CD45$^+$ liver mononucleated cells isolated at different time points. The percent values refers to the number of cells gated as CD11b$^+$. The data were from 4-6 animals per group.

Figure 2. CX_3CR1-positive monocyte-derived cells associated with steatohepatitis show feature of monocyte-derived dendritic cells.
Mice were fed a control or a methionine-choline deficient (MCD) diet for 8-weeks and liver CD45$^+$ mononucleated cells were analyzed by flow cytometry.
(Panel A) F4-80$^+$/CX_3CR1^+ cells were characterized for the relative distribution of inflammatory monocyte markers CD11b and Ly6C as well as for the expression of dendritic cell markers CD11c and MHCII. Grey lines refers to isotypic controls. One experiment representative of three. (Panel B) Distribution of Ly6C expression among hepatic F4-80$^+$/CX_3CR1^+ cells. One experiment representative of three. (Panel C) Liver CD45$^+$ mononucleated cells were evaluated for the relative prevalence of CD11c^{high}/MHCII$^+$ dendritic cells. The percent values refers to the number of cells gated as CD45$^+$. Quantitative data represent 4 animals per group.

Figure 3. Dentritic cells expansion during the evolution of steatohepatitis involved a pool of CX_3CR1-positive cells with features of monocyte-derived dendritic cells.
Mice were fed a control or a methionine-choline deficient (MCD) diet for 8-weeks and liver CD45$^+$ mononucleated cells were analyzed by flow cytometry.
(Panel A) CD11c^{high}/MHCII$^+$ hepatic dendritic cells were characterized for the expression of inflammatory monocyte markers F4-80 and Ly6C and for that of CX_3CR1. The percent values refers to the number of cells gated as CD11c^{high}/MHCII$^+$. Quantitative evaluation were from 4 animals per group. (Panel B) Distribution of CX3CR1^{high} expressing cells among CD11c^{high}/F4-80$^+$ and CD11c^{low}/F4-80$^+$ cells obtained from the livers of 8 week MCD-fed mice. The percent values refers to the number of cells gated. One experiment representative of three.

Figure 4. The progression of steatohepatitis is characterized by the increase in CX_3CR1-positive monocyte-derived dendritic cells producing TNF-α.
Mice were fed a control or a methionine-choline deficient (MCD) diet for up to 8-weeks.
(Panels A & B) Hepatic mRNA expression and circulating levels of TNF-α were evaluated in control and MCD-fed mice. 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. Data were from 6-8 animals per group. (Panel C) TNF-α expression by CD11c^{high}/F4-80$^+$ and CD11c^{low}/F4-80$^+$ cells was evaluated by flow cytometry along with (Panels D & E) the differential TNF-α expression by F4-80$^+$ positive or genitive for CX_3CR1 and the proportion of TNF-α-producing CX_3CR1^+ cells in control and NASH livers. The percent values refers to the number of cells gated in

the areas indicated by the arrows. Grey lines refers to isotypic controls. Quantitative data were from 3-4 animals per group.

Figure 5. Mice treatment with the H_2S donor NaHS reduces hepatic CX3CL1 expression and CX_3CR1-positive monocyte-derived dendritic cells associated with the progression of steatohepatitis.

Mice were fed a methionine-choline deficient (MCD) diet for 8 weeks. NaHS (1mg/kg body wt) was administered to MCD-fed mice starting from the fourth week of treatment.

(Panel A) The hepatic expression of CX_3CL1 and CX_3CR1 was evaluated by RT-PCR in mRNA extracted from control or MCD-fed mice with or without NaHS supplementation. The values are expressed as fold increase over control values after normalization to the β-actin gene. Data are from 6-9 animals per group; boxes include the values within 25^{th} and 75^{th} percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10^{th}-90^{th} percentile) comprise the eighty percent of the values.

(Panel B) The effect of NaHS supplementation on CX_3CR1 expression by F4-80^+ cells and $CD11c^{high}$ cells was evaluated by flow cytometry. Isotypic controls are shown as dotted lines. (Panel C) Changes in the distribution of CX_3CR1^{high} moDCs following NaHS supplementation of MCD-fed mice. The percent values refers to the number of cells gated as F4-80^+/$CD11c^{high}$. Data are from 3-4 animals per group.

Figure 6. Mice treatment with the H_2S donor NaHS reduces hepatic TNF-α production and improves hepatic injury during the progression of steatohepatitis.

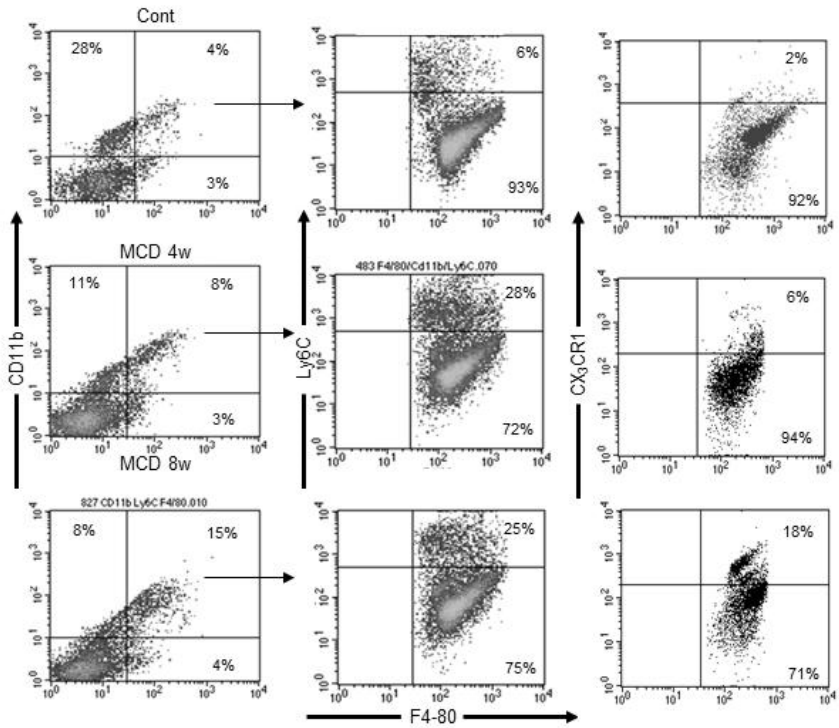
Mice were fed a methionine-choline deficient (MCD) diet for 8 weeks. NaHS (1mg/kg body wt) was administered to MCD-fed mice starting from the fourth week of treatment.

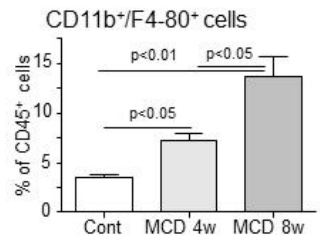
(Panel A) TNF-α expression and the relative prevalence of liver F4-80^+/TNF-α^+ cells was evaluated by flow cytometry. Data are from 3-4 animals per group. Isotypic controls are shown as dotted lines. (Panel B) Hepatic TNF-α mRNA and circulating TNF-α levels were evaluated in MCD-fed mice with or without NaHS supplementation. Liver TNF-α mRNA levels were measured by RT-PCR and expressed as fold increase over control values after normalization to the β-actin gene. Circulating TNF-α levels were determined by ELISA in the same animals. Data are from 6-9 animals per group; boxes include the values within 25^{th} and 75^{th} percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10^{th}-90^{th} percentile) comprise the eighty percent of the values.

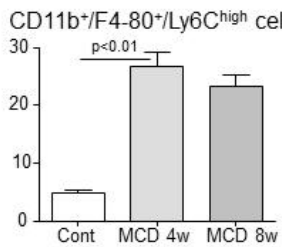
(Panels C & D) Liver histology was evaluated in hematoxilin/eosin stained sections from control or MCD-fed animals (magnification 200x). Necro-inflammatory foci and apoptotic cells were counted as reported in [17]. (Panel E) Liver damage was assessed by circulating alanine aminotransferase (ALT) release.

Summary statement

The data presented indicate that monocyte-derived dendritic cells (moDCs) sustain hepatic inflammation during the progression of experimental nonalcoholic steatohepatitis (NASH).


Cont
28%
4%
3%
6%
93%
2%
92%
MCD 4w
11%
8%
3%
28%
72%
6%
94%
MCD 8w
8%
15%
4%
25%
75%
18%
71%
CD11b
Ly6C
CX3CR1
F4-80


CD11b+/F4-80+ cells
% of CD45+ cells
p<0.01
p<0.05
p<0.05
Cont
MCD 4w
MCD 8w


CD11b+/F4-80+/Ly6Chigh cells
p<0.01
Cont
MCD 4w
MCD 8w

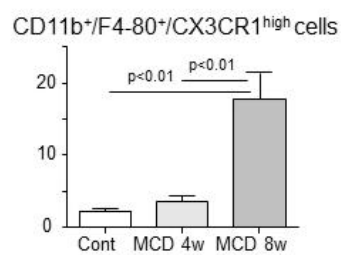

CD11b+/F4-80+/CX3CR1high cells
p<0.01
p<0.01
Cont
MCD 4w
MCD 8w

Figure 1

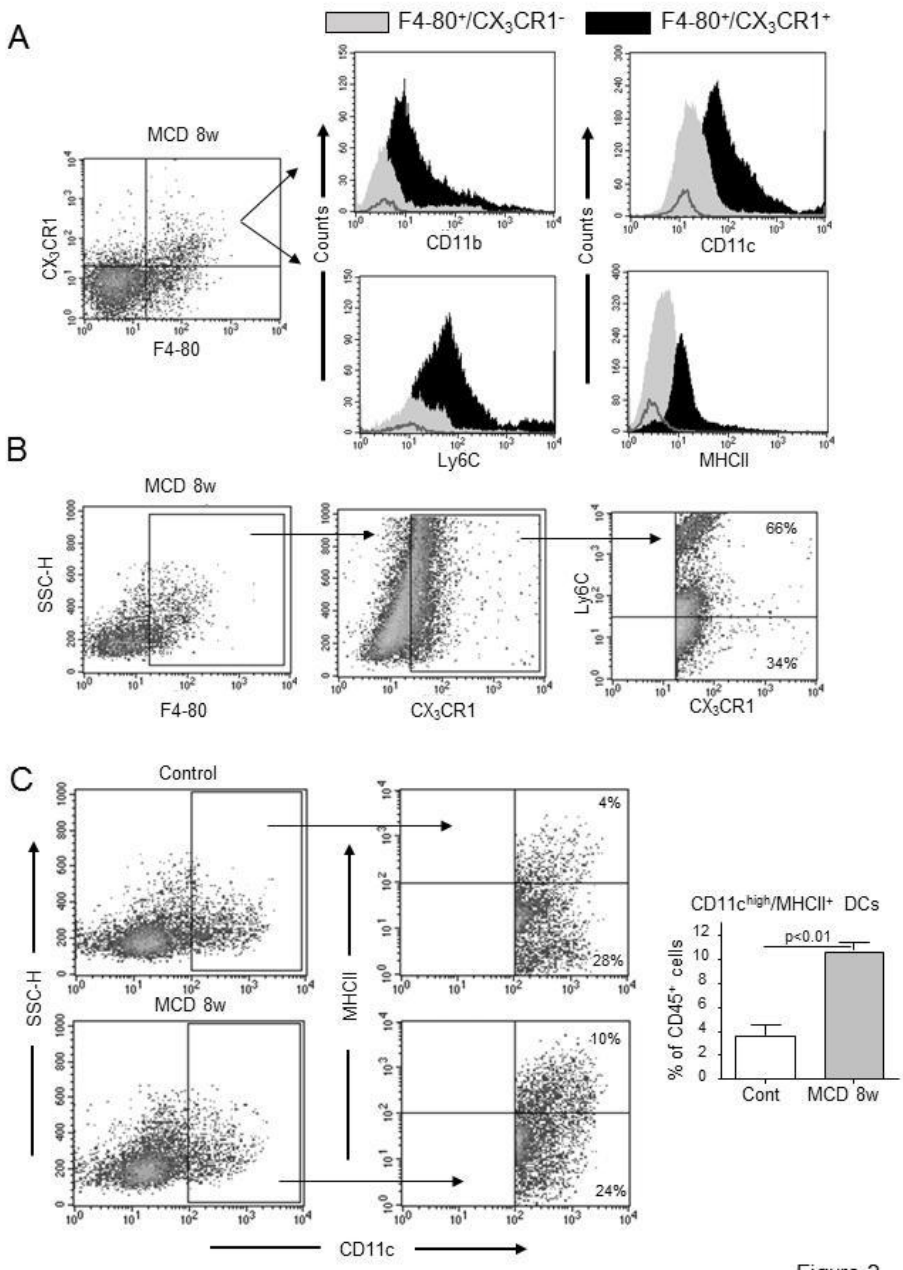

A
F4-80+/CX3CR1-
F4-80+/CX3CR1+
MCD 8w
CX3CR1
F4-80
Counts
CD11b
CD11c
Ly6C
MHCII
B
MCD 8w
SSC-H
F4-80
CX3CR1
Ly6C
66%
34%
C
Control
MCD 8w
SSC-H
MHCII
CD11c
4%
28%
10%
24%
CD11chigh/MHCII+ DCs
p<0.01
% of CD45+ cells
Cont
MCD 8w

Figure 2

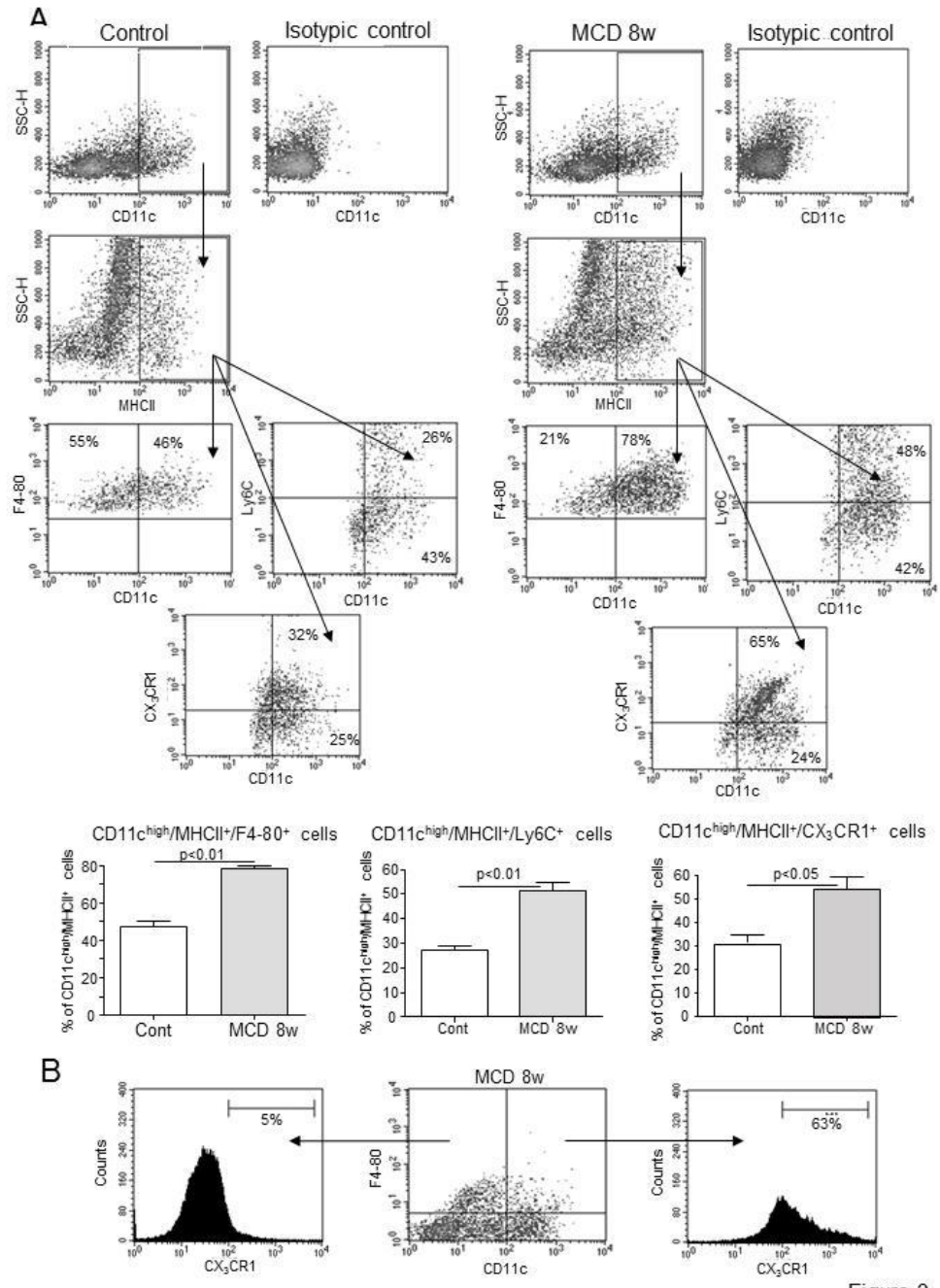

A
Control
Isotypic control
MCD 8w
Isotypic control
SSC-H
CD11c
MHCII
F4-80
Ly6C
CX3CR1
55%
46%
26%
43%
32%
25%
21%
78%
48%
42%
65%
24%
CD11c^high/MHCII+/F4-80+ cells
CD11c^high/MHCII+/Ly6C+ cells
CD11c^high/MHCII+/CX3CR1+ cells
p<0.01
p<0.01
p<0.05
% of CD11c^high/MHCII+ cells
Cont
MCD 8w
B
MCD 8w
5%
63%
Counts
F4-80
CD11c
CX3CR1

Figure 3

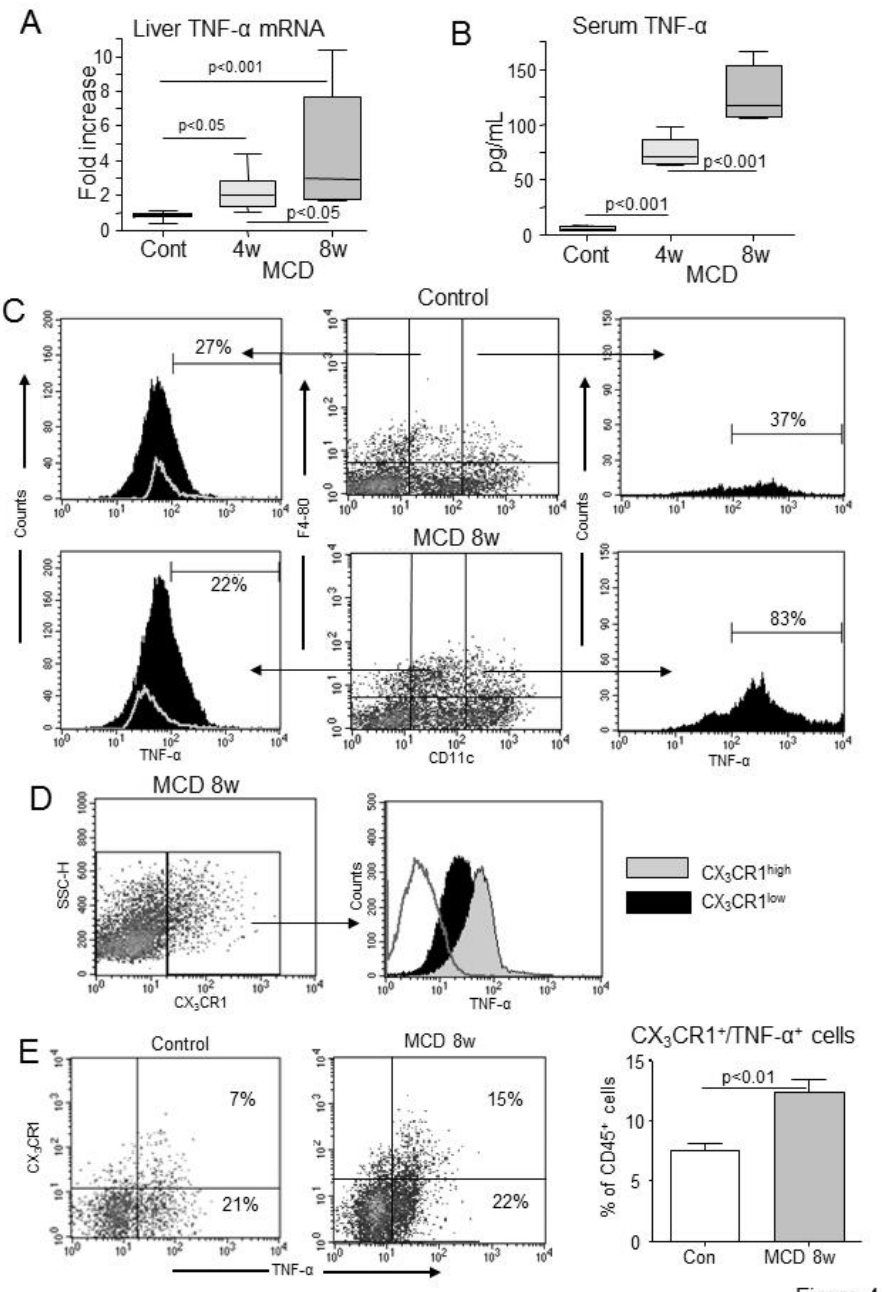

A
Liver TNF-α mRNA
Fold increase
p<0.001
p<0.05
p<0.05
Cont
4w
8w
MCD
B
Serum TNF-α
pg/mL
p<0.001
p<0.001
Cont
4w
8w
MCD
C
Control
27%
37%
Counts
F4-80
MCD 8w
22%
83%
TNF-α
CD11c
TNF-α
D
MCD 8w
SSC-H
CX3CR1
Counts
TNF-α
CX3CR1high
CX3CR1low
E
Control
MCD 8w
CX3CR1
7%
21%
15%
22%
TNF-α
CX3CR1+/TNF-α+ cells
% of CD45+ cells
p<0.01
Con
MCD 8w

Figure 4

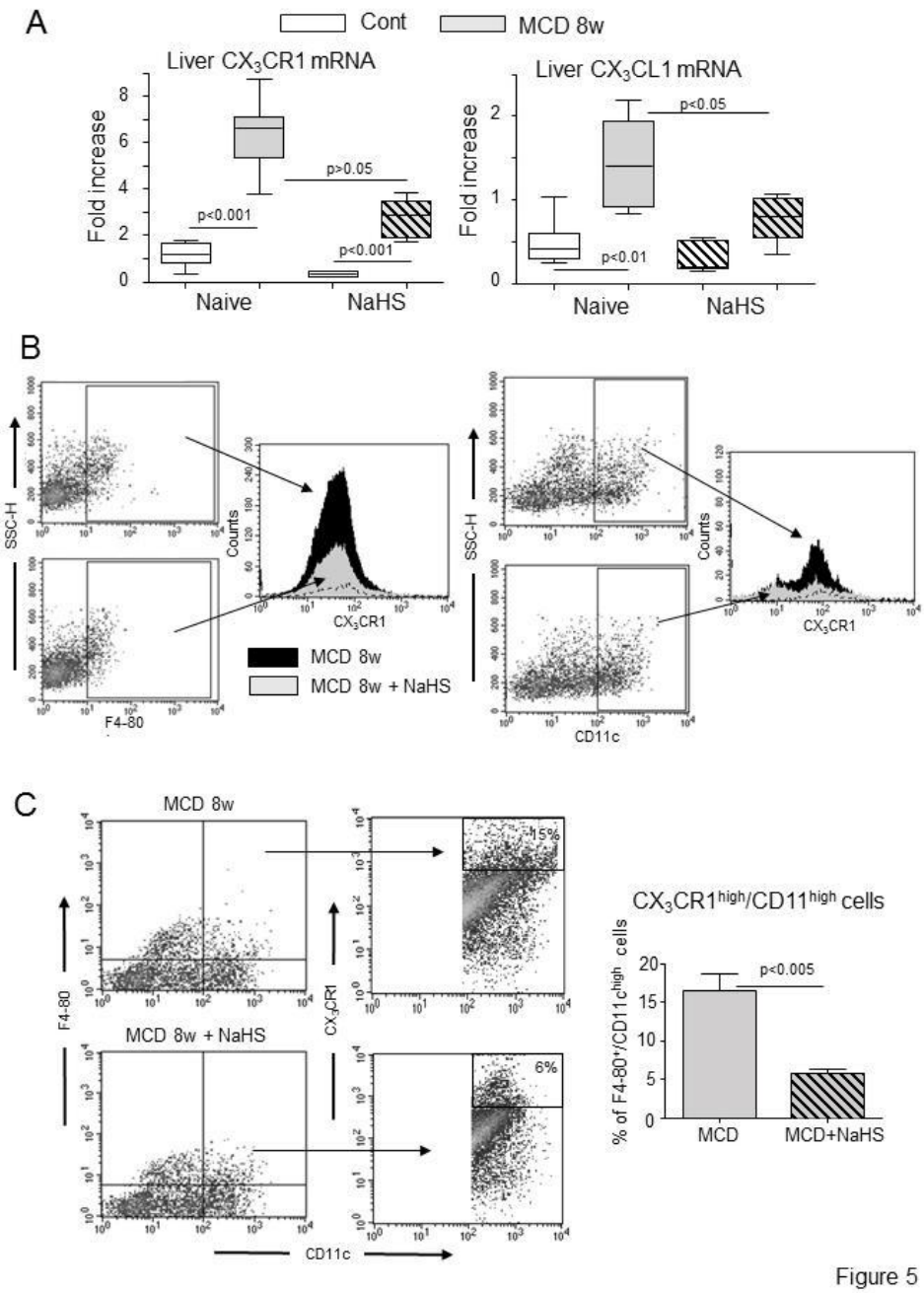

A
Cont
MCD 8w
Liver CX3CR1 mRNA
Fold increase
p<0.001
p>0.05
p<0.001
Naive
NaHS
Liver CX3CL1 mRNA
p<0.05
p<0.01
B
SSC-H
F4-80
Counts
CX3CR1
MCD 8w
MCD 8w + NaHS
CD11c
C
MCD 8w
MCD 8w + NaHS
15%
6%
CX3CR1high/CD11high cells
% of F4-80+/CD11chigh cells
p<0.005
MCD
MCD+NaHS

Figure 5

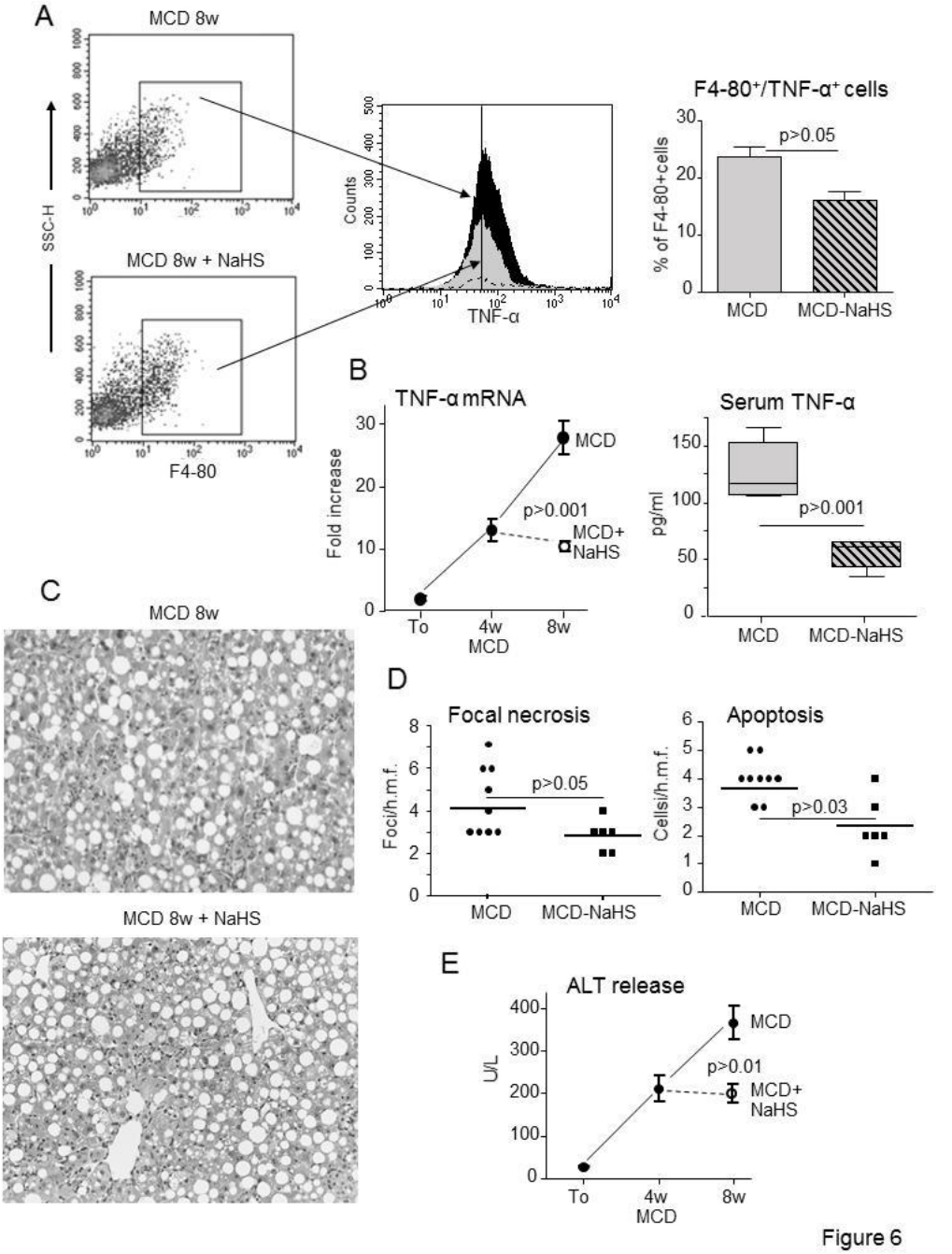

A
MCD 8w
MCD 8w + NaHS
SSC-H
F4-80
Counts
TNF-α
F4-80+/TNF-α+ cells
% of F4-80+cells
p>0.05
MCD
MCD-NaHS
B
TNF-α mRNA
Fold increase
MCD
p>0.001
MCD+ NaHS
To
4w MCD
8w
Serum TNF-α
pg/ml
p>0.001
MCD
MCD-NaHS
C
MCD 8w
MCD 8w + NaHS
D
Focal necrosis
Foci/h.m.f.
p>0.05
MCD
MCD-NaHS
Apoptosis
Cells/h.m.f.
p>0.03
MCD
MCD-NaHS
E
ALT release
U/L
MCD
p>0.01
MCD+ NaHS
To
4w MCD
8w

Figure 6