Fat-laden macrophages modulate lobular inflammation in nonalcoholic steatohepatitis (NASH).

Fat-laden macrophages modulate lobular inflammation in nonalcoholic steatohepatitis (NASH)

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Highlights

- Liver macrophages have an important role in promoting hepatic inflammation in nonalcoholic steatohepatitis (NASH)
- In both mice and human NASH liver macrophages are enlarged and contain lipid vesicles
- Despite inflammatory features, enlarged macrophages in NASH show an increased production of anti-inflammatory mediators
- Enlarged macrophage accumulation in NASH may influence hepatic inflammatory responses

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Abstract

Nonalcoholic steatohepatitis (NASH) is characterized by extensive hepatic monocyte infiltration and monocyte-derived macrophages have an important role in regulating the disease evolution. However, little is known about the functional changes occurring in liver macrophages during NASH progression. In this study, we investigated phenotypic and functional modifications of hepatic macrophages in experimental NASH induced by feeding C57BL/6 mice with a methionine–choline deficient (MCD) diet up to 8 weeks.

In mice with steatohepatitis liver F4/80-positive macrophages increased in parallel with the disease progression and formed small clusters of enlarged and vacuolated cells. At immunofluorescence these cells contained lipid vesicles positive for the apoptotic cell marker Annexin V suggesting the phagocytosis of apoptotic bodies derived from dead fat-laden hepatocytes. Flow cytometry revealed that these enlarged macrophages expressed inflammatory monocyte (CD11b, Ly6C, TNF-α)

markers. However, as compared to regular size macrophages the enlarged sub-set was characterized by an enhanced production of arginase-1 and of the anti-inflammatory mediators IL-10 and annexin A1. Similar vacuolated macrophages producing annexin A1 were also evident in liver biopsies of NASH patients. In mice with NASH, the accumulation of enlarged F4/80^{+} cells paralleled with a decline in the expression of the macrophage M1 activation markers iNOS, IL-12 and CXCL10, while the levels of M2 polarization markers arginase-1 and MGL-1 were unchanged. Interestingly, the lowering of IL-12 expression mainly involved the macrophage sub-set with regular size.

We conclude that during the progression of NASH fat accumulation within liver macrophages promotes the production of anti-inflammatory mediators that influence hepatic inflammatory responses.

Abbreviations

- NAFLD, nonalcoholic fatty liver disease;
- NASH, nonalcoholic steatohepatitis;
- MCD, methionine–choline deficient diet;
- (iNOS), inducible NO synthase;
- (AnxA1), annexin A1

Keywords

- Nonalcoholic fatty liver disease;
- Macrophages;
- Annexin A1;
- Liver inflammation

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1. Introduction

Growing evidence indicates that macrophages are important players in the evolution of hepatic inflammation in NASH. At the onset of the disease, Kupffer cell activation significantly contributes to the production of pro-inflammatory mediators and, by releasing chemokines such as CCL1, CCL2, and CCL5, they stimulate liver infiltration by circulating Ly6C^{high} monocytes (Tosello-Trampont et al., 2012 and Leroux et al., 2012). In turn, these latter rapidly differentiate to M1 polarized macrophages further contributing in expanding inflammatory responses (Maina et al., 2012). Moreover, macrophage interaction with CD4^{+} helper T-lymphocytes and NKT cells has an important role in sustaining lobular inflammation during the disease progression (Sutti et al., 2014 and Wehr et al., 2013). Accordingly, Kupffer cell depletion at the onset of NASH or the interference with monocyte recruitment through CCL2/CCR2 signaling prevents hepatic injury and inflammation in experimental models of NASH (Baeck et al., 2012 and Miura et al., 2012). What is less clear is how liver macrophages behave during the disease progression particularly in relation with the development of fibrosis (Tacke and Zimmermann, 2014). 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 death hepatocytes forming crown-like aggregates similar to those present in the inflamed visceral adipose tissue of obese patients (Rensen et al., 2009, Caballero et al., 2012 and Ioannou et al., 2013). Furthermore, these macrophages appear enlarged and contained lipid vesicles and cholesterol crystals resembling foam cells of atherosclerotic plaques (Ioannou et al., 2013). Interestingly, clusters of foamy macrophages are also evident in several mice models of

experimental NASH in association with lobular inflammation and hepatic fibrosis (Itoh et al., 2013 and Ioannou et al., 2015).

As recent evidence indicates that lipid accumulation in macrophages of either the adipose tissue or the liver promotes pro-inflammatory responses and primes these cells to lymphocyte recruitment (Leroux et al., 2012 and Prieur et al., 2011), we have investigated the phenotype and the possible role of foamy macrophages in modulating lobular inflammation during to the evolution of steatohepatitis to fibrosis.

2. Material and methods

2.1. Animals 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). The experimental protocols were approved by the University Commission for Animal Care and by the Italian Ministry of Health according to the current law for the use of laboratory animals.

2.2. 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 IL-12 was evaluated by a commercial ELISA kit (R&D Systems; Abingdon, UK).

2.3. Histology and immunohistochemistry

Liver biopsies from NASH patients were available from the Pathology Unit of the Ospedale Maggiore della Carità of Novara. Liver macrophages were identified in formalin-fixed sections using either anti-mouse F4/80 or anti-human CD68 antibodies (eBioscience, San Diego CA, USA) in combination with peroxidase-linked goat anti-rat IgG or horse-radish peroxidase polymer kit (Biocare Medical, Concord, CA, USA). 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-labeled annexin 5 (Roche Diagnostics, Penzberg, Germany) and Texas Red-labeled goat anti-rat IgG antibodies (Sigma, Milan, Italy).

2.4. mRNA extraction and real time PCR

Liver RNA was retro-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy). RT-PCR was performed in a Techne TC-312 thermacycler (TecneInc, Burlington NJ, USA) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNF-α, IL-1β, IL-12p40, iNOS, arginase-1, MGL-1, and beta-actin (Applied Biosystems Italia, Monza, Italy). All samples were run in duplicate and the relative gene expression was calculated as $2^{-\Delta Ct}$ and expressed as fold increase over control samples.

2.5. 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. (2003) 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).

2.6. Isolation of intrahepatic mononucleated cell 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 Laboratories Ltd. Burlington, Canada) as described in Crispe (1997). 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, MHCII, (eBiosciences, San Diego, CA, USA), F4/80 (Invitrogen, Abingdon, UK) and analyzed with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Intracellular staining for TNF-α, IL-12 and IL-10 was performed using specific fluorochrome-conjugated antibodies supplied by (eBiosciences, San Diego, CA, USA). AnxA1- and arginase-1-producing cells were detected using polyclonal rabbit antisera against, respectively AnxA1 (Millipore, Temecula, CA, USA) and arginase-1 (Genetex, San Antonio, TX, USA) in combination with phycoerythrin-conjugated anti-rabbit IgG (Sigma-Aldrich, Milan, Italy).

2.7. 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 test.

3. Results

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 (Supplementary 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 5 (Fig. 1), suggesting the phagocytosis of apoptotic bodies originating from dying fat-laden hepatocytes. 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 increased in the livers 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).

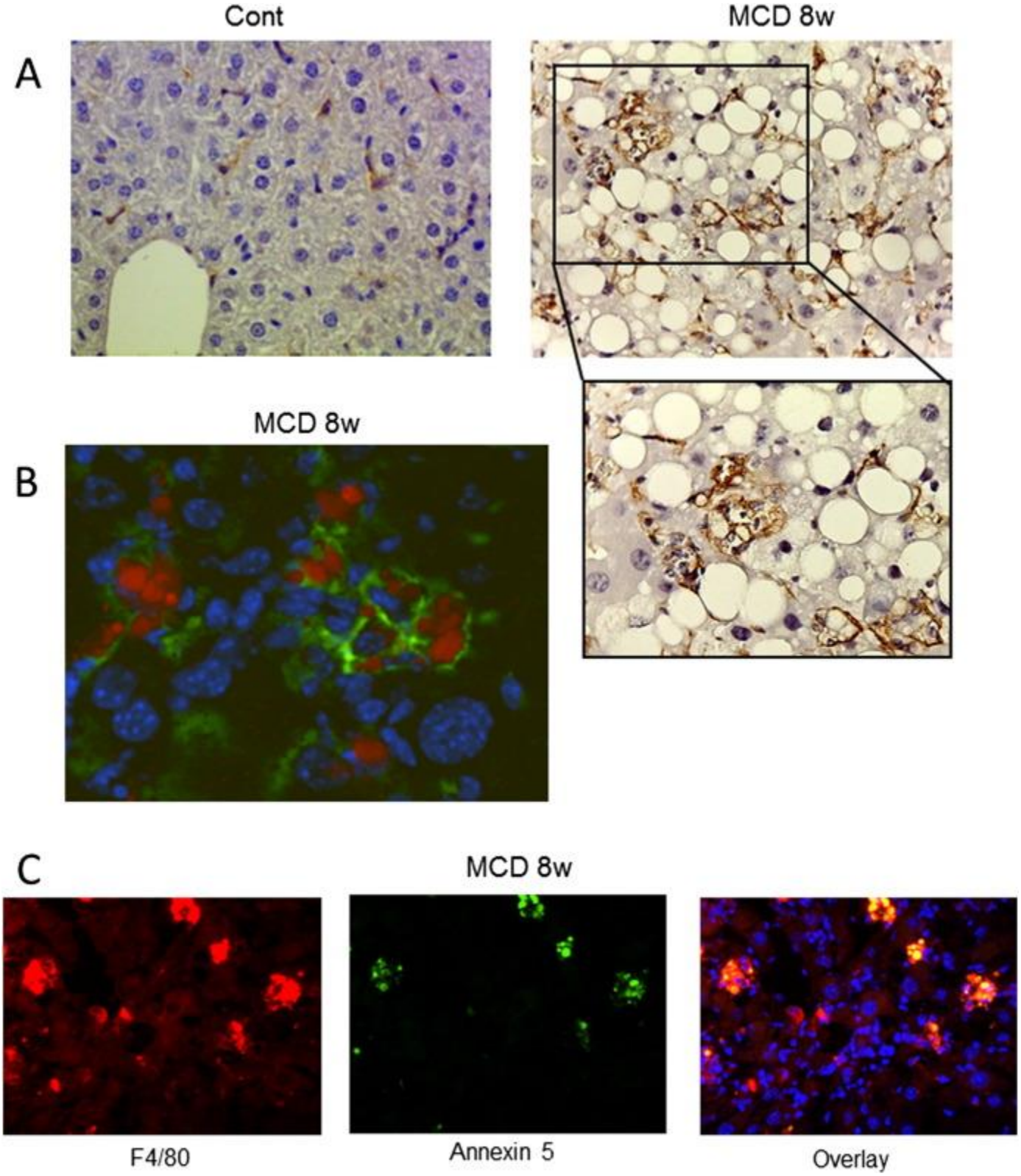

Fig. 1.

Morphological changes in liver macrophages during the evolution of steatohepatitis.

Mice were fed methionine–choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. (Panel A) Hepatic macrophages were evidenced by immunohistochemical staining with anti-F4/80 antibodies (magnification 40 ×). (Panel B) Double staining of frozen liver sections with the lipid dye Oil Red O (red) and anti-F4/80 antibody (green

immunofluorescence; magnification 40 ×). (Panel C) Co-localization of macrophages stained with Texas Red anti-F4/80 antibodies (red) and fluorescein-labeled annexin 5 (green) in frozen sections from NASH livers. Cell nuclei were courter-stained with DAPI. Images are representative of 3–4 distinct samples.

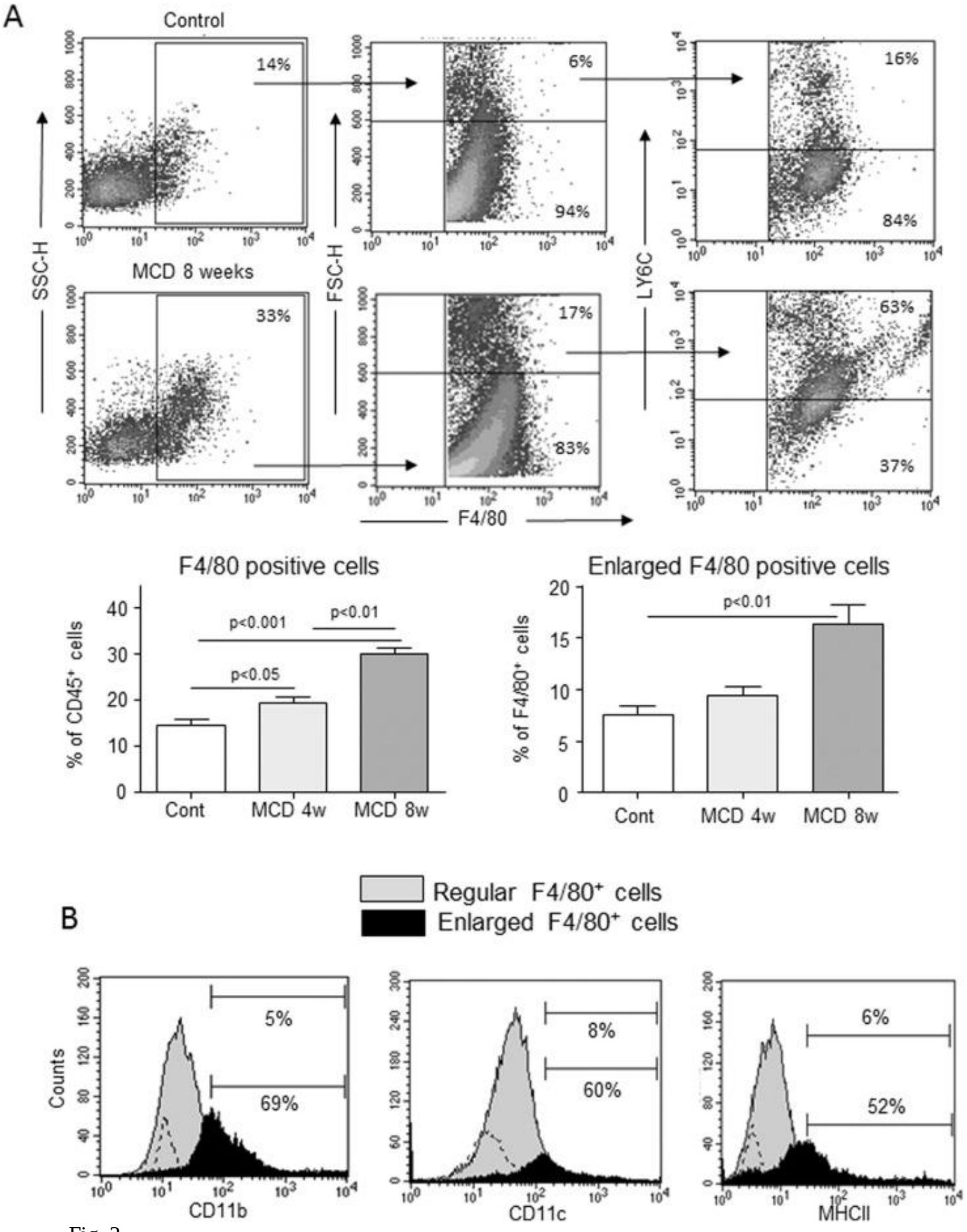

Fig. 2.

Flow cytometry analysis of hepatic macrophages during the evolution of steatohepatitis.

$CD45^+$ mononucleated cells were isolated from the livers of mice fed methionine–choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. (Panel A) $F4/80^+$ macrophages were analyzed for cell volume (forward scatter; FSC-H) and the monocyte marker Ly6C distribution. The percent values refer to the number of cells gated as $F4/80^+$. The data were from 3–4 animals per group. (Panel 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. One experiment representative of three.

Previous studies have shown that lipid-laden macrophages in human NASH had pro-inflammatory features and stained positive for myeloperoxidase and TNF-α (Rensen et al., 2009). We observed that enlarged $F4/80^+$ cells not only express more TNF-α but also had a higher production of interleukin-12 (IL-12), a marker of M1 activation (Supplementary Fig. 2). 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 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 declined thereafter (Supplementary Fig. 3). In line with these findings, macrophages isolated from the livers 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 phase of the disease (Fig. 3). 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. 3). Interestingly, the lowering of IL-12 expression mainly involved the macrophage sub-set with regular size (Fig. 3). On the other hand, macrophage expression of the M2 polarization markers arginase-1 and galactose-type C-type lectin-1 (MGL-1/CD301) was not affected in advanced NASH (Supplementary Fig. 4). It is noteworthy, that the up-regulation in macrophage arginase-1 that characterized steatohepatitis prevalently involved enlarged cells (Supplementary Fig. 4).

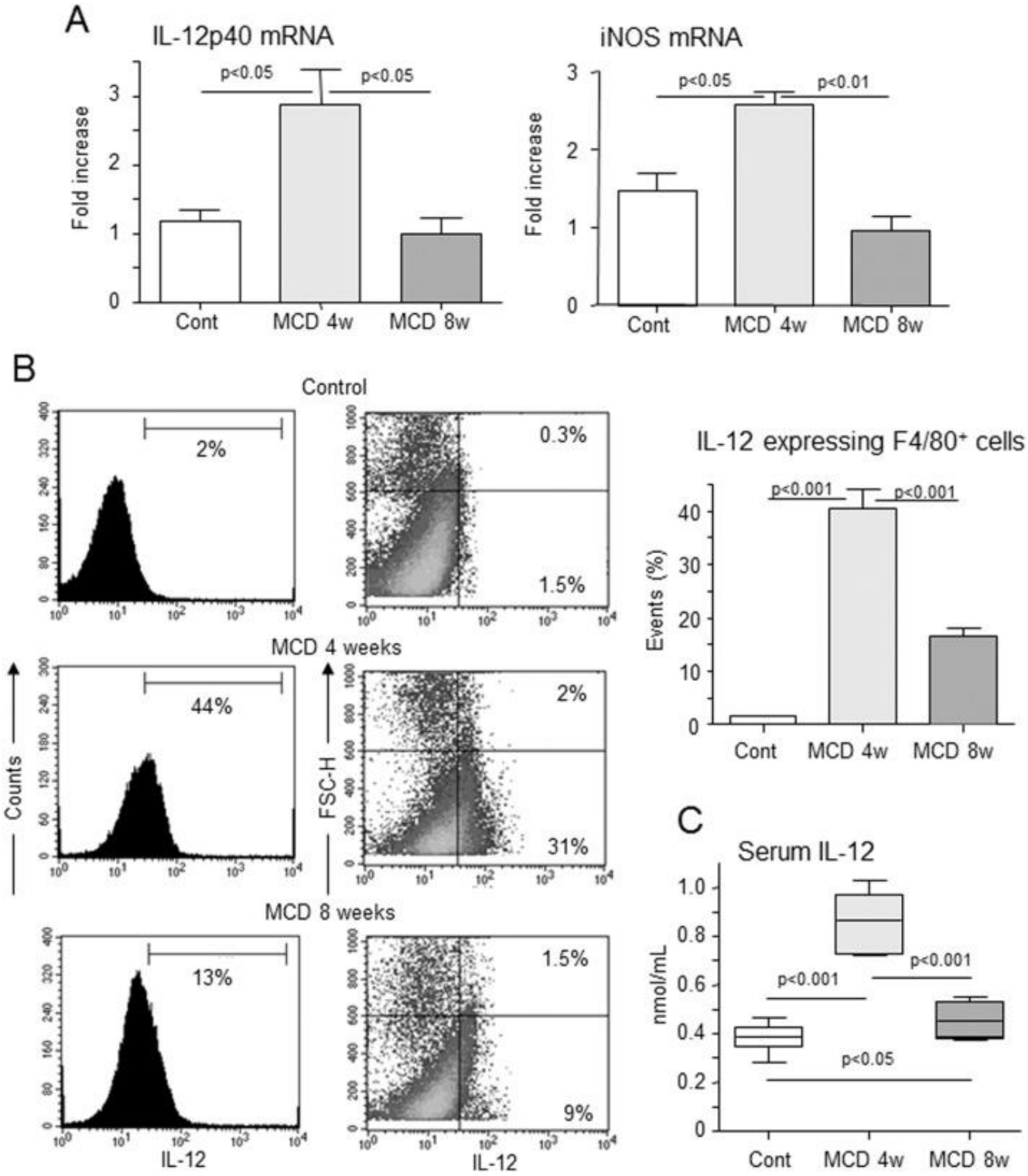

Fig. 3.

The evolution of steatohepatitis is associated with a down-modulation in the M1 activation of liver macrophages.

Mice were fed methionine–choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. (Panels 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 to the β-actin gene. The

data are from 4 animals per group. (Panel B) intrahepatic F4/80^{+} macrophages were analyzed by flow cytometry for intracellular IL-12 expression and IL-12 distribution in relation to cell volume (forward scatter; FSC-H). The values refer to the percent of cells gated as F4/80^{+} and represent 3–4 animals per group. (Panel C) Circulating IL-12 levels were determined control and MCD fed mice by immunoenzymatic assay. The data are from 5–6 animals per group; boxes include the values within 25th and 75th percentiles, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentiles) comprise the eighty percent of the values.

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 (Wan et al., 2014, Moschen et al., 2012 and Locatelli et al., 2014). 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. 4). Interestingly, the expression of both these anti-inflammatory mediators was 3–7 folds higher in the enlarged F4/80^{+} cell sub-set (Fig. 4). This suggested that AnxA1 and IL-10 released by enlarged lipid-laden macrophages can down-regulate M1-polarized responses.

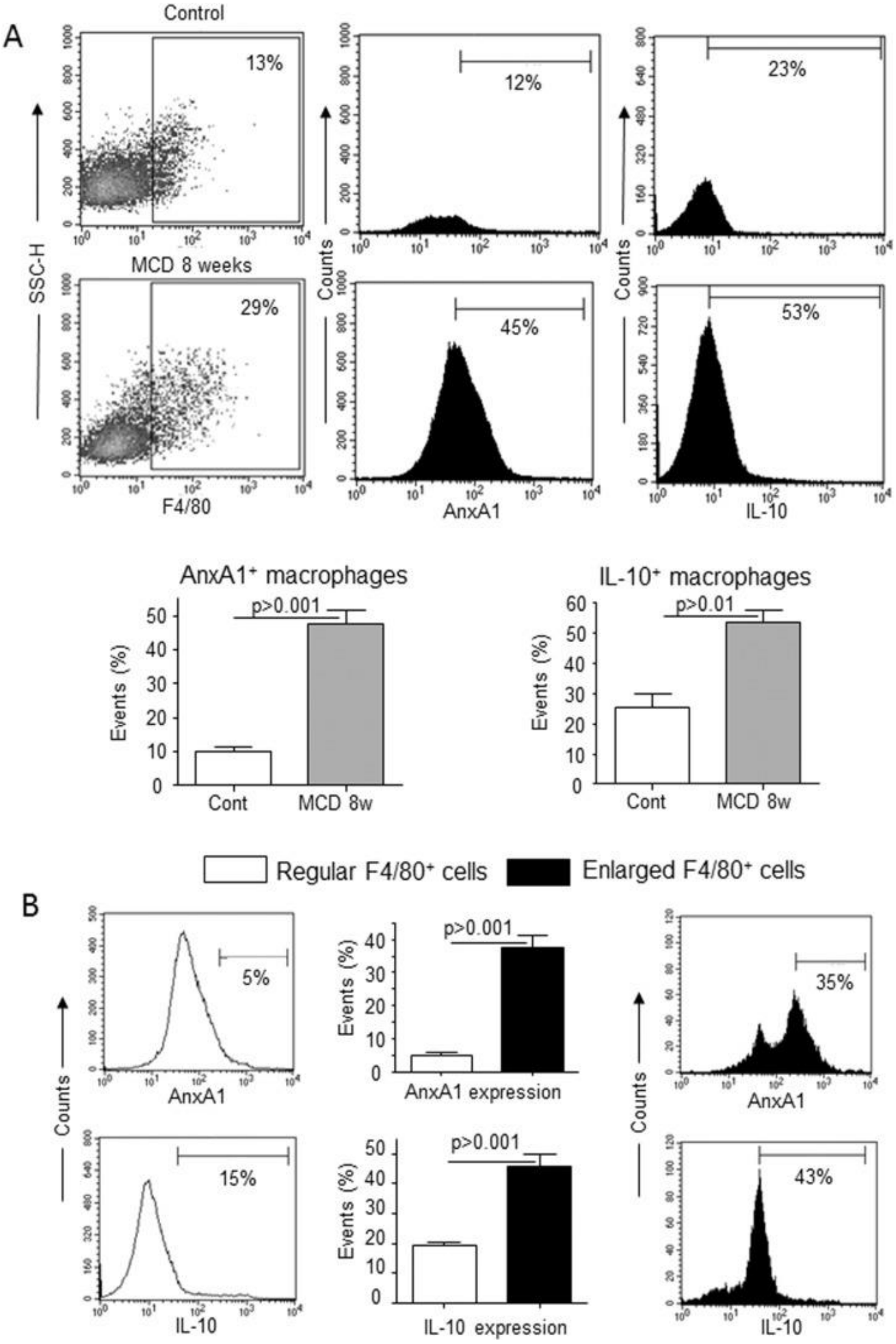
A
Control
MCD 8 weeks
SSC-H
F4/80
13%
29%
12%
45%
23%
53%
Counts
AnxA1
IL-10
AnxA1⁺ macrophages
IL-10⁺ macrophages
p>0.001
p>0.01
Events (%)
Cont
MCD 8w
Regular F4/80⁺ cells
Enlarged F4/80⁺ cells
B
5%
15%
35%
43%
AnxA1 expression
IL-10 expression

Fig. 4.

Enlarged macrophages associated with the advanced phases of steatohepatitis show increased production of anti-inflammatory mediators interleukin-10 (IL-10) and annexin A1 (AnxA1).

$CD45^+$ mononucleated cells were isolated from the livers of mice fed methionine–choline supplemented (Cont) or deficient (MCD) diets over an 8-week time period. (Panel A) Dotted lines refer to isotypic controls. $F4/80^+$ macrophages were analyzed for the production of IL-10 and AnxA1. (Panel B) Expression of IL-10 and AnxA1 among regular or enlarged $F4/80^+$ cells. The percent values refer to the number of cells gated as $F4/80^+$. The data were from 3–4 animals per group.

Figure options

According to previous studies (Rensen et al., 2009, Caballero et al., 2012, Ioannou et al., 2013 and Itoh et al., 2013), 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. 5). These cells were also selectively stained with anti-AnxA1 antibodies (Fig. 5), confirming that also in human NASH lipid-laden macrophages contributed to AnxA1 production.

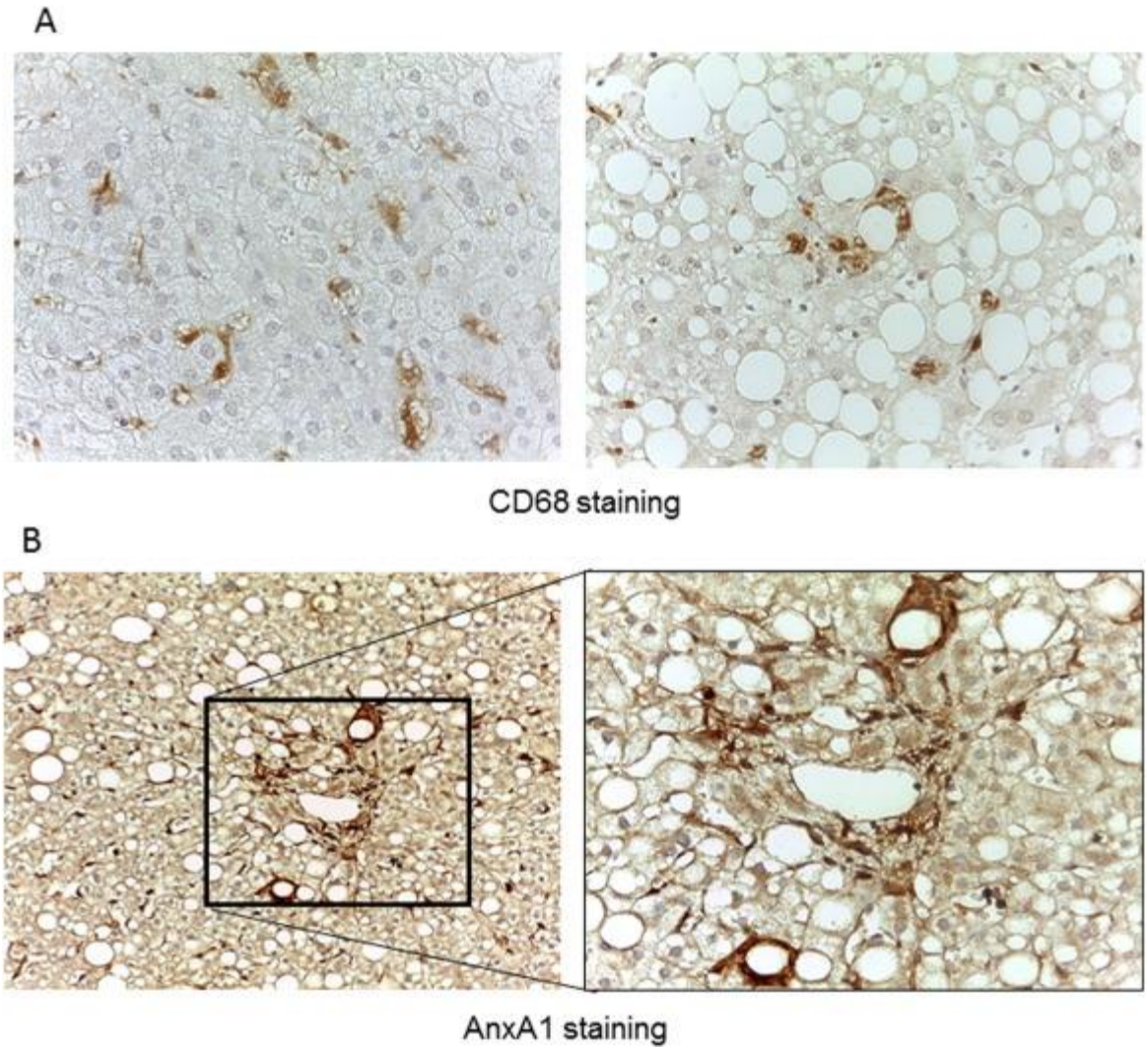

Fig. 5.

Immunohistochemical detection of enlarged-foamy macrophages in human NASH.

Formalin-fixed sections of liver biopsies from NASH patients were immunostained with anti-human CD68 (Panel A) or anti-AnxA1 (Panel B) antibodies in combination with horseradish peroxidase polymer kit (magnification 20 ×).

4. Discussion

Small macrophage clusters around lipid vesicles or surrounding fat-containing hepatocytes, generally referred as lipogranulomas, are common in adult human NASH (Yeh and Brunt, 2014) as well as in many experimental models of the disease (Itoh et al., 2013). The macrophages in these clusters are enlarged and have a foamy appearance due to the accumulation of cytoplasmic lipid droplets and cholesterol crystals (Ioannou et al., 2013 and Ioannou et al., 2015). These histological features are reminiscent of crown-like structures detectable in the adipose tissue of obese subjects that are characterized by macrophages forming aggregates around dead adipocytes and scavenging cell debris and residual lipids (Shapiro et al., 2013). Although fat-laden macrophages in the crown-like structures have been associated with the evolution of adipose tissue inflammation (McNelis and Olefsky, 2014), the actual significance of similar cells in NASH is less well defined.

The recruitment of circulating monocyte through CCL2/CCR2 signaling is considered the main responsible for the expansion of liver macrophage pool in NASH (Tacke and Zimmermann, 2014 and Zimmermann et al., 2012). Circulating monocytes are currently differentiated in two subsets. The first include inflammatory monocytes characterized as $Ly6C^{high}/CCR2^{+}/CX3CR1^{-}$ in mice or $CD14^{+}/CD16^{-}$ in humans that migrate to tissues in early phase of the response to injury producing pro-inflammatory mediators (Murray and Wynn, 2011). The second population, defined as $Ly6C^{-}/CCR2^{-}/CX3CR1^{+}$ in mice or $CD14^{-}/CD16^{+}$ in humans has less characterized functions and it is though to contribute to tissue healing (Zimmermann et al., 2012 and Murray and Wynn, 2011). Immunohistochemical studies in human liver biopsies have shown that fat-laden macrophages in NASH express leucocyte activation marker CD11b and CD11c along with TNF-α and myeloperoxidase suggesting pro-inflammatory capability (Rensen et al., 2009 and Itoh et al., 2013). On the same line, Ioannou and co-workers have shown that the presence of cholesterol crystals drives the activation of NLPR3 inflammasome in crown-like macrophages associated with experimental NASH (Ioannou et al., 2015). Our present data add on these findings by showing that beside an increased expression of CD11b, 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 (Zigmond et al., 2014) 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. AnxA1 is a 37 kDa calcium-phospholipid-binding protein that is produced by myeloid cells in response to glucocorticoids (Perretti and D'Acquisto, 2009). By interacting with its receptor formyl peptide receptor 2/Lipoxin A_4 receptor (FPR2/ALX) AnxA1 reduces phagocyte recruitment/activation, down-regulates the production of pro-inflammatory mediators and promotes IL-10 production (Perretti and D'Acquisto, 2009 and Cooray et al., 2013). In inflamed tissues AnxA1 is mainly produced by infiltrating macrophages following the phagocytosis of apoptotic bodies and plays an important role in driving

the termination of acute inflammation and in promoting tissue healing (Ariel and Timor, 2013 and Ortega-Gómez et al., 2013).

Hepatocyte apoptosis is a common in NASH as a result of lipotoxicity and endoplasmic reticulum stress (Leamy et al., 2013 and Gentile et al., 2011). We have observed that annexin 5 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. Furthermore, intracellular lipid accumulation in foam cells of atherosclerotic plaques has also been shown to promote macrophage functional changes by stimulating liver X receptors (LXPs) and peroxisome proliferator activated receptors (PPARs) (Leitinger and Schulman, 2013). Thus, it is possible that as a result of these events fat-laden macrophages accumulating in NASH livers might acquire an enhanced capacity of producing anti-inflammatory mediators in spite of having a phenotype similar to that of $Ly6C^{high}$ macrophages present in the early phases of acute injury (Cooray et al., 2013).

As a result of AnxA1 and IL-10 up-regulation in enlarged lipid-laden macrophages we have observed a down-modulation of liver M1-polarized responses that mainly involves the macrophage sub-set with regular size, suggesting that AnxA1 and IL-10 act in an autocrine/paracrine loop affecting pro-inflammatory responses by hepatic macrophages. Accordingly, we have recently reported that the induction of NASH in AnxA1-deficient mice is characterized by enhanced lobular inflammation due to increased macrophage recruitment and the exacerbation of the M1 phenotype (Locatelli et al., 2014). Furthermore, the addition of recombinant AnxA1 to hepatic macrophages isolated from NASH livers promotes IL-10 production and the down-modulation of M1 responses (Locatelli et al., 2014). In the same vein, Wan and co-workers (Wan et al., 2014) have shown that in mice with NAFLD IL-10 secretion induces the apoptosis of M1-activated macrophages hampering the severity of lobular inflammation.

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.

Disclosures

The authors do not have conflict of interests to disclose.

Acknowledgments

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References

Ariel, A., Timor, O., 2013. Hanging in the balance: endogenous anti-inflammatory mechanisms in tissue repair and fibrosis. J. Pathol. 229, 250–263.

Baeck,C.,Wehr,A.,Karlmark,K.R.,etal.,2012. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. Gut 61, 416–426.

Caballero, T., Gila, A., Sánchez-Salgado, G., et al., 2012. Histological and immunohisto-chemical assessment of liver biopsies in morbidly obese patients. Histol. Histopathol. 27, 459–466.

Cooray, S.N., Gobbetti, T., Montero-Melendez, T., et al., 2013. Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines pro-resolving functional responses. Proc.Natl.Acad.Sci.U.S.A.110,18232–18237.

Crispe, N.I., 1997. Isolation of mouse intrahepatic lymphocytes. Current Protocols in Immunology (3.21.1-321.8).

Froh, M., Konno, A., Thurman, R.G., 2003. Isolation of Kupffer cells. Curr. Protoc. Toxicol. (14.4.1-14.4.12). http://dx.doi.org/10.1002/0471140856.tx1404s14.

Gentile, C.L., Frye, M., Pagliasotti, M.J., 2011. Endoplasmic reticulum stress and unfolded protein response in nonalcoholic fatty liver disease. Antioxid. Redox Signal. 15, 505–521.

Ioannou, G.N., Haigh, W.G., Thorning, D., et al., 2013. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. J. Lipid Res. 54, 1326–1334.

Ioannou, G.N., Van Rooyen, D.M., Savard, C., et al., 2015. Cholesterol-lowering drugs cause dissolution of cholesterol crystals and disperse Kupffer cell crown-like structures during resolution of NASH. J. Lipid Res. 56, 277–285.

Itoh, M., Kato, H., Suganami, T., et al., 2013. Hepatic crown-like structure: a unique histological feature in non-alcoholic steatohepatitis in mice and humans. PLoS One 8, e82163. http://dx.doi.org/10.1371/journal.pone.0082163.

Leamy, A.K., Egnatchik, R.A., Young, J.D., 2013.Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. Prog. Lipid Res. 52, 165–174.

Leitinger, N., Schulman, I.G., 2013. Phenotypic polarization of macrophages in atheroscle-rosis. Arterioscler. Thromb. Vasc. Biol. 33, 1120–1126.

Leroux, A., Ferrere, G., Godie, V., et al., 2012.Toxic lipids stored by Kupffer cells correlates with their pro-inflammatory phenotype at an early stage of steatohepatitis. J. Hepatol. 57, 141–149.

Locatelli, I., Sutti, S., Jindal, A., et al., 2014. Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. Hepatology 60, 531–544.

Maina, V., Sutti, S., Locatelli, I., et al., 2012. Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin. Sci. (Lond.) 122,545–553.

McNelis, J.C., Olefsky, J.M., 2014. Macrophages, immunity, and metabolic disease. Immunity 41 (1), 36–48 (Jul 17).

Miura, K., Yang, L., van Rooijen, N., et al., 2012. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G1310 –G1321.

Moschen, A.R., Wieser, V., Tilg, H., 2012. Adiponectin: key player in the adipose tissue-liver crosstalk. Curr. Med. Chem. 19, 5467–5473.

Murray, P.J., Wynn, T.A., 2011. Protective and pathogenetic functions of macrophagesubsets. Nat. Rev. Immunol. 11, 723–737.

Ortega-Gómez, A., Perretti, M., Soehnlein, O., 2013.Resolution of inflammation: anintegrated view.EMBOMol.Med.5,661–674.

Perretti, M., D'Acquisto, F., 2009. AnnexinA1andglucocorticoidsaseffectorsoftheresolution of inflammation. Nat.Rev.Immunol.9,62–70.

Prieur, X., Mok, C.Y., Velagapudi, V.R., et al., 2011. Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. Diabetes 60, 797–809.

Rensen, S.S., Slaats, Y., Nijhuis, J., et al., 2009. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. Am. J. Pathol. 175, 1473–1482.

Shapiro, H., Pecht, T., Shaco-Levy, R., et al., 2013. Adipose tissue foam cells are present in human obesity. J. Clin. Endocrinol. Metab. 98, 1173–1181.

Sutti, S., Jindal, A., Locatelli, I., et al., 2014. Adaptive immune responses triggered by oxidative

stress contribute to hepatic inflammation in NASH. Hepatology 59, 886–897.

Tacke, F., Zimmermann, H.W., 2014. Macrophage heterogeneity in liver injury and fibrosis. J. Hepatol. 60, 1090–1096.

Tosello-Trampont, A.C., Landes, S.G., Nguyen, V., et al., 2012. Kupffer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-α production. J. Biol. Chem. 287, 40161–40172.

Wan, J., Benkdane, M., Teixeira-Clerc, F., et al., 2014. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology 59, 130–142.

Wehr, A., Baeck, C., Heymann, F., et al., 2013. Chemokine receptor CXCR6-dependent hepatic NK T Cell accumulation promotes inflammation and liver fibrosis. J. Immunol. 190, 5226–5236.

Yeh, M.M., Brunt, E.M., 2014. Pathological features of fatty liver disease. Gastroenterology 147, 754–764.

Zigmond, E., Samia-Grinberg, S., Pasmanik-Chor, M., et al., 2014. Infiltrating monocyte-derived macrophages and resident Kupffer cells display different ontogeny and functions in acute liver injury. J. Immunol. 193, 344–353.

Zimmermann, H.W., Trautwein, C., Tacke, F., 2012. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. Front. Physiol. 3. http://dx.doi.org/10.3389/fphys.2012.00056.

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