



**HAL**  
open science

## The deficit in folate and vitamin B12 triggers liver macrovesicular steatosis and inflammation in rats with dextran sodium sulfate-induced colitis

Zeinab Atieh Harb, Valérie Deckert, Aude Marchal Bressenot, Christo Christov, Rosa-Maria Gueant-Rodriguez, Jérémie Raso, Jean Marc Alberto, Jean-Paul Pais de Barros, Remy Umoret, Laurent Peyrin Biroulet, et al.

### ► To cite this version:

Zeinab Atieh Harb, Valérie Deckert, Aude Marchal Bressenot, Christo Christov, Rosa-Maria Gueant-Rodriguez, et al.. The deficit in folate and vitamin B12 triggers liver macrovesicular steatosis and inflammation in rats with dextran sodium sulfate-induced colitis. *Journal of Nutritional Biochemistry*, 2020, pp.108415. 10.1016/j.jnutbio.2020.108415 . hal-02873803

HAL Id: hal-02873803

<https://hal.univ-lorraine.fr/hal-02873803>

Submitted on 18 Jul 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

**The deficit in folate and vitamin B12 triggers liver macrovesicular steatosis and inflammation in rats with dextran sodium sulfate-induced colitis**

Zeinab Harb<sup>1</sup>, Valérie Deckert<sup>2</sup>, Aude Marchal Bressenot<sup>1,3\*</sup>, Christo Christov<sup>1\*</sup>, Rosa-Maria Guéant-Rodriguez<sup>1,4</sup>, Jérémie Raso<sup>1</sup>, Jean Marc Alberto<sup>1</sup>, Jean-Paul Pais de Barros<sup>2</sup>, Remy Umoret<sup>1</sup>, Laurent Peyrin Biroulet<sup>1,5</sup>, Laurent Lagrost<sup>2</sup>, Jean-Pierre Bronowicki<sup>1,5</sup>, Jean-Louis Guéant<sup>1,4,5</sup>

<sup>1</sup>INSERM U1256, Nutrition Génétique et Exposition aux Risques Environnementaux, Medical Faculty, University of Lorraine and Regional University Hospital Center of Nancy, Vandœuvre les Nancy, France.

<sup>2</sup>INSERM UMR1231 Lipides, Nutrition, Cancer, University of Bourgogne Franche-Comté, LipSTIC LabEx, Dijon, France

<sup>3</sup>Division of Anatomic-Pathology, Robert Debré University Hospital, Reims,

<sup>4</sup>Biochemical and Molecular biology lab, Regional University Hospital Center of Nancy, Vandoeuvre les Nancy, France.

<sup>5</sup>Division of Hepatogastroenterology, Regional University Hospital Center of Nancy, Vandoeuvre les Nancy, France

\*These authors contributed equally

**Correspondence:**

Pr Jean-Louis Guéant, MD, DSc, AGAF, Inserm UMRS 1256 N-GERE (Nutrition-Genetics-Environmental Risks), University of Lorraine, BP 184, 54511 Vandoeuvre les Nancy: Jean-Louis.Gueant@univ-lorraine.fr, Tel: +33 6 75 65 29 85, Fax: +33 3 72 74 61 31

Authors have no conflict of interest to declare.

**Funding:**

This work was supported by a doctoral contract MESRI, by FHU ARRIMAGE and by the French PIA project « Lorraine Université d'Excellence », reference ANR-15-IDEX-04-LUE

**Abbreviations**

ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase;  $\alpha$  SMA, alpha smooth muscle actin; CD, choline deficient; CDHF, choline deficient high fat; CD14, cluster of differentiation 14; CCL2, chemokine ligand 2; CCR2, c-c chemokine receptor type 2; DSS, dextran sodium sulfate ; DAI, disease activity index; HFD, high fat diet; IBD, inflammatory bowel disease; IHC, immunohistochemistry; IKK, inhibitory *kappa* B kinases; I $\kappa$ B $\alpha$ , inhibitory *kappa* B alpha; IL-1 $\beta$ , interleukin 1-beta; IRAK 4, interleukin 1 associated kinase 4; IRF 3 interferon regulatory factor 3; LPS, lipopolysaccharide; MDD, methyl donor deficient; MCP-1, monocyte chemoattractant protein 1; MMP2, matrix metalloproteinase 2; MD2, myeloid differentiation factor 2 ; Myd88 myeloid differentiation primary response 88; MCD, methionine choline deficient; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease, NF $\kappa$ B, nuclear factor *kappa* B; pIKK  $\alpha/\beta$ , phospho inhibitory *kappa* B kinases alpha/beta; pI $\kappa$ B  $\alpha$ , phospho inhibitory *kappa* B alpha; RIPA, radioimmunoprecipitation assay buffer; RNA, ribonucleic acid; TNF- $\alpha$ , tumor necrosis factor alpha; TLR4, toll-like receptor 4; TRAF 6, tnf receptor associated factor; TGF  $\beta$ , transforming growth factor beta; VEGF, vascular endothelial growth factor.

**Abstract**

The risks of non-alcoholic steatohepatitis (NASH) and deficiency in vitamin B12 and folate (methyl donor deficiency, MDD) are increased in inflammatory bowel disease (IBD). We investigated the influence of MDD on NASH in rats with DSS induced colitis. Two-month-old male Wistar rats were subjected to MDD diet and/or ingestion of DSS and compared to control animals. We studied steatosis, inflammation, fibrosis, plasma levels of metabolic markers, cytokines and LPS, and inflammatory pathways in liver. MDD triggered a severe macrovesicular steatosis with inflammation in DSS animals that was not observed in animals subjected to DSS or MDD only. The macrovesicular steatosis was closely correlated to folate, vitamin B12, homocysteine plasma level and liver SAM/SAH ratio. Liver inflammation was evidenced by activation of NF $\kappa$ B pathway and nuclear translocation of NF $\kappa$ B phospho-p65. MDD worsened the increase of IL-1 $\beta$  and abolished the increase of IL10 produced by DSS colitis. It increased monocyte chemoattractant protein 1 (MCP-1). MDD triggers liver macrovesicular steatosis and inflammation through imbalanced expression of IL-1  $\beta$  vs. IL10 and increase of MCP-1, in DSS colitis. Our results suggest to evaluating whether IBD patients with MDD and increase of MCP-1 are at higher risk of NASH.

**Key words:**

Non-alcoholic steatohepatitis, inflammatory bowel disease, vitamin B12, folate, monocyte chemoattractant protein 1 (MCP-1)

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) covers a spectrum of conditions ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) with pathological features including steatosis, hepatocyte injury, inflammation and various degrees of fibrosis [1]. NAFLD is one of the main manifestations in obese patients with metabolic syndrome [2,3]. It is rare and more severe in lean subjects [1-3]. Inflammatory bowel diseases (IBD) encompass Crohn's disease (CD) and ulcerative colitis (UC). NASH is more frequent in IBD patients than in the general non-obese population [4, 5]. This suggests that the systemic inflammation triggered by IBD could increase the risk of NASH in a subset of patients through risk factors and mechanisms that need to be dissected.

Different diets have been used in animal models for the study of NASH, including methionine/choline deficient (MCD), methyl donor deficient (MDD), choline deficient (CD) and high fat (HF) diets [6]. We showed that pups born from MDD rats subjected to MDD diet during foetal life and lactation developed NAFLD through impaired beta-oxidation of fatty acids [7]. Furthermore, the subsequent exposure of these pups to a high energy diet at adulthood triggered fibrosis and inflammation in the liver [8]. The MDD diet during adulthood aggravated the severity of IBD in rats exposed to dextran sodium sulfate (DSS) [9, 10].

Increased intestinal permeability of the so called "leaky gut syndrome" is one of the mechanisms of inflammatory bowel disease that produces systemic manifestations, including liver diseases. The induction of intestinal inflammation promotes LPS translocation, hepatic inflammation and fibrogenesis through dysbiosis and activation of TLR4/NF $\kappa$ B signaling pathway, underscoring the pathophysiological role of the gut-liver axis [11, 12, 13, 14, 15]. MDD weakens the intestinal barrier in rats exposed to DSS [16]. MDD affects also fetal programming of digestive cell organization and produce homocysteinylation of cytoskeleton proteins, cellular stress, and inflammation [16, 17, 18].

The deficiency in vitamin B12 and folate (methyl donor deficiency, MDD) is relatively frequent in IBD. In particular, the ileitis of Crohn's disease produces a malabsorption of vitamin B12 that may lead to increased homocysteine [19-21]. The prevalence of B12 deficiency and folate in Crohn's disease has been estimated respectively to 15.6% and 22.2 % in a study performed in Spain [21]. Folate and B12 deficits are also associated to an increased risk of NASH. A recent study of 83 NASH patients showed a correlation between low levels of folate and vitamin B12 and the histological severity of NASH [22]. It may be therefore

hypothesized that folate and B12 deficits may contribute to the increased risk of NASH in IBD.

In this study, we investigated the respective and synergic influence of MDD and DSS colitis on liver macrovesicular steatosis and inflammation in rats. In particular we evaluated whether the aggravation of DSS colitis by MDD could trigger liver inflammation. We evaluated We dissected the specific and synergic effects related to MDD and DSS, with a focus on pro-inflammatory cytokines and TLR4 pathways.

## 2. Materials and methods

### 2.1. Animal Treatments

*In vivo* manipulations were performed as recommended by the National Committee on Ethics Reflectin Experiment (described in the Guide for care and use of laboratory animals, NIH, MD, 1985) and the EU Directive 2010/63/EU for animal experiments. The experiments were performed in two-months old adult male Wistar rats (Janvier Labs, Le Genest-St-Isle, France). The Ethical agreement for the animal experiment was obtained from the French Ministry for Higher Education and Research (registration under APAFIS#1521-2015082611014308). Rats were divided into two distinct groups: a control group receiving a normal diet (maintenance diet A04, Scientific Animal Food and Engineering, France) and a group receiving a deficient diet without methyl donors, vitamin B12 and folate (Special Diet Service, Argenteuil, France) according to Blaise et al [23]. These diets were maintained for 6 months.

### 2.2. Induction of colitis, disease activity index and endoscopy

Colitis was induced by administration of 5% DSS (molecular weight 36 000–50 000, MP Biomedical, Strasbourg, France) dissolved in water for 7 days. Six months after the beginning of the deficiency, male rats were separated into four groups: [1] rats fed with standard diet, not treated with DSS and used as controls (noted C, n=6); [2] rats fed with deficient diet and not treated with DSS (noted MDD, n=6); [3] Rats fed with standard diet, treated with DSS and used as controls (noted C/DSS, n=6) and [4] Rats fed with deficient diet with DSS (noted MDD/DSS, n=6). Daily weight, physical condition, stool consistency, water/food consumption and the presence of gross and occult blood in excreta and at the anus were determined. The score of colitis disease activity index (DAI) was also calculated as described, using weight loss, stool consistency and blood in the stool [24]. The distal 3 cm of the colon and the rectum were examined using a rigid Storz Hopkins II miniendoscope (Storz, Tuttlingen, Germany) coupled to a basic Coloview system (xenon 175 light source and Endovision SLB Telecam; Storz) as described previously [10]. Endoscopic score was calculated by a blinded gastroenterologist as previously described [25].

### 2.3. Blood samples, colon tissue and hepatic tissue

## Harb, methyl donor deficit and NASH in colitis

The rats were sacrificed by decapitation six days after beginning DSS treatment. Blood was collected at sacrifice into a tube containing heparin. Plasma was collected and stored at  $-80^{\circ}\text{C}$  and the colon and the liver were quickly removed, gently washed in PBS1X (2.7 mmol/L KCl, 140 mmol/L NaCl, 6.8 mmol/L  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 mmol/L  $\text{KH}_2\text{PO}_4$ , pH 7.4), frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until protein and RNA analysis. The tissue samples for histological studies were recovered by dissection and kept in formalin-fixed paraffine-embedded (FFPE) blocs.

### *2.4. Biochemical analysis*

Plasma concentrations of vitamin B12 and folate were determined by radio-dilution isotope assay (simulTRAC-SNB, MP Biomedicals, Eschwege, Germany). Homocysteine, methylmalonic acid, succinic acid, SAM and SAH concentrations were measured in plasma by High Performance Liquid Chromatography (Waters, St Quentin, France) coupled to mass spectrometry (Api 4000 Qtrap Applied Biosystems, Courtaboeuf, France), as described previously [26, 27]. Lipids, glycemia, ASAT, ALAT and other routine biochemical parameters were determined in plasma as described previously [7]. We used a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (RECYTMAG-65k Merck Millipore) to measure the plasma concentrations of the following cytokines and chemokines: EGF, Eotaxin/CCL11, Fractalkine, G-CSF, GM-CSF, GRO/KC, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-18, IP-10, Leptin, LIX, MCP-1, MIP-1 $\alpha$ , MIP-2, RANTES, TNF- $\alpha$ , VEGF.

### *2.5. Histological and immunohistochemical analysis (IHC) in the liver*

To detect extracellular matrix proteins, the slides were stained with 0.1% Sirius Red. Images (x 200, 413 x 311  $\mu\text{m}$ ) from Sirius Red colored slides were acquired on a BX63 microscope equipped with a DP70 color camera (both from Olympus, France). Each image was centered by a central lobular vein (terminal hepatic venule) thus covering converging acinar zones 3 where fatty accumulation begins and is most severe later in disease [28]. Segmentation of lipid accumulation was carried out using 3 image processing software packages: KS400 3.0 (Zeiss, France), Ilastik, version 1.3.2 developed by the European Molecular Biology Laboratory, Heidelberg and FIJI [29, 30]. Identification of fat vacuoles was preceded by segmentation of all hepatocytes present and performed by applying arithmetic logical



operators to the binarized and original image. The central hepatic veins were also excluded in a similar way to calculate hepatic parenchyma area. Two parameters were thereby calculated: (i) percentage of hepatic parenchyma area occupied by steatosis and (ii) percentage of macrovesicular steatosis of all steatosis present. Immunohistochemical (IHC) analysis to test for nuclear translocation of nuclear factor *kappa* B (NF $\kappa$ B) was performed as described [9]. IHC analysis was also performed to check for the chemokine, monocyte chemoattractant protein -1(MCP-1), in the liver and in the intestine as described [7,9]

### 2.6. *Endotoxin assay*

LPS concentration in plasma was determined by direct quantitation of 3 $\beta$ -hydroxymyristate, a major component of the Lipid A moiety, as previously described [31]. We used quantitative lipopolysaccharide analysis using HPLC/MS/MS and its combination with the limulus amebocyte lysate assay [31].

### 2.7. *Protein extraction and Western Blotting*

The extraction of total proteins from the liver was completed by lysing homogenized tissue in RIPA buffer (sodium phosphate anhydrous dibasic, potassium dihydrogen phosphate, 150 mM NaCl, 1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Protease Inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) after washing twice with ice-cold 1X PBS) and then quantified using the bicinchoninic acid assay method (BCA) (Thermo-Fisher Scientific, Illkirch, France). The Western blots were performed as described previously [10, 26].

### 2.8. *Statistics*

Statistical analyses of biochemical markers were performed using Statview V.5 software for Windows (SAS Institute, Berkley, California, USA). Densitometry analyses of western blots were reported as mean  $\pm$  SEM. Raw data were compared using Kruskal-Wallis test and the one-way analysis of variance (ANOVA) with Fisher's test. The Spearman *Rho*-test was used to study correlation between variables. The level of significance was set at *p*-value < 0.05. Results were indicated by asterisks in figures (\**P*-value < 0.05; \*\**P*-value < 0.01 and \*\*\**P*-value < 0.001).

### 3. Results

#### 3.1. Efficacy of the methyl donor deficient diet

As expected, the MDD diet decreased dramatically the plasma concentrations of vitamin B12 and folate (Table 1). The B12/folate deficit was reflected by an increase of plasma concentration of homocysteine and a decrease of SAM/SAH ratio in MDD animals compared with controls (Table 1). The treatment of rats with DSS in drinking water did not influence folate, vitamin B12 and homocysteine plasma levels (Table 1). It decreased dramatically the SAM/SAH ratio in MDD rats. It induced also a severe colitis in MDD rats illustrated by the highest DAI and endoscopic scores reported in MDD/DSS group compared to other groups controls (Fig. 1A and B), as previously described [9, 10, 32, 33, 34].

#### 3.2. The colitis of MDD/DSS rats triggers a liver macrovesicular steatosis

Liver histological quantification of steatosis in a defined microscopic area showed no significant macrovesicular steatosis in the control and MDD deficient rats and a mild macrovesicular steatosis in rats treated only by DSS. In contrast, we observed a dramatic increase of macrovesicular steatosis in the MDD deficient rats treated with DSS (Fig. 2A and B). The percentage of macrovesicular steatosis was positively correlated with homocysteine ( $Rho=0.616$ ,  $P=0.0022$ ) and negatively correlated with folate ( $Rho=-0.498$ ,  $P=0,0184$ ), vitamin B12 ( $Rho=-0.612$ ,  $P=0.0025$ ) ( $Rho=-0,583$ ,  $P=0.0044$ ) and SAM/SAH ratio ( $Rho=-0.583$ ,  $P=0.0044$ ) among groups. The SAH/SAM ratio was the single determinant of macrovesicular steatosis, in multiple correlation analysis ( $P<0.001$ ). We observed a very mild fibrosis in the histological examination of liver tissue, in deprived rats treated with DSS (Fig. 2A) and an increased expression of the profibrotic marker MMP2 but no change of COL1A1 and  $\alpha$ SMA (Fig. 2C).

#### 3.3. The deficit in folate and vitamin B12 activates the NF $\kappa$ B pathway in the liver of rats with DSS Colitis

We observed an increased nuclear translocation of NF $\kappa$ B p65 only in liver tissues of deficient rats exposed to DSS (Fig. 3A). This was consistent with the higher expression and/or

phosphorylation of proteins involved in the activation pathway of NF $\kappa$ B, in western blot analysis (Fig. 3B). The protein expression of NF $\kappa$ B p65 and its phosphorylated form were markedly increased in MDD/DSS group. Consistently, we observed an increased expression of IKK $\alpha$  and a decreased expression and increased phosphorylation of I $\kappa$ B- $\alpha$  (Fig. 3B).

*3.4. DSS treatment increases the plasma level of IL-1 $\beta$  and IL10 and the deficit in vitamin B12 and folate increases monocyte chemoattractant protein 1 (MCP-1, also named CCL2, c-c chemokine ligand type 2) and impairs the increase of IL10 in DSS treated rats*

We observed an increased plasma level of IL-1 $\beta$  in rats subjected to DSS and MDD diet/DSS and an increase of MCP-1 and VEGF plasma levels in the MDD/DSS treated rats, compared to the other groups (Fig. 4A). Conversely, the increase of plasma level of IL-10 produced by DSS treatment in DSS rats was impaired by the deficit in vitamin B12 and folate in MDD/DSS rats. We observed a significant association between folate and IL10 ( $Rho=0.582$ ,  $P=0.0045$ ) and between homocysteine and IL4 ( $Rho=0.591$ ,  $P=0.0038$ ) among groups.

*3.5. The deficit in vitamin B12 and folate increases IL-1 $\beta$  and MCP-1 expression and impairs the increase of IL10 in DSS treated rats*

The increased plasma level of MCP-1 was consistent with its histological expression in liver tissue. We observed a more pronounced expression of MCP-1 in the liver of MDD/DSS treated rats, compared to other groups (Fig. 4). We observed an increased expression of IL-1 $\beta$  in the liver of MDD/DSS group compared to control and control/DSS animals (Fig. 5A) and an increased expression of MCP-1 and no significant change of TNF $\alpha$  and IL6 in the same group compared to the 3 others (Fig. 5B). The lowest expression of anti-inflammatory IL-10 in the liver was reported in the animals subjected to MDD and DSS (Fig. 5B). A similar result was obtained in the colon. IL-10 expression among groups mirrored the severity of DSS induced colitis (Fig. 1 and Fig. 5C). In contrast, we observed an increased expression of MCP-1 in the colon of C/DSS and MDD/DSS rats, in comparison to the control group (Fig. 5C). IL-1 $\beta$  was gradually increased in the colon of C/DSS, MDD and MDD/DSS groups, compared to the control group (Fig. 5C).

*3.6. LPS/TLR4 pathway in the liver is not activated by colitis in MDD/DSS rats*

The plasma LPS concentration was slightly decreased in C/DSS and MDD groups and was not different in MDD/DSS group, compared to C control animals (Fig. 6A). This observation was consistent with the absence of LPS related-activation of the TLR4 pathway, despite increased expression of CD14, the TLR4 partner needed for LPS binding. We did not observe any increase in the expression of phosphorylated and non-phosphorylated TLR4 and downstream proteins, including IRF 3, TRIF, TRAF 6, IRAK 4 and MYD 88 (Fig. 6B).

#### **4. Discussion**

Steatohepatitis was triggered by the deficit in vitamin B12 and folate in MDD rats with DSS colitis. The animals exposed to DSS and MDD exhibited a macrovesicular steatosis and an activation of pro-inflammatory pathways that was not found in those exposed to MDD diet or DSS only. The dual exposure produced inflammation but no histological fibrosis, despite increased expression of the profibrotic marker MMP2. The macrovesicular steatosis and inflammation in liver from rats with DSS colitis and subjected to MDD was related to altered expression of IL-1 $\beta$ , IL-10, MCP-1 in intestine, blood and liver, as summarized in fig.7.

As expected, MDD produced a more severe colitis in adult rats treated by DSS, compared to the control diet [9, 10, 16]. The severity of lesions was assessed by the recommended routine methods, including analysis of body weight, high-resolution mini-endoscopy and HE staining in 0.5 cm segments [35]. We did not measure the colon length but we did not notice any visible length reduction in this model. MDD alone did not produce macrovesicular steatosis (Fig. 2). This model was therefore adapted to study whether the deficit in folate and vitamin B12 may influence the development of NASH in DSS colitis. We showed histological and molecular hallmarks of NASH only in the MDD animals with DSS colitis. The macrovesicular steatosis and the SAH/SAM ratio were both dramatically increased in these animals, compared to those subjected to either DSS or MDD. The SAH/SAM ratio is a direct consequence of the deficit in folate and vitamin B12 [32, 33] and it was the single metabolic determinant of macrovesicular steatosis, in multiple correlation analysis. The liver inflammation of MDD/DSS rats was evidenced by the activation of NF $\kappa$ B pathway and the translocation of phospho-p65 of NF $\kappa$ B active form into the nucleus (Fig. 3) [34]. The activation of the classical NF $\kappa$ B pathway is usually triggered by pro-inflammatory cytokines,

including IL-1 $\beta$  [34], including in animal models of colitis [36]. Both colitis and MDD deficient diet increased IL-1 $\beta$  but the highest expression was reported in the animals exposed to both conditions. The concentration of IL10 mirrored that of IL-1 $\beta$  in liver, colon and blood of the rats from the 4 experimental groups (Fig. 4 and 5). DSS colitis produced an increase of IL10 that was abolished in MDD animals. The decreased IL10 may explain in part why MDD produced a more severe colitis than normal diet, in animals subjected to DSS intake [9,10]. IL-10 is involved in the pathomechanisms of IBD as illustrated by its association with IBD in GWAS and the enterocolitis observed in IL10 knockout mice [37]. IL10 and IL-1 $\beta$  have opposite effects. IL10 directly inhibits while IL-1 $\beta$  triggers the nuclear localization of the NF- $\kappa$ B p65/p50 heterodimer [38,39]. These opposite effects suggest that the decrease of IL10 amplifies the effects of the increase of IL-1 $\beta$  in the liver and the colon of the animals with DSS colitis, which are deficient in folate and vitamin B12. We concluded therefore that the imbalanced expression of both cytokines could contribute to NASH pathomechanism, at least in our animals with DSS colitis and deficit in vitamin B12 and folate.

Our results are consistent with the association previously observed between IL-1 $\beta$ , MCP-1 and homocysteine [40, 41, 42, 43] and the association between homocysteine and vitamin B12 and folate in IBD patients [19]. The increased level of MCP-1 in MDD/DSS rats could be a cause and consequence of the liver activation of NF $\kappa$ B [39]. The MCP1-CCR2 pathway plays a critical role in hepatic recruitment of pro-inflammatory macrophages that release TNF $\alpha$ , IL-1 $\beta$  and IL-6 [44, 45]. MCP1 and its receptor (CCR2) recruit monocyte subsets in the liver. The macrophages derived from these monocytes activate hepatic myofibroblasts and these activated myofibroblasts release VEGF and matrix metalloproteases such as MMP2 [46]. Experimental studies have pointed MCP-1 as a possible target of NASH. The pharmacological inhibition of MCP-1 diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury produced by MCD diet [47]. In another study, the inhibition of MCP-1 receptor resulted in attenuation of diet induced insulin resistance, adipose tissue inflammation and NASH [48]. The role of the translocation of LPS and subsequent activation of TLR4 pathway has been shown as a prominent pathological mechanisms of NASH in mice exposed to MCD diet [49, 50]. In contrast, we did not find any activation of TLR4 pathway in the liver and no increased LPS serum level in our model of MDD/DSS colitis. In agreement with our data, NASH is observed in a subset of patients with normal serum level of endotoxins [50–54].

In conclusion, the deficit in folate and vitamin B12 triggers NASH through a prominent role of IL-1 $\beta$ , IL10 and MCP-1 in the gut-liver axis of animals with DSS colitis. We found that the deficit in vitamin B12 and folate produced a dramatic macrovesicular steatosis and activation of pro-inflammatory pathways mediated by the imbalanced and mirrored expression of IL-1 $\beta$  and IL10 and the subsequent increase of MCP-1 and VEGF in the DSS-induced colitis of rats. These data suggest investigating further whether IBD patients with deficit in folate and vitamin B12 and systemic increase of MCP-1 are at higher risk for developing NASH.

### References:

- [1] Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010;53:372–84.
- [2] Younossi Z, Tacke F, Arrese M, Sharma BC, Mostafa I, Bugianesi E, et al. Global Perspectives on Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. *Hepatol Baltim Md* 2018.
- [3] Hamaguchi M, Kojima T, Takeda N, Nakagawa T, Taniguchi H, Fujii K, et al. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann Intern Med* 2005;143:722–8.
- [4] McGowan CE, Jones P, Long MD, Barritt SA. Changing shape of disease: Nonalcoholic fatty liver disease in Crohn's disease—A case series and review of the literature: *Inflamm Bowel Dis* 2012;18:49–54.
- [5] Sourianarayanan A, Garg G, Smith TH, Butt MI, McCullough AJ, Shen B. Risk factors of non-alcoholic fatty liver disease in patients with inflammatory bowel disease. *J Crohns Colitis* 2013;7:e279-285.
- [6] Lau JKC, Zhang X, Yu J. Animal models of non-alcoholic fatty liver disease: current perspectives and recent advances: NAFLD animal models. *J Pathol* 2017; 241:36–44.
- [7] Pooya S, Blaise S, Moreno Garcia M, Giudicelli J, Alberto J-M, Guéant-Rodriguez R-M, et al. Methyl donor deficiency impairs fatty acid oxidation through PGC-1 $\alpha$  hypomethylation and decreased ER- $\alpha$ , ERR- $\alpha$ , and HNF-4 $\alpha$  in the rat liver. *J Hepatol* 2012;57:344–51.

- [8] Bison A, Marchal-Bressenot A, Li Z, Elamouri I, Feigerlova E, Peng L, et al. Foetal programming by methyl donor deficiency produces steato-hepatitis in rats exposed to high fat diet. *Sci Rep* 2016; 6:37207.
- [9] Chen M, Peyrin-Biroulet L, George A, Coste F, Bressenot A, Bossenmeyer-Pourie C, et al. Methyl deficient diet aggravates experimental colitis in rats. *J Cell Mol Med* 2011;15:2486–97.
- [10] Melhem H, Hansmannel F, Bressenot A, Battaglia-Hsu S-F, Billioud V, Alberto JM, et al. Methyl-deficient diet promotes colitis and SIRT1-mediated endoplasmic reticulum stress. *Gut* 2016;65:595–606.
- [11] Matsushita N, Osaka T, Haruta I, Ueshiba H, Yanagisawa N, Omori-Miyake M, et al. Effect of Lipopolysaccharide on the Progression of Non-Alcoholic Fatty Liver Disease in High Caloric Diet-Fed Mice. *Scand J Immunol* 2016;83:109–18.
- [12] Gäbele E, Dostert K, Hofmann C, Wiest R, Schölmerich J, Hellerbrand C, et al. DSS induced colitis increases portal LPS levels and enhances hepatic inflammation and fibrogenesis in experimental NASH. *J Hepatol* 2011;55:1391–9.
- [13] Achiwa K, Ishigami M, Ishizu Y, Kuzuya T, Honda T, Hayashi K, et al. DSS colitis promotes tumorigenesis and fibrogenesis in a choline-deficient high-fat diet-induced NASH mouse model. *Biochem Biophys Res Commun* 2016;470:15–21.
- [14] Miura K. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. *World J Gastroenterol* 2014;20:7381.
- [15] Cani PD, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* 2009;15:1546–58.
- [16] Bressenot A, Pooya S, Bossenmeyer-Pourie C, Gauchotte G, Germain A, Chevaux J-B, et al. Methyl donor deficiency affects small-intestinal differentiation and barrier function in rats. *Br J Nutr* 2013;109:667–77.
- [17] Bossenmeyer-Pourie C, Blaise S, Pourié G, Tomasetto C, Audonnet S, Ortiou S, et al. Methyl donor deficiency affects fetal programming of gastric ghrelin cell organization and function in the rat. *Am J Pathol* 2010;176:270–7.
- [18] Bossenmeyer-Pourie C, Pourié G, Koziel V, Helle D, Jeannesson E, Guéant J-L, et al. Early methyl donor deficiency produces severe gastritis in mothers and offspring through N-homocysteinylation of cytoskeleton proteins, cellular stress, and inflammation. *FASEB J Off Publ Fed Am Soc Exp Biol* 2013;27:2185–97.

- [19] Peyrin-Biroulet L, Rodriguez-Guéant R-M, Chamaillard M, Desreumaux P, Xia B, Bronowicki J-P, et al. Vascular and cellular stress in inflammatory bowel disease: revisiting the role of homocysteine. *Am J Gastroenterol* 2007;102:1108–15.
- [20] Oussalah A, Guéant J-L, Peyrin-Biroulet L. Meta-analysis: hyperhomocysteinaemia in inflammatory bowel diseases. *Aliment Pharmacol Ther* 2011;34:1173–84.
- [21] Bermejo F, Algaba A, Guerra I, Chaparro M, De-La-Poza G, Valer P, et al. Should we monitor vitamin B<sub>12</sub> and folate levels in Crohn's disease patients? *Scand J Gastroenterol* 2013;48:1272–7.
- [22] Mahamid M, Mahroum N, Bragazzi NL, Shalaata K, Yavne Y, Adawi M, et al. Folate and B12 Levels Correlate with Histological Severity in NASH Patients. *Nutrients* 2018;10.
- [23] Blaise S, Alberto J-M, Nédélec E, Ayav A, Pourié G, Bronowicki J-P, et al. Mild neonatal hypoxia exacerbates the effects of vitamin-deficient diet on homocysteine metabolism in rats. *Pediatr Res* 2005;57:777–82.
- [24] Murthy SN, Cooper HS, Shim H, Shah RS, Ibrahim SA, Sedergran DJ. Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. *Dig Dis Sci* 1993;38:1722–34.
- [25] Becker C, Fantini MC, Wirtz S, Nikolaev A, Kiesslich R, Lehr HA, et al. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* 2005;54:950–4.
- [26] Battaglia-Hsu S, Akchiche N, Noel N, Alberto J-M, Jeannesson E, Orozco-Barrios CE, et al. Vitamin B12 deficiency reduces proliferation and promotes differentiation of neuroblastoma cells and up-regulates PP2A, proNGF, and TACE. *Proc Natl Acad Sci U S A* 2009;106:21930–5.
- [27] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313–21.
- [28] Sakhuja P. Pathology of alcoholic liver disease, can it be differentiated from nonalcoholic steatohepatitis? *World J Gastroenterol* 2014;20:16474–9.
- [29] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9:676–82.
- [30] Sommer C, Strähle C, Köthe U, Hamprecht FA. Ilastik: Interactive learning and segmentation toolkit. Eighth IEEE International Symposium on Biomedical Imaging (ISBI). Proceedings, 2011, 230-33.



- [31] Pais de Barros J-P, Gautier T, Sali W, Adrie C, Choubley H, Charron E, et al. Quantitative lipopolysaccharide analysis using HPLC/MS/MS and its combination with the limulus amoebocyte lysate assay. *J Lipid Res* 2015;56:1363–9.
- [32] Guéant JL, Elakoum R, Ziegler O, Coelho D, Feigerlova E, Daval JL, Guéant-Rodriguez RM. Nutritional models of foetal programming and nutrigenomic and epigenomic dysregulations of fatty acid metabolism in the liver and heart. *Pflügers Arch.* 2014;466:833-50.
- [33] Guéant JL, Namour F, Guéant-Rodriguez RM, Daval JL. Folate and fetal programming: a play in epigenomics? *Trends Endocrinol Metab.* 2013;24:279-89.
- [34] Viatour P, Merville M-P, Bours V, Chariot A. Phosphorylation of NF- $\kappa$ B and I $\kappa$ B proteins: implications in cancer and inflammation. *Trends Biochem Sci* 2005;30:43–52.
- [35] Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* 2017;12:1295-1309.
- [36] Eichele DD, Kharbanda KK. Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. *World J Gastroenterol* 2017; 23: 6009-6196
- [37] Marlow GJ, van Gent D, Ferguson LR. Why interleukin-10 supplementation does not work in Crohn's disease patients. *World J Gastroenterol* 2013; 19: 3931-3941
- [38] Schottelius AJ, Mayo MW, Sartor RB, Baldwin AS. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem* 1999;274: 31868-31874
- [39] Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J Biol Chem* 1995; 270:9558-9563
- [40] Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, Leonard EJ. Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett* 1989;244:487–93.
- [41] Thompson WL, Van Eldik LJ. Inflammatory cytokines stimulate the chemokines CCL2/MCP-1 and CCL7/MCP-3 through NF $\kappa$ B and MAPK dependent pathways in rat astrocytes [corrected]. *Brain Res* 2009;1287:47–57.

- [42] Danese S, Sgambato A, Papa A, Scaldaferrri F, Pola R, Sans M, et al. Homocysteine triggers mucosal microvascular activation in inflammatory bowel disease. *Am J Gastroenterol* 2005;100:886–95.
- [43] Poddar R, Sivasubramanian N, DiBello PM, Robinson K, Jacobsen DW. Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease. *Circulation* 2001;103:2717–23.
- [44] Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol* 2016;13:316–27.
- [45] Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J Interferon Cytokine Res* 2009;29:313–26.
- [46] Bocca C, Novo E, Miglietta A, Parola M. Angiogenesis and Fibrogenesis in Chronic Liver Diseases. *Cell Mol Gastroenterol Hepatol* 2015;1:477–88.
- [47] Baeck C, Wehr A, Karlmark KR, Heymann F, Vucur M, Gassler N, et al. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. *Gut* 2012;61:416–26.
- [48] Mulder P, van den Hoek AM, Kleemann R. The CCR2 Inhibitor Propagermanium Attenuates Diet-Induced Insulin Resistance, Adipose Tissue Inflammation and Non-Alcoholic Steatohepatitis. *PloS One* 2017;12:e0169740.
- [49] Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;13:1324–32.
- [50] Zhu L, Baker RD, Baker SS. Gut microbiome and nonalcoholic fatty liver diseases. *Pediatr Res* 2015;77:245–51.
- [51] Volynets V, Küper MA, Strahl S, Maier IB, Spruss A, Wagnerberger S, et al. Nutrition, intestinal permeability, and blood ethanol levels are altered in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Dis Sci* 2012;57:1932–41.
- [52] Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, et al. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr* 2008;138:1452–5.
- [53] Harte AL, da Silva NF, Creely SJ, McGee KC, Billyard T, Youssef-Elabd EM, et al. Elevated endotoxin levels in non-alcoholic fatty liver disease. *J Inflamm Lond Engl* 2010;7:15.

[54] Alisi A, Manco M, Devito R, Piemonte F, Nobili V. Endotoxin and plasminogen activator inhibitor-1 serum levels associated with nonalcoholic steatohepatitis in children. *J Pediatr Gastroenterol Nutr* 2010;50:645–9.

### Figure Legends:

**Fig. 1. Methyl donor deficiency (MDD) aggravates dextran sulfate sodium (DSS) induced colitis.** (A) Disease Activity Index (DAI): Deficient animals treated with DSS (MDD/DSS) vs. control animals treated with DSS (C/DSS)  $**P < 0.01$ . (B) The endoscopic examination of the colon from C, C/DSS, MDD, MDD/DSS groups shows more severe colitis in the MDD/DSS than in the C/DSS group,  $**P < 0.01$ . Data are reported as means  $\pm$  SEM (n= 6 in each group).

**Fig. 2. Methyl donor deficiency (MDD) induces macrovesicular steatosis in sodium dextran sulfate (DSS) -dependent colitis.** (A) Representative histological sections of liver treated with hematoxylin and eosin (H & E), Masson trichrome and Sirius red. In bottom, segmentation of lipid accumulation was carried out using image processing software, showing macrovesicles (arrows) segmented in green (image x 200 centered by a centrolubular vein), especially in methyl donor deficient rats treated with DSS (MDD / DSS). Trichrome staining showed no fibrosis in these rats. (B) Quantification of steatosis in a defined microscopic area showed a significantly increased percentage of steatosis in deficient rats treated with DSS in comparison to controls ( $*P$ -value = 0.01) and MDD and to C/DSS treated rats ( $P$ -value= 0.05). Quantification of macrosteatosis vesicles (%) among all steatotic vesicles shows a significantly increased number of macrovacuoles in the deficient rats treated with DSS, compared to other groups (MDD/DSS vs. C,  $***P$ -value = 0.00003, MDD/DSS vs. MDD,  $**P$ -value= 0.001, MDD/DSS vs. DSS,  $**P$ -value = 0.002, C/DSS vs. C,  $*P$ -value = 0.02). Statistical data measured by Kruskal-Wallis test followed by Conover post-test, further adjusted by the Benjamini-Hochberg FDR method. (C) A higher protein expression of the fibrotic marker MMP2 was reported in the MDD/DSS treated rats, whereas other fibrotic markers ( $\alpha$  SMA and COL1A1) showed no change in expression among the different groups. The protein bands were quantified densitometrically, normalized with  $\alpha$  tubulin and expressed

as arbitrary units. Data are reported as means  $\pm$  SEM (n=6 in each group), \* $P$ <0.05, \*\* $P$ <0.01. OD: optical.

Abbreviations: Control rats (C), C/DSS (control treated with DSS), MDD (methyl donor deficient), and MDD/DSS (methyl donor deficient treated with DSS).

**Fig. 3. Induced colitis in deficient rats exposed to dextran sodium sulfate (DSS) activates NF $\kappa$ B pathway in the liver.**

(A) Immunohistochemical analysis showed significant translocation of NF $\kappa$ B p65 into the nucleus in deficient rats treated with DSS (MDD/DSS) in comparison to other groups: Control rats (C), C/DSS (control treated with DSS), MDD (methyl donor deficient), and MDD/DSS (methyl donor deficient treated with DSS). (B) NF $\kappa$ B and its different downstream modulators are activated as shown by western blots in the liver of MDD/DSS treated rats in comparison to other groups. The protein expression of NF $\kappa$ B p65 and its phosphorylated form were markedly increased in MDD/DSS group. Consistently, decreased expression and increased phosphorylation of I $\kappa$ B- $\alpha$  is observed. The protein bands were quantified densitometrically, normalized with  $\alpha$  tubulin and expressed as arbitrary units. Data are reported as means  $\pm$  SEM (n= 6 in each group), \* $P$ <0.05, \*\* $P$  <0.01. OD: optical density

**Fig. 4. Cytokines and chemokines in plasma, liver and intestine of 8 months-aged rats separated into 4 different groups, control (C), control treated with dextran sodium sulfate (C/DSS), methyl donor deficient (MDD) and methyl donor deficient treated with DSS (MDD/DSS).**

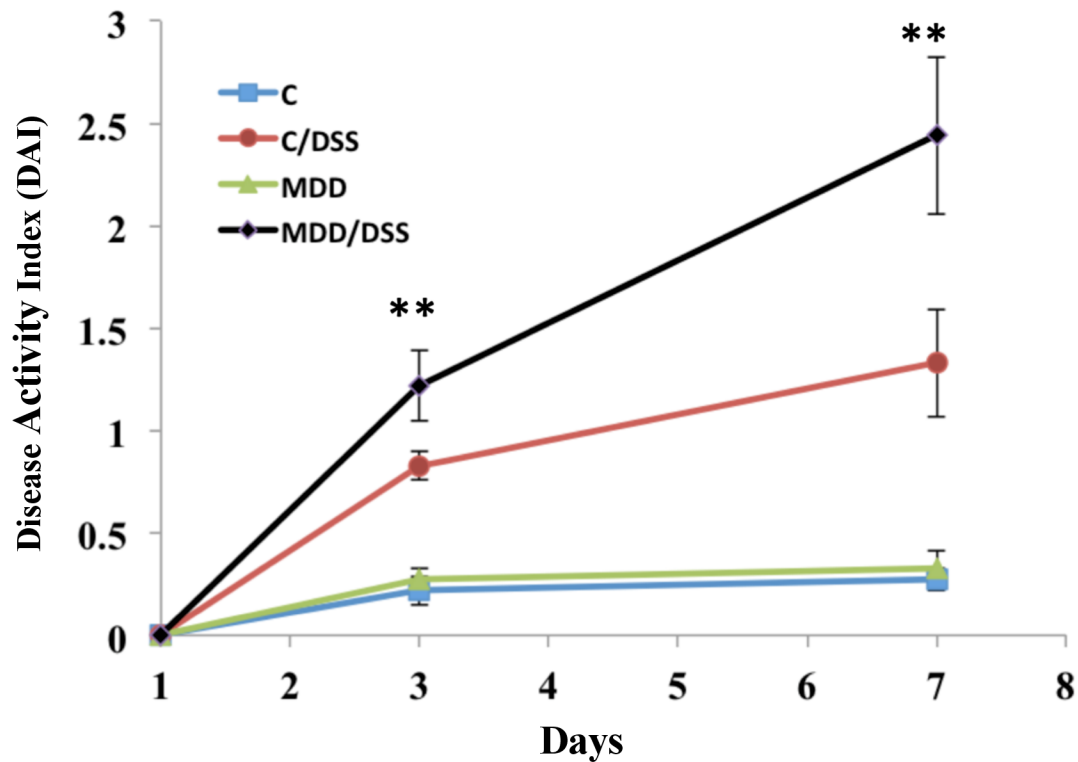
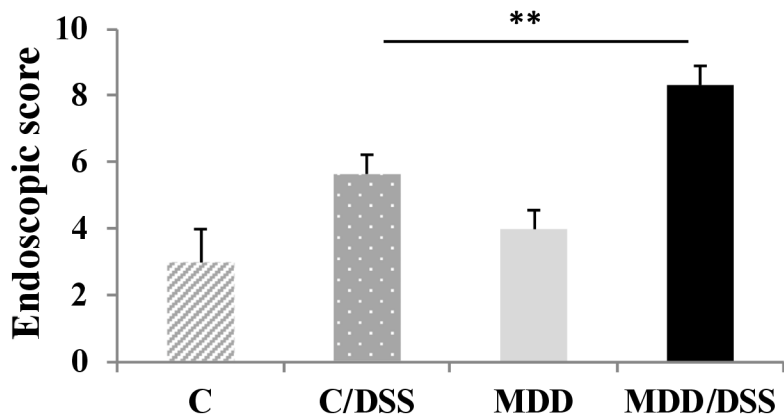
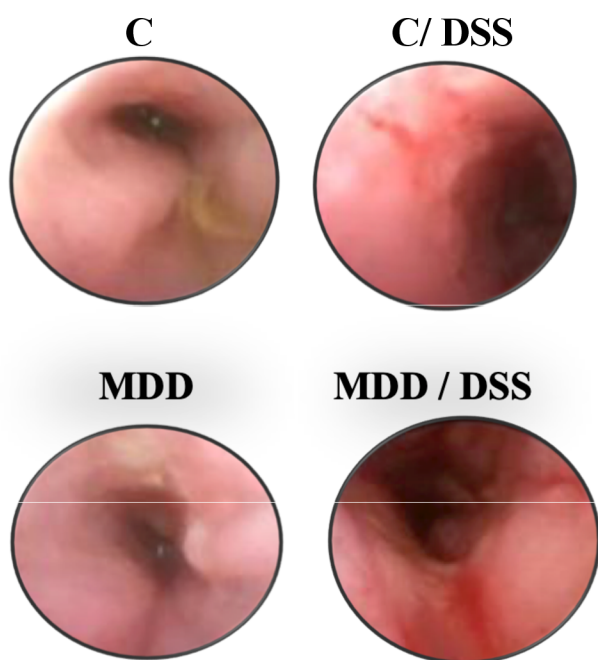
(A) Plasma level of cytokines and chemokines. The chemokine MCP-1 showed a dramatic increase in the MDD/DSS treated rats in comparison to other groups. Among cytokines, IL-1 $\beta$  showed a significant increase in the DSS treated groups and IL10 a significant increase in the DSS treated control group, but not in the DSS treated MDD group. VEGF also showed a significant increase in the MDD/DSS treated rats. Data are reported as means  $\pm$  SEM (n= 6 in each group), \* $P$ <0.05, \*\* $P$ <0.01. (B) Immunohistochemical (IHC) examination of the expression of MCP1 in the liver and the colon. IHC examination showed an increased expression of MCP-1 in the MDD/DSS treated rats with a more pronounced increase in the liver.

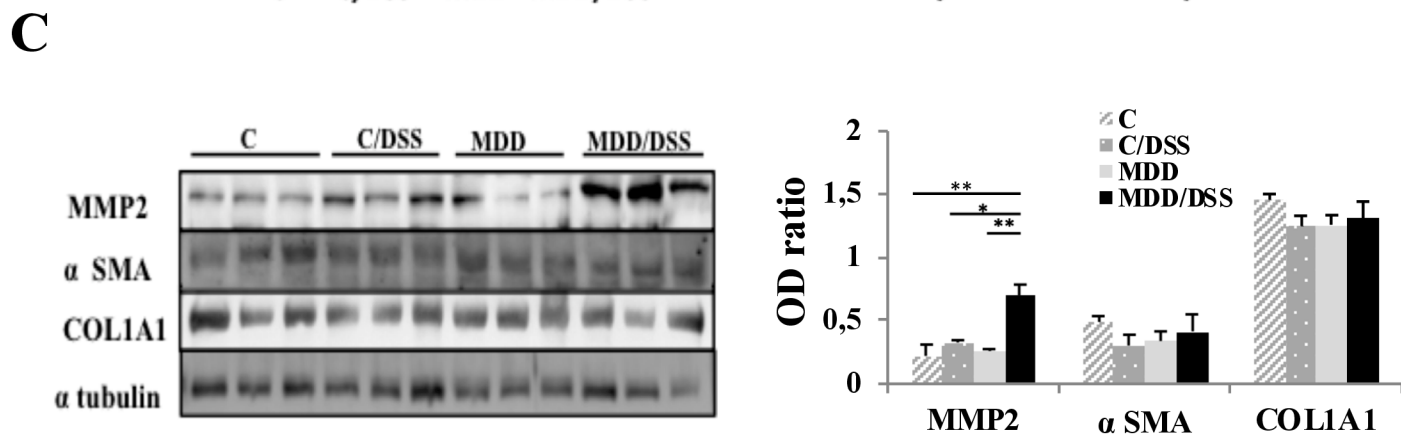
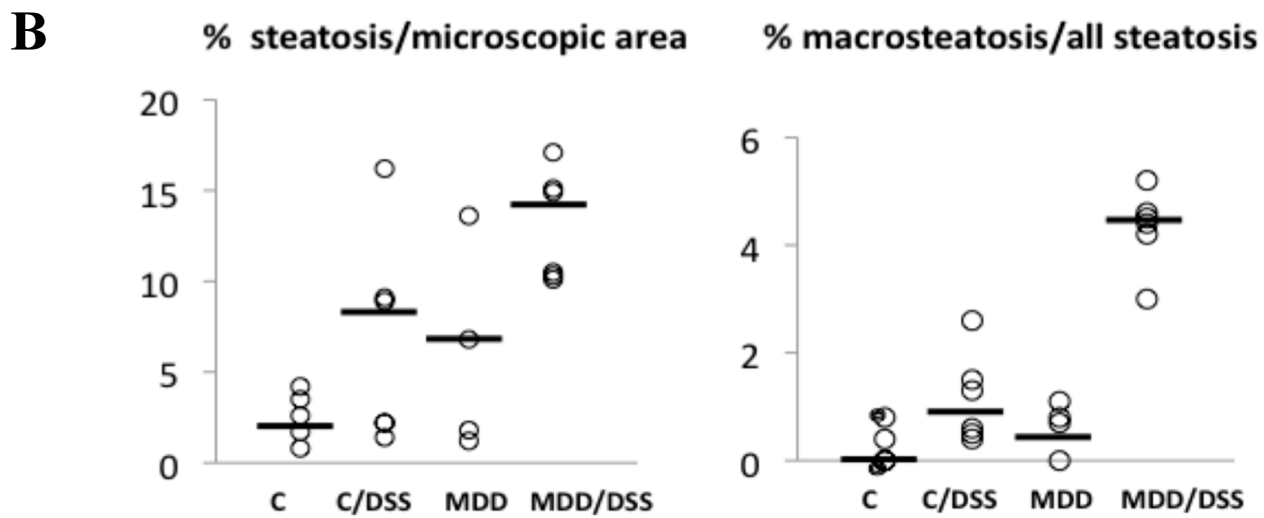
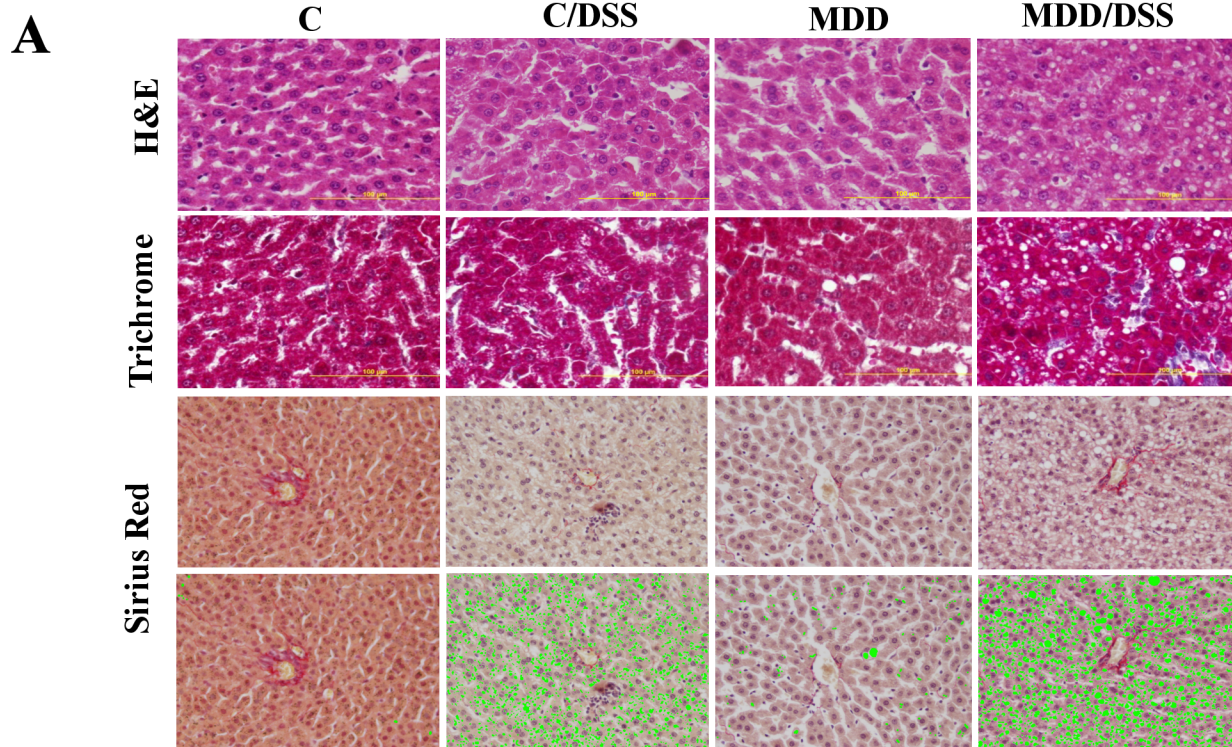
**Fig. 5. Expression of pro-inflammatory mediators in the liver and colon of methyl donor deficient (MDD) rats treated with dextran sodium sulfate (MDD/DSS).**

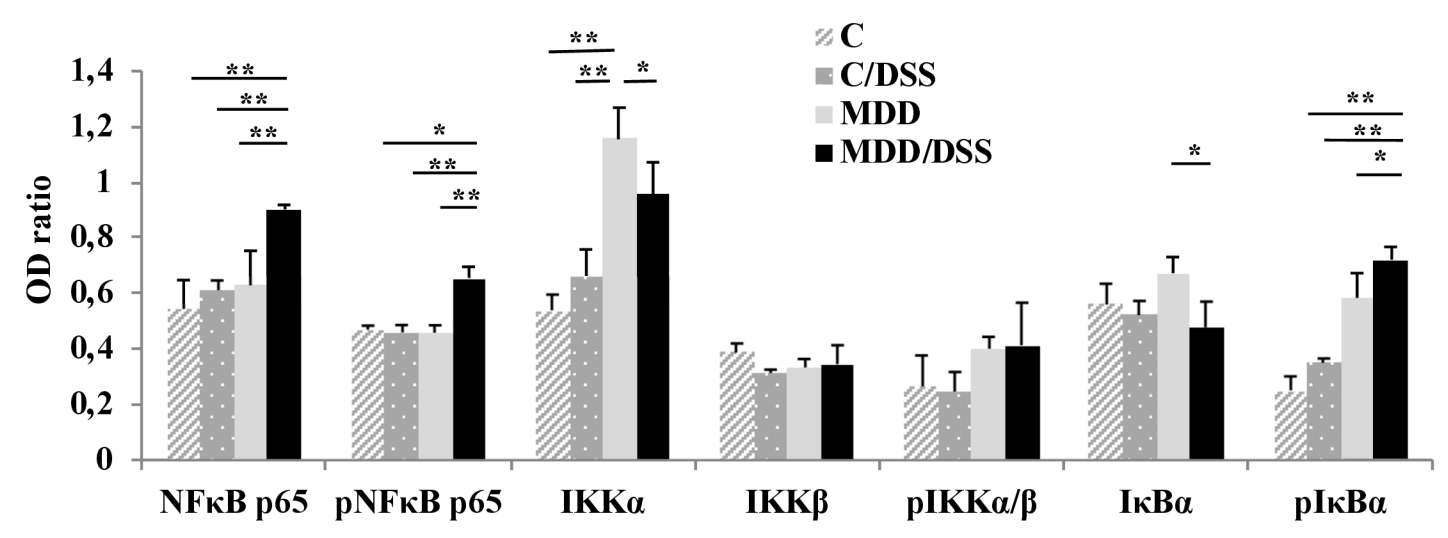
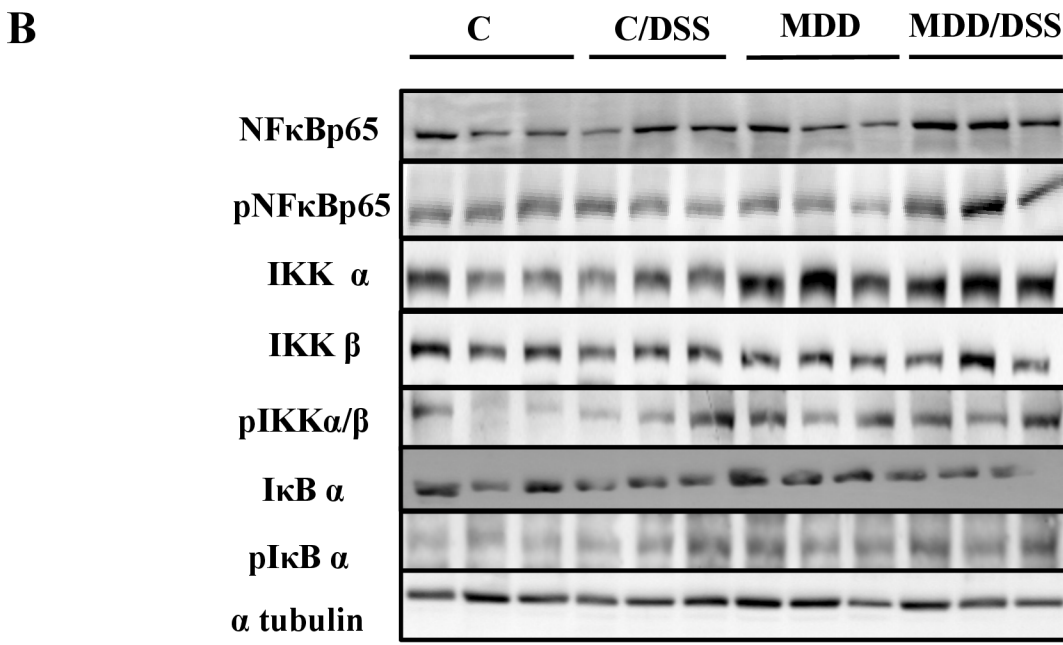
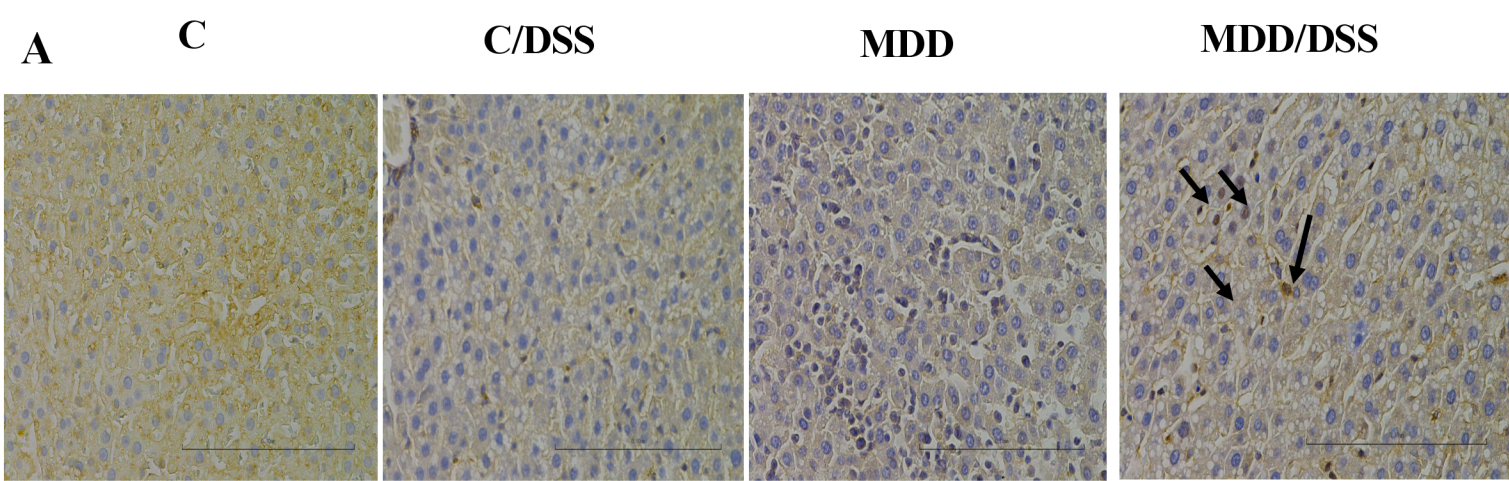
(A) IL-1 $\beta$  reported a gradual increase in liver expression among control (C), control treated with DSS (C/DSS), methyl donor deficient (MDD) and MDD treated with DSS (MDD/DSS) groups respectively. The expression of cleaved caspase-1 was increased in deficient groups (MDD and MDD/DSS) in comparison to control groups (C and C/DSS). (B) MCP-1 showed a gradual increase in the liver of control rats treated with DSS (C/DSS), methyl donor deficient (MDD) and methyl donor deficient ones treated with DSS (MDD/DSS) respectively in comparison to control rats (C). No change in expression was observed for the cytokines TNF alpha, IL-6 and their receptors among the different groups in the liver. (C) MCP-1 expression was also increased in the colon of control rats treated with DSS (C/DSS) and in the colon of deficient rats treated with DSS (MDD/DSS). IL-1beta was increased in the colon of C/DSS, MDD and MDD/DSS groups in comparison to controls while IL-10 showed a gradual decrease in these groups in comparison to controls.

**Fig. 6. Steatohepatitis is not related to activation of TLR4 pathway by its ligand (lipopolysaccharide, LPS).** (A) Neither the administration of dextran sodium sulfate (DSS) nor the methyl deficient diet (MDD) increased the plasma LPS level. (B) TLR4 pathway and its downstream mediators reported no change in expression among the different groups except for CD14. The protein bands were quantified densitometrically, normalized with  $\alpha$  tubulin and expressed as arbitrary units. Data are reported as means  $\pm$  SEM (n= 6 in each group), \* $P$ <0.05, \*\* $P$ <0.01. OD: optical density.

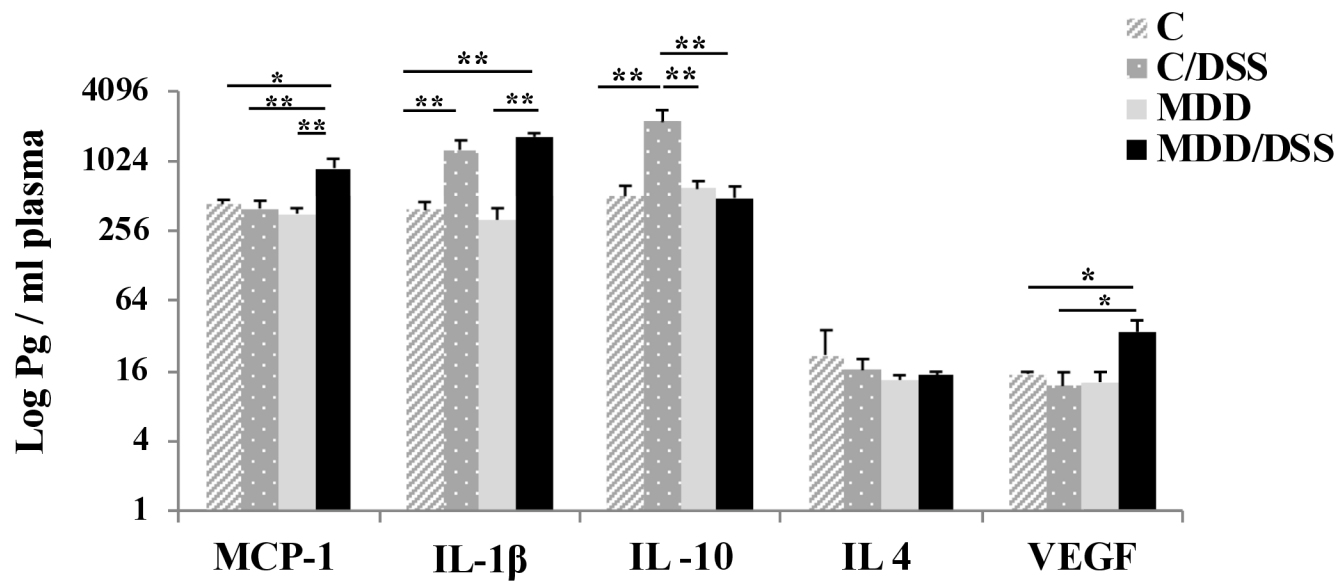
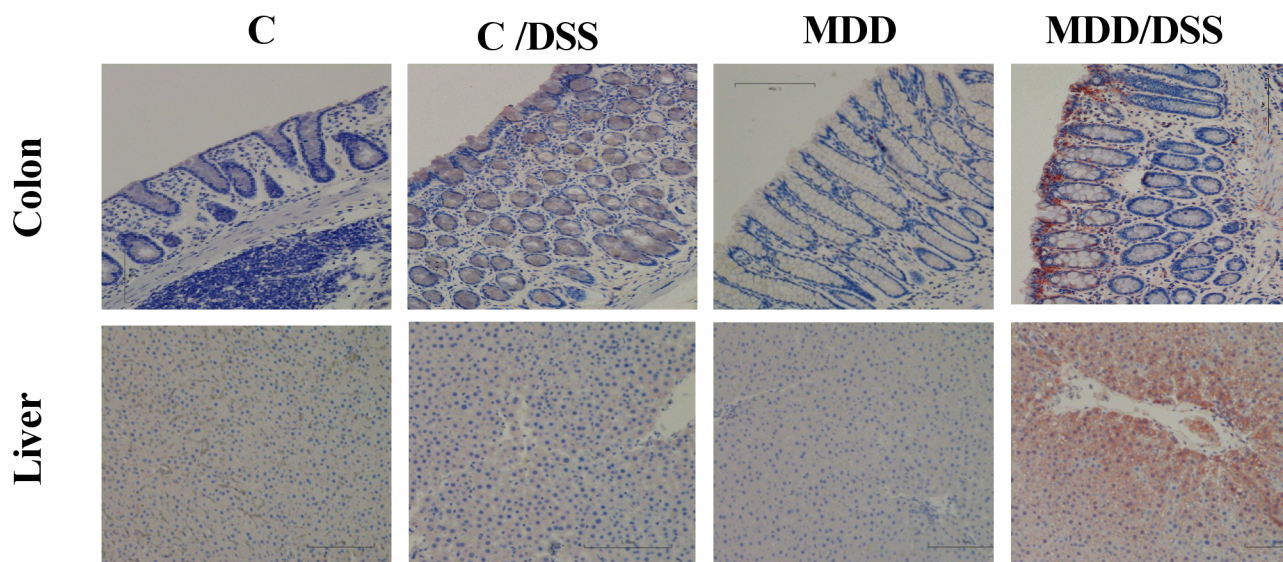
**Fig. 7. Relationship between methyl donor deficiency (MDD) and steatohepatitis in rats with DSS colitis.** The increased macrovesicular steatosis and inflammation observed in liver of rats with DSS colitis and subjected to methyl donor deficient diet was associated with altered expression of IL-1 $\beta$ , IL-10, MCP-1 in intestine, blood and liver.

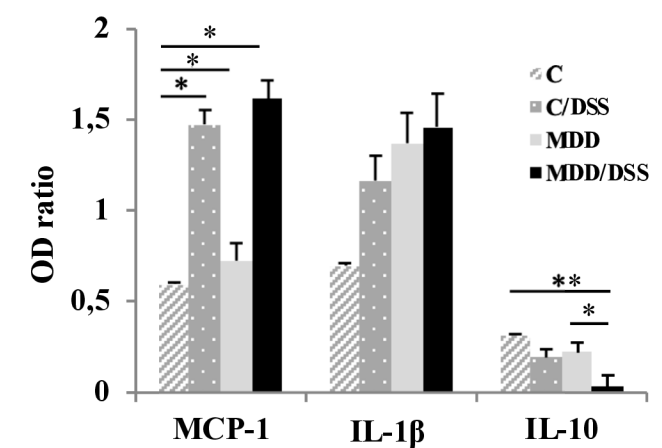
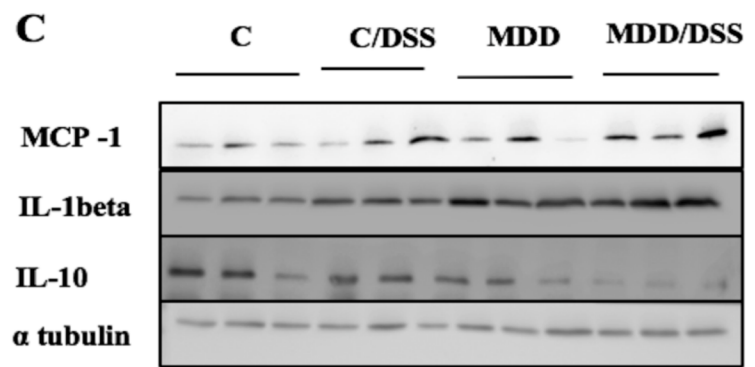
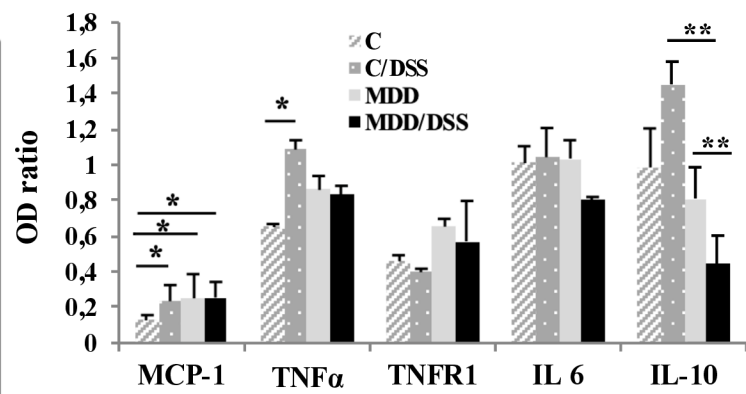
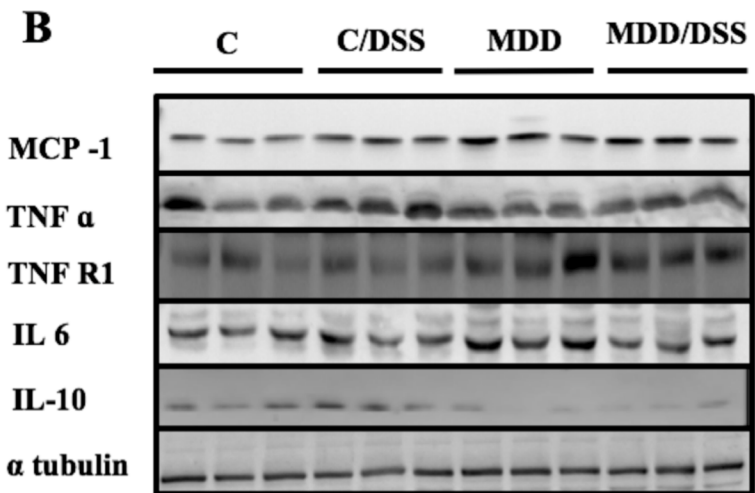
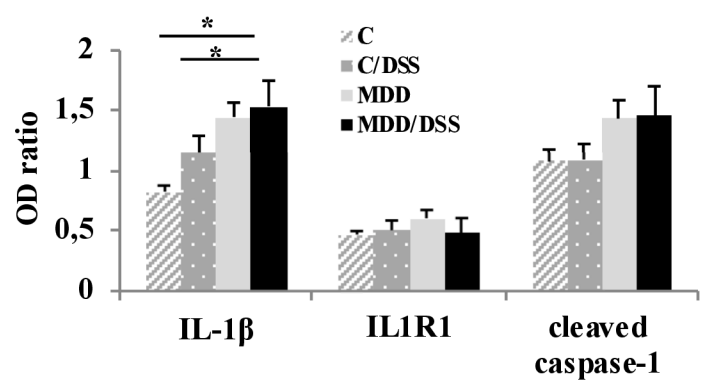
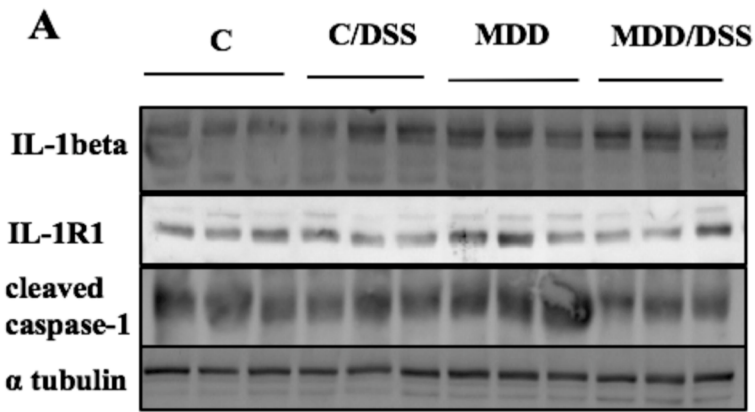
**A****B**

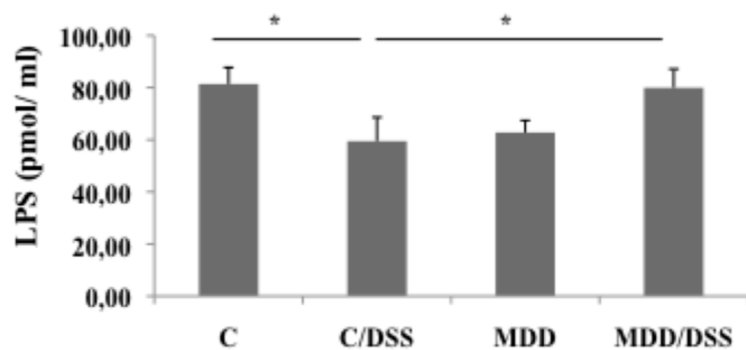
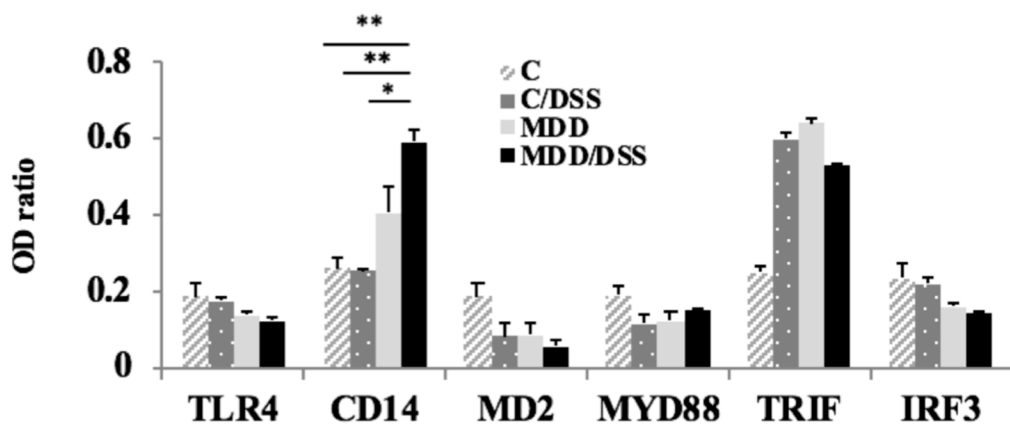
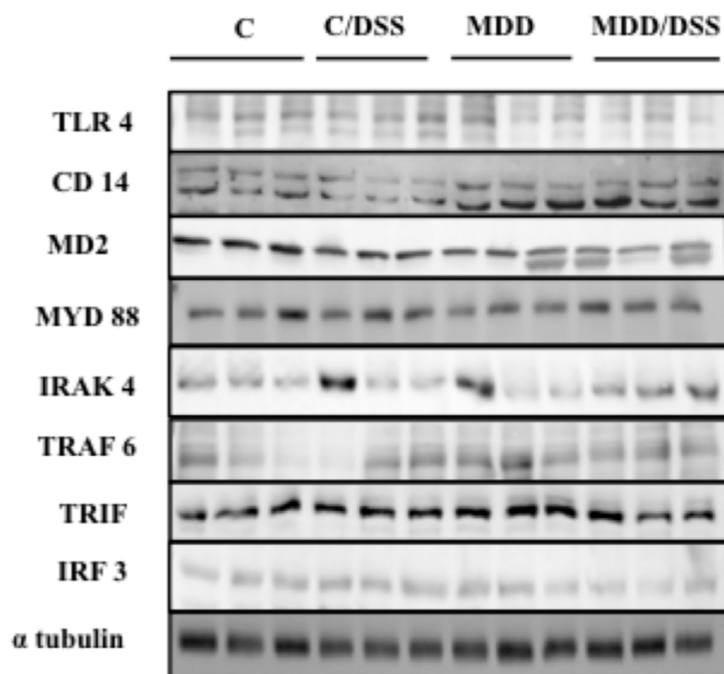


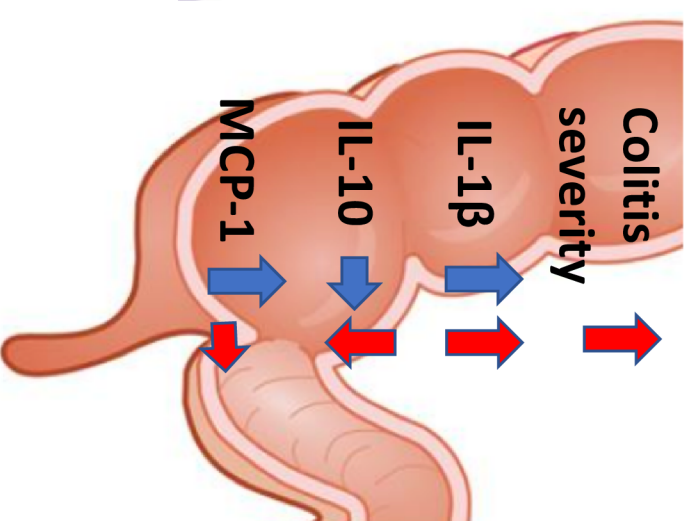
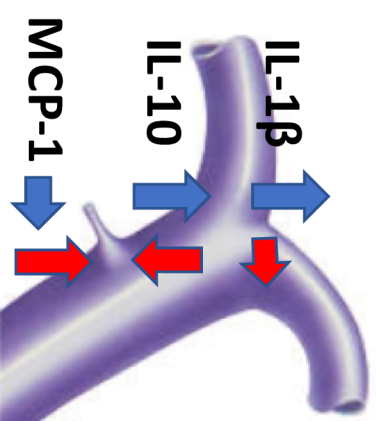
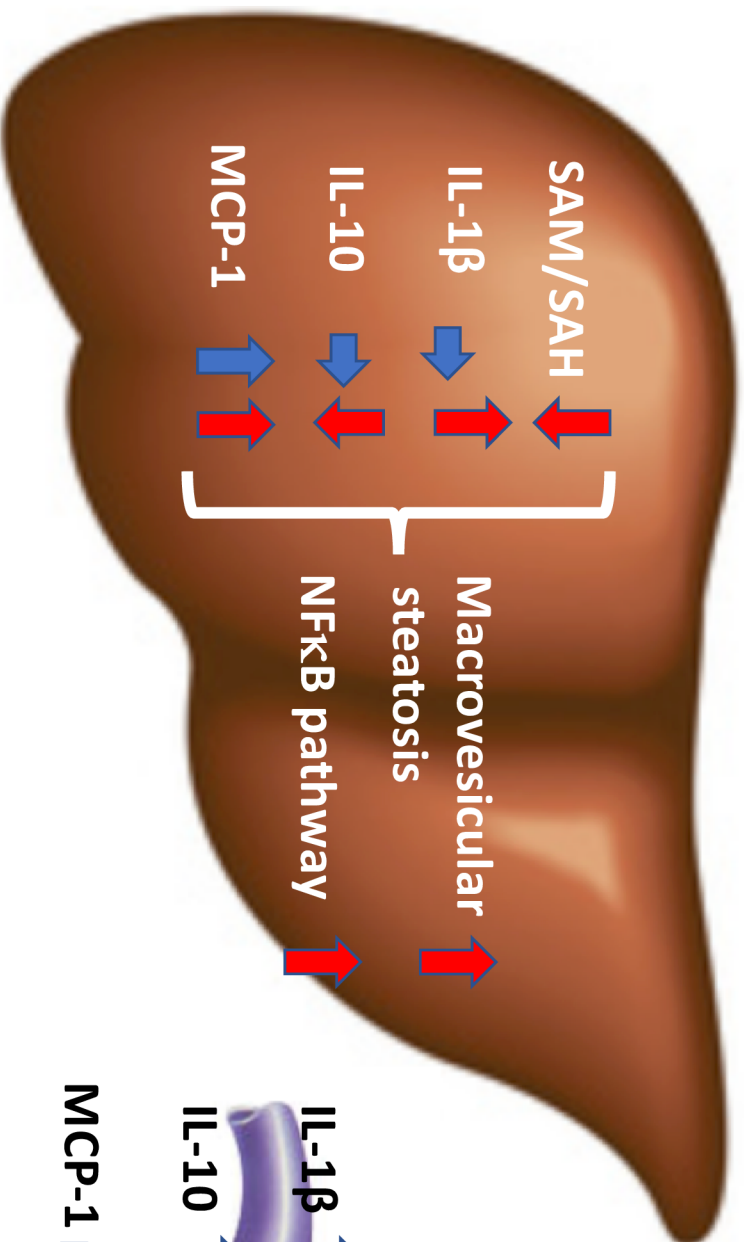




**A****B**



**A****B**



■ DSS colitis vs. control rats

■ DSS colitis vs. DSS/MDD rats



**Table 1: Biological markers in the plasma of 8 months aged rats separated into 4 different groups C (control), C/DSS (control treated with DSS), MDD (methyl donor deficient) and MDD/DSS (methyl donor deficient treated with DSS)**

Parameters	C	C/DSS	MDD	MDD/DSS	P value C/DSS vs MDD/DSS
<b>Vitamin B12 (pmol/l)</b>	357±52	334±24	66±40	53±2	0,0013 **
<b>Folate (nmol/l)</b>	67±13	95 ±20	15±10	13,5±5	<0,0001***
<b>Homocysteine (µmol/l)</b>	7±1,8	6,5±1,4	34±8,2	31±1,3	0,0001***
<b>SAM (nmol/l)</b>	228±48	182±51	187±74	210±28	0,54
<b>SAH (nmol/l)</b>	56±22	34±14	99±59	147±36	0,006**
<b>SAM/SAH Ratio</b>	4,41±1,5	5,6±0,95	2,4±1,5	1,45±0,15	0,0032**

Data are reported as means ±SD (n=3 in each group), \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$