Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice

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Abstract

In humans, there is large inter-individual variability in the evolution of NAFLD (non-alcoholic fatty liver disease) to NASH (non-alcoholic steatohepatitis). To investigate this issue, NASH was induced with an MCD (methionine–choline-deficient) diet in C57BL/6 and Balb/c mice that are characterized by different biases in Th1/Th2 and macrophage (M1/M2) responses. Following 4 weeks on the MCD diet, steatosis and lobular inflammation were prevalent in C57BL/6 (Th1, M1 oriented) than in Balb/c (Th2, M2 oriented) mice. Consistently, hepatic TNFα (tumour necrosis factor α) mRNA expression and circulating TNFα levels were higher in MCD-fed C57BL/6 than in MCD-fed Balb/c mice. The Th1/Th2 bias did not account for the increased NASH severity, as in both strains MCD feeding did not significantly modify the liver mRNA expression of the Th1 markers IFNγ (interferon γ) and T-bet or that of the Th2 markers IL-4 (interleukin 4) and GATA-3. Conversely, MCD-fed C57BL/6 mice displayed higher liver mRNAs for the macrophage M1 activation markers iNOS (inducible NO synthase), IL-12p40 and CXCL10 (CXC chemokine ligand 10) than similarly treated Balb/c mice, without effects on the M2 polarization markers IL-10 and MGL-1 (macrophage galactose-type C-type lectin-1). Circulating IL-12 was also higher in MCD-fed C57BL/6 than in MCD-fed Balb/c mice. The analysis of macrophages isolated from the livers of MCD-treated mice confirmed an enhanced expression of M1 markers in C57BL/6 mice. Among all of the MCD-treated mice, liver iNOS, IL-12p40 and CXCL10 mRNA levels positively correlated with the frequency of hepatic necro-inflammatory foci. We concluded that the macrophage M1 bias in C57BL/6 mice may account for the increased severity of NASH in this strain, suggesting macrophage responses as important contributors to NAFLD progression.

Introduction

NAFLD (non-alcoholic fatty liver disease) is characterized by TAG (triacylglycerol) accumulation within the liver and is, at present, the most frequent hepatic lesion in Western countries in relation to the growing prevalence of obesity and the metabolic syndrome [1]. Although fatty liver is often benign, approximately 10–25% of NAFLD patients develop NASH (non-alcoholic steatohepatitis) characterized by parenchymal injury, inflammation and

Key words: cytokine, Kupffer cell, liver inflammation, liver injury, non-alcoholic fatty liver disease, strain difference.

Abbreviations: ALT, alanine aminotransferase; CCL2, CC chemokine ligand 2; CXCL10, CXC chemokine ligand 10; IFNy, interferon γ; iIL, interleukin; iNOS, inducible NO synthase; MCD, methionine–choline-deficient; MGL-1, macrophage galactose-type C-type lectin-1. Circulating IL-12 was also higher in MCD-fed C57BL/6 than in MCD-fed Balb/c mice. The analysis of macrophages isolated from the livers of MCD-treated mice confirmed an enhanced expression of M1 markers in C57BL/6 mice. Among all of the MCD-treated mice, liver iNOS, IL-12p40 and CXCL10 mRNA levels positively correlated with the frequency of hepatic necro-inflammatory foci. We concluded that the macrophage M1 bias in C57BL/6 mice may account for the increased severity of NASH in this strain, suggesting macrophage responses as important contributors to NAFLD progression.

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fibrosis that can progress to cirrhosis and, in some case, to hepatocellular carcinoma [1]. A still open question in this field concerns the mechanisms responsible for NAFLD progression to more severe liver injury [2]. NAFLD is characterized by an increase in circulating NEFAs (non-esterified fatty acids), hepatic oxidative damage, endoplasmic reticulum stress and adipokine imbalances [3–5]. The current view suggests that all these events promote hepatic inflammation by stimulating Kupffer cells to secrete pro-inflammatory mediators and to recruit leucocytes within the liver [3–5]. Consistently, NASH patients show an increased hepatic expression of cytokine genes that correlates with the severity of liver lesions [6]. However, little is known about the factors determining why some NAFLD patients progress to NASH and others not.

Inter-strain variability in both innate and adaptive immunity is well documented in mice and has been exploited for elucidating the pathogenesis of several inflammatory diseases [7,8] as well as of multifactorial diseases with an inflammatory component, such as atherosclerosis [9]. In recent years, several groups reported differences among mouse strains in the susceptibility to experimental NASH [10–14]. In particular, C57BL/6 and 129/SVJ develop NASH and hepatic fibrosis upon receiving a high fat diet, whereas A/J mice are resistant [12]. Steatohepatitis induced by feeding an MCD (methionine–choline-deficient) diet is also more severe in A/J and C57BL/6 than in C3H/HeN or Balb/c mice [10,11]. So far, the mechanisms responsible for these differences have not been investigated in detail. In particular, little is known about the influence of the genetic background on the multiple mechanisms controlling hepatic inflammation. Studies in obese subjects have shown that chemokines released by fat-resident macrophages recruit to the adipose tissue T-lymphocytes and NKT cells (natural killer T-cells) [15]. In turn, Th1-activated CD4+ stimulate macrophage M1 polarization with the production of pro-inflammatory mediators [TNFα (tumour necrosis factor α), IL (interleukin)-12, CCL2 (CC chemokine ligand 2) and NO] causing insulin resistance and further recruitment of inflammatory cells into the adipose tissue [15,16]. Interestingly, an increase in M1 cytokines is also associated with the development of NASH in both experimental animals and humans [4–6].

To investigate whether the variability in the severity of NASH among mice strains might give some clue to understand the factors influencing the progression of the human disease, we compared liver inflammation in C57BL/6 and Balb/c mice that are characterized by a different bias in cytokine production by CD4+ Th and macrophages. In fact, C57BL/6 mice show a prominent Th1 cytokine production [IL-2, TNFα and IFNγ (interferon γ)] as opposed to a prevalence of Th2 cytokine response (IL-4, IL-5 and IL-13) in Balb/c mice [17]. Such a Th1 bias has a major role in determining the increased susceptibility to atherosclerosis of C57BL/6 mice, being the driving force for plaque inflammation [17,18]. Conversely, a Th2 bias is associated with an increased fibrosis in Balb/c mice [19]. Independently from the Th1/Th2 responses, C57BL/6-derived macrophages also show a bias to M1 polarized activation, whereas macrophages from Balb/c mice have a tendency to the so-called ‘alternative’ M2 activation [20]. This latter condition is characterized by anti-inflammatory, profibrogenic and immunosuppressive responses [21]. Since Th1 lymphocytes and M1 macrophages are responsible for adipose tissue inflammation and insulin resistance in obesity [15,16], in the present study we avoided possible interferences due to fat inflammation by using the MCD model of NASH that allows to reproduce the liver features of the human disease in the absence of obesity and insulin resistance [22].

MATERIAL AND METHODS

Animals and experimental protocol

Male C57BL/6 and Balb/c mice (8-weeks old) were purchased from Harlan–Nossan, and the dietary treatment was initiated after 1 week acclimatization. Mice were fed for 4 weeks with either an MCD diet or a control diet supplied by Laboratorio Dottori Piccioni. Body weight was recorded weekly throughout the experiment. Mice were anaesthetized with sevoﬂurane and blood was collected by cardiac puncture. Livers were rapidly removed and, after rinsing in ice-cold saline, cut in pieces. Aliquots were immediately frozen in liquid nitrogen and kept at −80 °C until analysed. Two portions of each liver were fixed in 10% formalin or snap-frozen in OCT (optimal cutting temperature) compound for histology respectively.

Animal experiments were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

Biochemical analysis

Plasma ALT (alanine aminotransferase) and total liver TAG content were determined by spectrometric kits supplied by Radim and Sigma Diagnostics respectively. Circulating TNFα and IL-12 levels were evaluated in the sera using commercial ELISA kits (Peprotech).

Isolation and purification of liver macrophages

Liver macrophages were isolated from the livers of MCD-fed mice by collagenase perfusion according to Froh et al. [23]. The cells were suspended in serum-free RPMI 1640 medium and purified by 1 h of adhesion on plastic disks. Cell purity, as estimated by immunofluorescence and flow cytometry,
was above 80% (see Supplementary Figure S1 at http://www.clinsci.org/cs/122/cs1220545add.htm). The cells were then processed for mRNA and protein extraction as outlined below.

mRNA extraction and real-time PCR
RNA was extracted with TRI reagent (Applied Biosystems) according to the manufacturer’s instructions and was quantified at an absorbance of 270 nm with a Nanodrop spectrometer (Thermo Scientific). RNA (1 μg) was retro-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. Real-time PCR was performed in a TC-312 thermalcycler (Thecne), using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNFα, IL-12p40, IL-4, IFNγ, IL-17a, IL-10, T-bet, GATA-3, RORγT (retinoic acid-receptor-related orphan receptor γT), iNOS (inducible NO synthase), CXCL10 (CXC chemokine ligand 10), MGL-1 (macrophage galactose-type C-type lectin-1), arginase-1, 18S and β-actin (Applied Biosystems). The data were processed using 7000 System Software and normalized to the β-actin or 18S gene expression. All samples were run blind in duplicate and the results were expressed as arbitrary units.

Western blotting
Liver macrophages were homogenized in ice-cold lysis buffer [10 mM Tris/HCl (pH 7.4) containing 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 20 mM sodium pyrophosphate, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mM DTT (dithiothreitol), 1 mM PMSF, 10 mg/ml leupeptin, 1 mg/ml pepstatin and 60 mg/ml aprotinin], and protein extracts (100 μg) were electrophoresed by SDS/PAGE on 10% polyacrylamide gels. Nitrocellulose membranes were probed with monoclonal antibodies against mouse iNOS and arginase-1 (BD Biosciences and Genetex) and revealed with Western Lightning Chemiluminescence Reagent Plus [ECL (enhanced chemiluminescence); PerkinElmer] using the VersaDoc 3000 quantitative imaging system and Quantity One software (Bio-Rad Laboratories).

Histology and immunohistochemistry
Liver pathology was assessed in haematoxylin/eosin and Masson’s trichrome stained sections. The severity of steatosis and lobular inflammation was scored by an experienced pathologist, according to the method described by Kleiner et al. [24]. The number of necro-inflammatory foci was counted in ten different high-magnification microscopic fields. Kupffer cells and liver infiltrating T-cells were detected in frozen sections using anti-mouse F4/80 (eBioscience) or anti-mouse CD3 monoclonal antibodies (R&D Systems) in combination with peroxidase-linked goat anti-rat IgG and HRP (horseradish peroxidase) polymer kit (Biocare Medical) respectively. The numbers of F4/80-positive macrophages were counted in ten different microscopic fields. CD3-positive cells are expressed as a percentage of the inflammatory cells infiltrating liver parenchyma in ten different microscopic fields.

Data analysis and statistical calculations
Statistical analyses were performed by SPSS statistical software using a one-way ANOVA test with Tukey correction for multiple comparisons, or a Kruskal–Wallis test for non-parametric values. A Pearson’s r value was used for verifying correlations. Significance was taken at the 5% level. Normality distribution was preliminary assessed by the Kolmogorov–Smirnov test.

RESULTS
Feeding C57BL/6 and Balb/c mice with an MCD diet for 4 weeks resulted in the development of steatohepatitis characterized by liver TAG accumulation and ALT release (Figure 1). Liver histology revealed the presence of macrovesicular steatosis accompanied by lobular inflammation, lipogranulomas, hepatocyte apoptosis and focal necrosis (Figures 2A–2D). By comparing MCD-fed animals, C57BL/6 mice had higher (P < 0.05) hepatic TAGs than Balb/c mice, whereas no significant differences were appreciable in ALT release (Figure 1). The two strains also suffer similar weight loss upon the administration of the MCD diet (results not shown). Following blind semi-quantitative evaluation of hepatic histology, MCD-treated C57BL/6 mice had higher scores for steatosis (P < 0.05) and lobular inflammation (P < 0.002) and an increased prevalence of focal necrosis (P < 0.001) as compared with similarly treated Balb/c mice (Figures 2E–2G). Collagen deposition in MCD-fed mice was very modest and mostly localized to the perisinusoidal spaces of the centrilobular areas without appreciable strain differences (results not shown). In accordance with the histopathology, liver mRNA expression and circulating levels of TNFα were significantly higher in C57BL/6 than in Balb/c MCD-treated mice (Figure 2).

Immunohistochemistry for CD3-positive cells showed that inflammatory infiltrates in MCD-treated C57BL/6 livers were characterized by an increased prevalence of T-lymphocytes (19.8 ± 7.0 compared with 13.3 ± 5.1%; P < 0.02) that were mostly localized around the necrotic foci (Figure 3). By measuring the liver mRNA content of IFNγ and IL-4 as representative for CD4+ T-cells Th1 and Th2 responses respectively, we observed that IFNγ and IL-4 expression were higher in control C57BL/6 than in Balb/c mice (Figure 3). The MCD diet did not significantly modify IFNγ expression in C57BL/6, whereas it lowered IFNγ mRNA in Balb/c mice (Figure 3). In both strains MCD feeding did not significantly affect IL-4 mRNA pattern (Figure 3).
Figure 1  Liver ALT release (A) and TAG content (B) evaluated in C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet
The values refer to 12–15 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

Figure 2  Hepatic inflammation in C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet
Liver histology was revealed by haematoxylin/eosin staining (A–D: magnification, ×200 and ×400). Lobular inflammation was scored semi-quantitatively according to Kleiner et al. [24] (E), while the number of necro-inflammatory foci (F) were counted in ten different high-magnification microscopic fields. The liver mRNA expression of TNFα (G) was measured by real-time PCR and expressed as arbitrary units after normalization to the actin gene expression. The circulating levels of TNFα were determined in the sera of the same animals (H). The values refer to 12–15 animals in each group and the boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.

No significant changes were also evident in the liver mRNAs of the Th1 regulator T-bet and of the Th2 transcription factor GATA-3 (Figure 3). IL-17-producing Th17 lymphocytes are a newly identified subset of effector helper T-cells, distinct from Th1 and Th2 CD4+ T-cells, which increasingly recognized to play a role in driving inflammation in chronic liver diseases [25]. As Th17 lymphocytes have been implicated in human alcoholic hepatitis [26], we investigated possible signs of Th17 response in experimental NASH. IL-17a mRNA was almost undetectable in the livers of control mice and increased just above detection limits.
upon MCD feeding, but because of the very low values the data were unreliable (results not shown). However, MCD-diet feeding did not modify the mRNA expression of RORγ/T, a transcription factor controlling Th17 lymphocyte differentiation (Figure 3). Taken together these findings suggest that lymphocyte polarized responses did not primarily account for strain differences in the onset of MCD-induced NASH in mice.

The possible influence of strain bias in macrophage activation was then investigated. Immunohistochemistry using anti-F8/40 antibodies did not reveal appreciable differences in the number of liver macrophages between MCD-fed C57BL/6 and Balb/c mice (results not shown). In both strains, the development of NASH was associated with an increased mRNA expression of M1 activation markers such as iNOS, IL-12p40 and CXCL10 (Figure 4). However, in the livers of MCD-treated C57BL/6 mice iNOS, IL-12p40 and CXCL10 mRNAs were respectively 3.3, 3.5 and 2.5 times higher than in MCD-treated Balb/c mice (Figure 4). Measurement of circulating IL-12 confirmed an increased production of this cytokine in MCD-fed C57BL/6 mice (Figure 4). On the other hand, despite the hepatic mRNAs for the M2 markers MGL-1 (CD301) and IL-10 were higher in control C57BL/6 than Balb/c mice, the MCD diet did not appreciably influence these markers (Figure 4). To further substantiate these observations, macrophages were isolated from the livers of MCD-fed mice of both strains. Figure 5 shows that the macrophages from C57BL/6 livers displayed an enhanced expression of iNOS and IL-12p40 as compared with those from Balb/c mice. Conversely, the M2 marker arginase-1 was greatly increased in Balb/c-derived macrophages, whereas no change was observed in MGL-1 expression (Figure 5).

The relationship between macrophage M1 bias and NASH severity was supported by the observation that, among all MCD-fed mice, the liver mRNA expression of iNOS ($r = 0.70, P = 0.017$), IL-12p40 ($r = 0.60, P = 0.009$) and CXCL10 ($r = 0.51, P = 0.018$) positively correlated with the frequency of hepatic necro-inflammatory foci.

**Figure 3** Evaluation of T-lymphocyte polarization markers in the liver of C57BL/6 and Balb/c mice with NASH induced by 4 weeks of feeding with an MCD diet

The mRNA expression of IFNγ and IL-4 and of the transcription factors T-bet, GATA-3 and RORγ/T were measured by real-time PCR. The values refer to 12–15 animals in each group and are expressed as arbitrary units after normalization to the actin gene expression. The boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians; 80 % of the values are between the extremes of the vertical bars (10–90th percentile). Cont, control.
DISCUSSION

To date, only a few studies have investigated the inter-strain differences in mice susceptibility to experimental NASH. Despite some variability related to the different dietary protocols, the following picture emerges: by using the MCD diet, the apparent rank in transaminase release is A/J>C57BL/6>C3H/HeN=Balb/c=DBA/2J [10,11], whereas long-term feeding of a diet deficient only in methionine causes more liver injury and hepatocarcinogenesis in DBA/2J than in C57BL/6 mice [14]. Conversely, C57BL/6 and 129/SVJ, but not A/J, mice develop NASH and hepatic fibrosis upon receiving a high fat diet [12,13]. At present, the factors responsible for such differences have been poorly characterized. QTL (quantitative trait locus) analysis has identified several loci in chromosomes 1, 2 and 7 that influence the extent of liver injury (ALT release) in seven mice strains receiving the MCD diet [10]. Furthermore, epigenetic mechanisms controlling DNA and histone methylation have been proposed to account for the differences in NASH progression between DBA/2J and C57BL/6 mice [14]. Our present results confirm and extend the above observations, showing that after 4 weeks on the MCD diet C57BL/6 mice developed more severe NASH than Balb/c mice.

Recent evidence indicates that the inflammatory process in NASH and atherosclerosis may share common mechanisms [27]. Interestingly, in experimental models of atherosclerosis C57BL/6 mice develop more extensive plaques than Balb/c mice [28,29]. Such a different behaviour depends upon a prevalent expression in the C57BL/6 strain of specific class II MHC molecules that regulate Th1 CD4+ T-cell activation [28]. In turn, IFNγ, TNFα and CD40 ligand produced by CD4+ T-cells drive plaque macrophages to produce ROS (reactive oxygen species), NO and pro-inflammatory cytokines [30]. Consistently, blunting CD4+ T-cells or their
Th1 responses decreases atherosclerosis in ApoE\(^{-/-}\) (apolipoprotein E-deficient) C57BL/6 mice fed on a high-cholesterol diet [30]. C57BL/6 Th1 bias does not appear to have a major influence on the severity of MCD-induced NASH. This result is rather unexpected, since previous studies have shown that fatty liver in mice fed on hypercaloric or choline-deficient diets promotes IFN\(\gamma\) production [31,32]. Nonetheless, it should be noted that in these latter studies IFN\(\gamma\) expression requires extensive T-cell activation by concanavalin A [31] or long-term (10–20 weeks) choline deficiency [32]. Thus it is possible that CD4\(^+\) T-cell recruitment might require longer than the 4-week treatment used in the present experiments.

During inflammation, macrophages can express different functional differentiation patterns in response to environmental stimuli such as bacterial products and cytokines [21]. Bacterial LPS (lipopolysaccarides) and IFN\(\gamma\) promote classic M1 activation characterized by the production of ROS, NO and pro-inflammatory cytokines and chemokines (IL-1\(\beta\), TNF\(\alpha\), IL-12, CXCL9 and CXCL10); conversely IL-4, IL-10 and TGF\(\beta\) (transforming growth factor \(\beta\)) induce alternative M2 macrophage activation associated with anti-inflammatory, pro-fibrogenic, angiogenetic and immuno-suppressive activities [21]. M1 activation characterizes not only bacterial infections, but is also evident in macrophages of atherosclerotic plaques and in those from the adipose tissue of obese subjects [16,33]. Our present results point to the importance of M1 responses in the development of NASH by showing that a prevalent liver expression of M1 markers correlates with increased hepatic inflammation in C57BL/6 mice with MCD-induced NASH. On the same line, a recent study in morbidly obese patients demonstrates that NASH, but not NAFLD, is associated...
with a specific increase in the liver expression of a wide array of pro-inflammatory M1 cytokines/chemokines (IL-1β, IL-18, CCL2–5 and CXCL9–11) [34]. Several mechanisms might contribute in promoting M1 responses in the early phase of NASH. For instance, TLR (Toll-like receptor)-4, TLR-2 and TLR-9 have been shown to trigger pro-inflammatory responses in fatty livers [35,36], being activated by dietary lipids, oxidation products and molecules released from damaged hepatocytes [5,37]. The complement cascade can also stimulate macrophage activation through C3b and C5b receptor interactions and the release of anaphylotoxins. In this latter context, Rensen et al. [38] have recently reported an extensive deposition of different complement fractions in liver biopsies from NASH patients that associates with increased hepatocyte apoptosis, granulocyte infiltration and higher liver expression of IL-1β, IL-6 and IL-8 mRNAs.

Altogether these results indicate that C57BL/6 mice M1 bias in liver macrophages responses contributes to the increased susceptibility to NASH of this strain, suggesting the importance of genetic/epigenetic factors regulating macrophage activation in influencing NAFLD progression to steatohepatitis. These observations point to the usefulness of exploring mice strain differences in the susceptibility to experimental NASH for obtaining new insights into the mechanisms responsible for inter-individual variability of NAFLD evolution in humans.

**AUTHOR CONTRIBUTION**

Virginia Maina and Salvatore Sutti designed the study and performed the experiments; Irene Locatelli, Matteo Vidali and Cristina Mombello contributed to the experiments and data analysis; Cristina Bozzola performed the histological analysis; and Emanuele Albano supervised the research and wrote the paper.

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Macrophage and NASH susceptibility in mice


Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice

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Figure S1 Purity of macrophages isolated from the livers of C57BL/6 and Balb/c mice fed on an MCD diet for 4 weeks was estimated by immunofluorescence using an anti-mouse F4/80 rat IgG and FITC-labelled anti-rat IgG serum (A and B) as well as by flow cytometry using allophycocyanin-labelled anti-mouse F4/80 and phycoerythrin-labelled anti-mouse CD45 antibodies (C and D).

In the immunofluorescence assays, nuclei were counterstained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole). Magnification, ×40.

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