NF-κB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses

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INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is becoming one of the most common liver diseases worldwide [1]. One still open issue in NAFLD pathogenesis concerns the mechanisms responsible for the switching from simple steatosis to NASH (non-alcoholic steatohepatitis). This aspect is clinically relevant because parenchymal injury and inflammation that characterize NASH are the driving forces for the disease evolution to fibrosis/cirrhosis [2,3].

Growing evidence indicates that the activation of the NF-κ B (nuclear factor κB) plays an critical role in the onset of adipose tissue inflammation in obesity [4]. Furthermore, studies in rodent models of NAFLD/NASH show that an increased NF-κ B activity is also associated with the development of steatosis, hepatic insulin resistance and inflammation [5,6]. A similar NF-κ B stimulation is also evident in the liver biopsies from NASH patients [7]. On the other hand, interfering with NF-κ B nuclear translocation ameliorates liver insulin resistance, steatosis and inflammation in...
NAFLD [6,8]. NF-κB are a family of dimeric proteins consisting of five Rel subunits (p50/NF-κB1, p52/NF-κB2, p65/RelA, p68/RelB and p75/c-Rel) that by binding with each other can form a variety of hetero- and homodimers regulating genes involved in immunity, inflammation and cell survival [9]. Among the different NF-κB units, p30/NF-κB1 and its precursor molecule p105 have been shown to have important regulatory activities that contribute in down-modulating NF-κB-mediated responses [10,11]. Accordingly, NF-κB-deficient animals show impaired macrophage polarization, develop more severe colitis and pneumonia and have a delayed resolution of glomerulonephritis [11,12]. Moreover, a functional polymorphism of human NF-κB1 gene (-94ins/delATTG, rs28720239) that reduces the protein production has been associated with a higher prevalence of inflammatory and autoimmune diseases [13–15]. The same polymorphism also increases the risk of cirrhosis in alcoholic patients [16]. From this background we investigated whether NF-κB1 deficiency might influence the progression of NASH. So far, experimental data regarding the role of NF-κB1 in liver injury have given conflicting results, as NF-κB1 deletion in mice does not affect acute liver injury and regeneration, while it enhances inflammation and fibrogenesis following chronic CCl4 (carbon tetrachloride) intoxication [17–19]. In the present study, NASH was induced by feeding mice on an MCD (methionine–choline-deficient) diet because...
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Figure 1  NF-κB1 deletion enhances liver injury and fibrosis in mice with NASH
WT and NF-κB1−/− (NFKB1 ko) mice were fed 4 weeks with either a control or a MCD diet. The livers were stained with haematoxylin/eosin (A–D; magnification ×200) or Masson’s trichrome (E, F; magnification ×400).

RESULTS

NF-κB1 deficiency worsens steatohepatitis and promotes liver fibrosis in mice fed on the MCD diet
The livers of NF-κB1−/− mice fed on the control diet had normal histological appearance, except for the presence of sporadic aggregates of mononuclear cells surrounding apoptotic hepatocytes (Figure 1). Feeding for 4 weeks on the MCD diet caused an appreciable weight loss in rodents. In line with the higher energy expenditure [20], NF-κB1−/− mice suffered more severe weight loss than the WT littermates (39.4 ± 5.7% compared with 27.5 ± 3.0%; P = 0.0002). Both WT and NF-κB1−/− mice fed on the MCD diet developed NASH, which was characterized by macrovesicular steatosis accompanied by lobular infiltration of inflammatory cells, hepatocytes ballooning and focal necrosis (Figure 1). In addition, granulomas consisting in aggregates of mononucleated cells surrounding fat-laden hepatocytes were also observed (Figure 1). Blinded semi-quantitative scoring for steatosis and lobular inflammation showed that MCD-fed NF-κB1−/− mice had more steatosis, lobular infiltration and increased hepatocyte apoptosis as compared with the WT littermates, whereas the prevalence of necrotic foci was comparable. The frequency of granulomas was also higher in NF-κB1−/− mice (Table 1). In accordance with histology, intrahepatic triacylglycerol accumulation, ALT release and oxidative stress were significantly higher in MCD-fed NF-κB1−/− mice than in MCD-fed WT animals (Figure 2). Furthermore, NF-κB1−/− mice fed on the MCD diet had a 2-fold increase in both liver TNFα mRNA and circulating TNFα levels compared with similarly treated WT animals (Figure 2). The stimulation in apoptosis observed in livers from MCD-fed NF-κB1−/− mice was not due to an impaired regulation of NF-κB-dependent anti-apoptotic factors, as the hepatic expression of Bcl-XL and A20 genes was not affected by NF-κB1 deficiency (results not shown). The steatohepatitis induced by the MCD diet is known to triggers hepatic fibrosis. However, in mice fibrillar matrix, accumulation becomes evident only after 8 weeks of treatment. Unexpectedly,
Masson’s trichrome staining for liver collagen revealed that already after 4 weeks on the MCD diet NF-κB1−/− mice developed appreciable centrilobular collagen deposition (Figure 1). Conversely, MCD-fed WT mice had a very modest increase in fibrillar matrix mainly localized in the peri-sinusoidal spaces (Figure 1). Immunohistochemistry for α-SMA also showed an increased number of α-SMA-positive activated myofibroblast-like HSCs only in the livers of MCD-fed NF-κB1−/− mice (Figure 2). Supporting these observations, the hepatic mRNAs for type 1 procollagen α1 and the TIMP-1 were expressed significantly more in NF-κB1−/− mice than in WT mice (Figure 2). Interestingly, an increased severity of NASH in NF-κB1−/− mice was already appreciable after 2 weeks of feeding on the MCD diet, as knockout animals had more extensive lobular inflammation and an increase in ALT release and TNFα and procollagen α1 mRNA expression than their WT littermates (see Supplementary Figure S1 at http://www.clinsci.org/cs/124/cs1240279add.htm).

**NF-κB1 deficiency does not influence macrophage responses in NASH**

NF-κB1-derived p105 and p50 proteins are known to be involved in regulating macrophage and lymphocyte responses [10,11]. In particular, p50 homodimers have an important role in down-modulating pro-inflammatory cytokine production in macrophages and HSCs as well as in driving macrophage ‘alternative’ M2 polarization [23–25]. Macrophage immunostaining with anti-F4/80 antibodies revealed that in both strains the development of NASH was associated with an increase in the number of F4/80-positive cells, but without significant differences between WT and NF-κB1−/− mice (see Supplementary Figure S2 at http://www.clinsci.org/cs/124/cs1240279add.htm). Furthermore, NF-κB1 deficiency did not influence the up-regulation of macrophage M1 markers IL-12p40 and iNOS promoted by NASH (Supplementary Figure S2). As expected, hepatic mRNAs for the M2 markers MGL1 (CD301) and IL-10 were lower in control NF-κB1−/− mice compared with the WT littermates. MDC feeding did not affect MGL1 and IL-10 expression, although IL-10 mRNA remained significantly lower in MCD-fed NF-κB1−/− mice (Supplementary Figure S2). Furthermore, despite the loss of NF-κB1 that has been reported to enhance the production of CCL2 and CXCL10 in HSCs [24], liver mRNAs for these chemokines were comparable in the MCD-fed WT and NF-κB1−/− animals (Supplementary Figure S2).

**NKT cell [NK (natural killer) T-cell] recruitment characterizes NASH in NF-κB1-deficient mice**

Lymphocytes are a common feature in NASH inflammatory infiltrates. Immunohistochemical staining for CD3 showed an increased prevalence of T-cells in the lobular infiltrates of MCD-fed NF-κB1−/− (27.9 ± 2.4 compared with 20.1 ± 4.7; *P* = 0.002). Flow-cytometry confirmed that CD3+ T-cells were increased in NASH livers and that T-cell recruitment was more pronounced in NF-κB1−/− mice (Figure 3). Such an increase involved effector CD8+ T-cells, without changes in the helper CD4+ T-cell fraction (Figure 3). We also observed that, although NASH in WT animals was characterized by the lowering of both liver NK (CD3−, NK1.1+) and NKT (CD3+, NK1.1+) cell populations (Figure 3), the NK cell fraction was unchanged in the livers of MCD-fed NF-κB1−/− mice (Figure 3). Furthermore, in these latter mice the development of NASH was associated with an increase in the NKT cell pool (Figure 3). The increase in liver NKT cells observed in NF-κB1−/− mice fed on the MCD diet was associated with a specific up-regulation in the hepatic mRNA expression of IL-15, a cytokine involved in NK and NKT cell survival and maturation [26] (Figure 4). IL-15 overexpression was evident already after 2 weeks of treatment (Figure 3) concomitantly with an increase in the mRNA of the NK/NKT cell marker NK1.1 (Supplementary Figure S1), suggesting that NF-κB1 deficiency might stimulate NKT cell recruitment by promoting IL-15 activity. Recent studies have proposed a role for NKT-cell-derived osteopontin in the progression of NASH [27,28]. In line with this view, MCD-fed NF-κB1−/− mice had a specific increase in the liver mRNAs for IFN-γ and osteopontin, two cytokines produced by NKT cells. The hepatic content of these cytokines was also 3- and 2-fold higher in NF-κB1−/− than in the WT mice. Moreover, approximately 40% of the osteopontin-expressing leukocytes isolated from NF-κB1−/− NASH livers were positive for NK1.1 (Figure 4).

**DISCUSSION**

Growing evidence indicates that the NF-κB1-gene-coded proteins p105 and p50 have important regulatory activities of inflammatory responses [10,11]. In particular, p105 forms complexes with p50 and the protein kinase TPL2 (tumour progression locus-2) blocking their functions, whereas p50 homodimers act as transcriptional activators promoting memory T-cell activation and macrophage M2 polarization [10,11,25]. Furthermore, p50 in combination with HDAC-1 (histone deacetylase-1) regulates pro-inflammatory genes [TNFα, CCL2, CXCL10 and GM-CSF (granulocyte/macrophage colony-stimulating factor)] expression in activated HSCs [24]. In the present study, we show that steatohepatitis induced by the MCD diet is more severe in NF-κB1−/− mice being characterized by extensive lobular infiltration by mononucleated cells, frequent lipogranulomas, higher
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Figure 2  NF-κB1 deletion enhances markers of liver injury and fibrosis in mice with NASH

WT and NF-κB1−/− (NFKB1 ko) mice were fed for 4 weeks on either a control or an MCD diet. ALT release (A) and liver triacylglycerol content (B) were evaluated by enzymatic methods. The liver mRNA expression of TNFα, α1-procollagen and TIMP-1 (C, F, G) were measured by RT-PCR (reverse transcription–PCR) and expressed as the fold increase after normalization to the β-actin gene. The circulating levels of TNFα were determined in the sera of the same animals (D). The values refer to 8–10 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile). Activated HSCs expressing α-SMA+HSC were evidenced by immunohistochemistry and counted in ten different high-magnification microscopic fields (E).

circulating TNFα levels and increased hepatocyte apoptosis. Furthermore, NASH in NF-κB1−/− mice associates with a more rapid progression of centrilobular fibrosis that is already evident after 4 weeks of treatment. Previous observations concerning the factors promoting chronic liver injury in CCl4-treated NF-κB1−/− mice have implicated an enhanced recruitment of α-SMA-positive activated HSCs that, beside producing procollagen 1α and TIMP-1, overexpress pro-inflammatory mediators such as TNFα, CCL2, CXCL10, GM-CSF in relation with an impaired down-modulation of these genes by NF-κB1/p50 and HDAC-1 [24]. Although an increase in activated HSCs characterizes NASH in NF-κB1−/− mice, the expression of CCL2 and CXCL10 genes in these animals is comparable with the WT mice, thus excluding that HSC-derived cyto/chemokines account for the promotion of liver injury.

Studies using mice models of glomerulonephritis have shown that a diffuse T-cell infiltration characterize delayed resolution of kidney inflammation in NF-κB1−/− mice [12]. In accordance with these findings, we have observed that T-cell recruitment is associated the rapid progression of NASH in NF-κB1−/− mice. Such an increase in T-cells specifically involves cytotoxic CD8+ T-cells and NKT cells, whereas CD4 helper T-cells are unaffected. The role of NKT cells in NASH is complex, as steatosis and the early phase of steatohepatitis is characterized by the lowering of the liver NKT cell pool as a consequence of IL-12 production [29–30], whereas an expansion of the NKT
cell population is evident in mice with more advanced NASH [27]. Furthermore, NKT cell depletion prevents hepatic inflammation and fibrosis induced by the MCD diet [28], but enhances the liver expression of inflammatory markers in mice fed on a high-fat diet [31]. We observed that an increase in NKT cells characterizes advanced NASH (steatohepatitis plus fibrosis) in MCD-fed NF-κB1−/− mice as opposed to NKT cell depletion present in WT animals that only show steatohepatitis. This is consistent with the correlation between the NAS score and hepatic NKT cell prevalence reported in NASH patients [32], supporting the importance of NKT cells in driving the progression of NASH towards fibrosis. So far, little is known about how NF-κB1 deficiency influences NKT cell responses. Stankovic et al. [33] recently reported that NF-κB1 loss moderately reduces hepatic NKT cell pool in C57BL/6 mice by affecting their maturation. However, we observed that already in the early phases of NASH NF-κB1−/− mice up-regulate the hepatic expression of IL-15. IL-15 is a pleiotropic cytokine belonging to the four α-helix bundle cytokine family and is responsible for macrophage, T-cell, NK cell and NKT cell survival and maturation [26].

Figure 3  NASH in NFκB1-deficient mice associates with an increased recruitment of hepatic T-cells
Liver leukocytes were isolated from the livers of WT and NFκB1−/− (NFKB1 ko) mice fed for 4 weeks on either a control or a MCD diet (B) and analysed by FACS. Representative plot of CD3-positive T-cells (A) and statistical analysis of total CD3+ and CD8+ or CD4+ T-cells subsets (B–D). Representative FACS plot of NK1.1 and CD3-positive lymphocytes (E) and statistical analysis of CD3− NK1.1+ (NK) and CD3+ NK1.1+ (NKT) cells (F, G). The values refer to 5–6 animals in each group and the bars represent medians ± S.D.
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Figure 4 Production of NKT-cell-derived cytokines characterizes NASH in NF-κB1-deficient mice

NASH was induced by feeding mice on an MCD diet for either 2 or 4 weeks. Liver mRNA expression of IL-15, IFN-γ and osteopontin (A–C) were evaluated by RT–PCR and expressed as the fold increase after normalization to the β-actin gene. The intrahepatic levels of IFN-γ (D) and osteopontin (E) were evaluated by ELISA. The values refer to 6–10 animals in each group and the boxes include the values within 25th and 75th percentile, whereas the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile). Representative FACS plot of osteopontin- and NK1.1-expressing leucocytes isolated from the livers of NF-κB1−/− (NFKB1 ko) mice fed on the MCD diet (F).

In the liver, IL-15 produced by hepatocytes has a key role in creating a T-cell-favourable microenvironment and it also induces the expression of NK cell markers on T-cells [34]. Although IL-15 is constitutively produced in the liver, its expression by hepatocytes and hepatic progenitor cells increases in response to injury [35]. Indeed, in WT mice IL-15 up-regulation is evident in advanced NASH induced by 8 weeks feeding on the MCD diet concomitantly with hepatic NKT cells expansion [27]. Thus we propose that NF-κB1 loss might favour IL-15 up-regulation at the onset of NASH, preventing NKT cell depletion caused by IL-12 [30] and leading to a more rapid increase in their number through a more efficient hepatic differentiation.

Concerning the mechanisms by which NKT cells could favour the progression of liver injury in NASH, we observed that MCD-fed NF-κB1−/− mice display a specific increase in the liver production of IFN-γ and osteopontin. IFN-γ can originate from both CD8+ T and NKT cells and is potent inducer of TNFα and ROS (reactive oxygen species) generation by macrophages. Osteopontin is a glucosylated cytokine produced by both immune and parenchymal cells that is increasingly recognized to play important roles in inflammation and tissue healing [36]. NKT cells are a relevant source of osteopontin in lymphocyte-mediated hepatic injury [37], and osteopontin-expressing NKT cells are evident in the livers of NF-κB1−/− mice with NASH. This does not
exclude the possibility that other cells such as T-cells, macrophages or cholangiocytes [38] might also contribute to osteopontin formation. A role for osteopontin in stimulating NASH evolution is in line with the observation that osteopontin-deficient mice are protected against steatohepatitis and fibrosis induced by feeding on an MCD diet [28,39]. Furthermore, with comparison with that reported by Sahai et al. [39] using A/J mice we did not observe changes in hepatic osteopontin expression in WT C57BL/6 mice fed on the MCD diet for 4 weeks. This discrepancy might reflect strain differences in hepatic inflammatory responses involved in the onset of NASH [41]. It is noteworthy that NF-κB-/- mice develop appreciable liver fibrosis despite the presence of elevated IFN-γ production that should antagonize fibrogenesis. In this context, the increase in liver osteopontin observed in these animals might have a relevant role, as osteopontin can efficiently stimulate HSCs to collagen production by engaging integrin αvβ3 and stimulating inositol 3-phosphate kinase and NF-κB signalling [42].

Altogether these results indicate that NF-κB1 down-modulation speed up NASH progression to fibrosis by stimulating the liver recruitment of osteopontin-producing NKT cells, thus supporting the importance of these cells in the evolution of NAFLD. Moreover, our results point to the possible relevance of NF-κB1 gene polymorphisms as a risk factor for the progression of the human disease.

**CLINICAL PERSPECTIVES**

- NF-κB activation is involved in the pathogenesis of NASH. Among the NF-κB subunits, p50/NF-κB1 has regulatory activities down-modulating NF-κB-mediated responses. Recently, a functional polymorphism in NF-κB1 gene (rs28720239) has been associated with a higher prevalence of inflammatory/autoimmune diseases as well as with an increased risk of alcoholic cirrhosis.

- By inducing experimental NASH feeding, we observed that liver injury, lobular inflammation and fibrosis developed more rapidly in NF-κB1-/- mice than in WT mice. Such effects were dependent on an increased liver recruitment of osteopontin-producing NKT cells through the up-regulation of IL-15 expression.

- These results stress on the importance of NKT cells in the evolution of NASH and point to NF-κB1 gene polymorphisms as a possible risk factor for the progression of NASH in humans.

**AUTHOR CONTRIBUTION**

Irene Locatelli and Salvatore Sutti conceived and carried out the experiments and analysed the data. Marco Vacchiano contributed to the experiments. Cristina Bozzola performed the histological analysis. Emanuele Albano wrote the paper. All authors had final approval of the submitted and published versions.

**FUNDING**

This study was supported by the Italian Ministry of Education, University and Research [grant number PRIN 2009ARYX4T_003].

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Received 31 May 2012/27 July 2012; accepted 12 September 2012
Published as Immediate Publication 12 September 2012, doi: 10.1042/CS201210289
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**SUPPLEMENTARY ONLINE DATA**

**Figure S1** Increased severity of NASH in NF-κB1-deficient mice fed for 2 weeks on an MCD diet
Liver histology was evidenced by haematoxilin/eosin staining in MCD-fed WT and NF-κB1−/− (NF-κB1-ko) mice (A, B; magnification ×200). ALT release (C) and liver triacylglycerol content (D) were evaluated by enzymatic methods. The liver mRNA expression of TNF-α, α1-procollagen and NK1.1 genes (E–G) were measured by RT–PCR and expressed as fold increase after normalization to the β-actin gene. The values refer to six animals in each group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile).

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Figure S2 The NF-κB1 deficiency did not influence macrophage responses in mice with NASH

Wild-type and NF-κB1−/− (NFκB1ko) mice were fed 4 weeks on either a control or an MCD diet. F4/80-positive macrophages were counted in ten different high-magnification microscopic fields (A). The liver mRNA expression of IL-12p40, iNOS, MGL1, IL-10, CCL2 and CXCL10 (B–G) were evaluated by RT–PCR and expressed as the fold increase after normalization to the β-actin gene. The values refer to 8–10 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. Of the values, 80% are contained between the extremes of the vertical bars (10th–90th percentile).