The balance between IL-17 and IL-22 produced by liver-infiltrating T-helper cells critically controls NASH development in mice

Simona Rolla*†[1], Elisa Alchera‡[1], Chiara Imarisio‡[1], Valentina Bardina*†, Guido Valente§,
Paola Cappello*†¶, Cristina Mombello§, Antonia Follenzi‡, Francesco Novelli*†‖¶[1] and Rita Carini‡[1]

*Center for Experimental Research and Medical Studies (CERMS), Azienda Ospedaliera Città della Salute e della Scienza di Torino, via Cherasco 15, 10126 Turin, Italy
†Department of Molecular Biotechnology and Health Sciences, via Nizza 56, University of Torino, 10126 Turin, Italy
‡Department of Health Sciences, University of Piemonte Orientale, via Solaroli 17, 28100 Novara, Italy
§Department of Translational Medicine, University of Piemonte Orientale, via Solaroli 17, 28100 Novara, Italy
‖Immunogenetics and Transplantation Biology Unit, Azienda Ospedaliera Città della Salute e della Scienza di Torino, via Cherasco 15, 10126 Turin, Italy
¶Molecular Biology Center, University of Turin, via Nizza 52, 10126 Turin, Italy

Abstract

The mechanisms responsible for the evolution of steatosis towards NASH (non-alcoholic steatohepatitis) and fibrosis are not completely defined. In the present study we evaluated the role of CD4$^+$ T-helper (Th) cells in this process. We analysed the infiltration of different subsets of CD4$^+$ Th cells in C57BL/6 mice fed on a MCD (methionine choline-deficient) diet, which is a model reproducing all phases of human NASH progression. There was an increase in Th17 cells at the beginning of NASH development and at the NASH–fibrosis transition, whereas levels of Th22 cells peaked between the first and the second expansion of Th17 cells. An increase in the production of IL (interleukin)-6, TNFα (tumour necrosis factor α), TGFβ (transforming growth factor β) and CCL20 (CC chemokine ligand 20) accompanied the changes in Th17/Th22 cells. Livers of IL-17$^{-/-}$ mice were protected from NASH development and characterized by an extensive infiltration of Th22 cells. *In vitro*, IL-17 exacerbated the JNK (c-Jun N-terminal kinase)-dependent mouse hepatocyte lipotoxicity induced by palmitate. IL-22 prevented lipotoxicity through PI3K (phosphoinositide 3-kinase)-mediated inhibition of JNK, but did not play a protective role in the presence of IL-17, which up-regulated the PI3K/Akt inhibitor PTEN (phosphatase and tensin homologue deleted on chromosome 10). Consistently, livers of IL-17$^{-/-}$ mice fed on the MCD diet displayed decreased activation of JNK, reduced expression of PTEN and increased phosphorylation of Akt compared with livers of wild-type mice. Hepatic infiltration of Th17 cells is critical for NASH initiation and development of fibrosis in mice, and reflects an infiltration of Th22 cells. Th22 cells are protective in NASH, but only in the absence of IL-17. These data strongly support the potentiality of clinical applications of IL-17 inhibitors that can prevent NASH by both abolishing the lipotoxic action of IL-17 and allowing IL-22-mediated protection.

Key words: hepatocyte, immune reactions, lipotoxicity, phosphoinositide 3-kinase signalling, steatosis.

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is the most frequent hepatic affliction in Western countries. In the general population, the prevalence of NAFLD ranges from 20 to 30%, but reaches 70% among overweight individuals [1,2]. Most patients with NAFLD remain asymptomatic, but 20% develop chronic hepatic inflammation and parenchymal injury [NASH (non-alcoholic steatohepatitis)], which can lead to cirrhosis and increased mortality [3–6].

Several alterations, such as oxidative stress and pro-inflammatory cytokine production, unbalanced adipokine

generation, mitochondrial dysfunctions and direct hepatocyte toxicity by non-esterified ('free') fatty acids are associated with NAFLD progression to NASH [7–9]. However, the comprehension of the mechanisms that drive the evolution of steatosis into more severe liver injury remains to be determined, and no effective therapeutic strategies are available.

Increasing evidence indicates the hepatic infiltration of CD4$^+$ T-helper (Th) cells during NASH development. CD4$^+$, together with CD8$^+$ T-cells have, indeed, been detected in the late phase of NASH development in mice [10], whereas CD4$^+$ Th17 cells were found in livers of NASH patients [11]. CD4$^+$ Th cells assist B-cells and CD8$^+$ cytotoxic cells in the activation and perpetuation of immune and autoimmune reactions [12]. Consistently, IgG against antigens originating from oxidative stress products are detectable in NAFLD/NASH patients [13,14] and immunization of mice with lipid peroxidation products exacerbated NASH development [10]. Inflammation is a main pathological feature of NASH, and cells of adaptive immunity, along with cells of innate immunity, can trigger and sustain hepatic inflammatory reactions [12]. CD4$^+$ T-lymphocytes can develop into Th and T$_{reg}$ (T-regulatory) cells [15]. Except for Th2 cells, which are mainly stimulated in response to allergies and in the clearance of extracellular pathogens, all Th1, Th17, Th22 and T$_{reg}$ cells can play a role in sterile inflammatory reactions associated with NASH. Th1 cells secrete IFNγ (interferon γ), a pro-inflammatory cytokine involved in the immune response to intracellular pathogens. T$_{reg}$ cells control effector T-cell responses and suppress the development of functional inflammatory and immune reactions. Th17 cells mainly secrete IL (interleukin)-17 and, to a lesser extent, IL-21, IL-22, IFNγ and TNFα (tumour necrosis factor α), which promote tissue inflammation by induction of pro-inflammatory chemokines and recruitment of leucocytes [16]. Th22 cells have been described as a new Th subset exclusively producing IL-22, but not IL-17 or IFNγ [17,18]. Consistent with a role of adaptive immunity in hepatic inflammation, NASH was found to be associated with a Th1 cytokine response characterized by IFNγ and TNFα secretion in mice [10,19], and genome-wide analysis showed that stimulation of primary hepatocytes with IL-17 induced the expression of several inflammation-associated genes, including chemokines and C-reactive protein [20]. Finally, adaptive immunity responses may directly affect different steps of NAFLD evolution towards more severe liver diseases. In addition to their pro-inflammatory activity, IL-17 and IL-22 cytokines can, in fact, directly signal to tissues inducing damaging or protective effects respectively. In particular, IL-17 treatment increases hepatocyte steatosis induced by fatty acids [11], whereas its neutralization attenuates LPS (lipopolysaccharide)-induced hepatitis of fatty liver in mice [11]. Additionally, IL-17RA (IL-17 receptor A)-deficient mice exhibit decreased hepatic damage upon feeding on a high-fat diet [21] or fibrosis upon bile duct ligation and carbon tetrachloride administration [22]. On the other hand, IL-22 up-regulates several antioxidant and anti-apoptotic genes in different epithelial cells, including hepatocytes [23].

The above observations indicate that innate immunity responses mediated by one or more of the CD4$^+$ Th cell types can act as a critical determinant in the pathogenesis of the progression of NAFLD/NASH/cirrhosis. To date, however, no data are avail-

able on the different CD4$^+$ Th cell subsets infiltrating the liver during NASH or on their possible variation during the different phases of NASH development. More importantly, no definitive demonstration of a critical role of CD4$^+$ Th cell reactions in inducing NASH development is available.

To investigate these issues, in the present study we evaluated the occurrence, the changes and the possible role of infiltrating Th1 (CD4$^+$IFNγ^+), Th17 (CD4$^+$IL-17$^+$), Th22 (CD4$^+$IL-22$^+$) and T$_{reg}$ (CD4$^+$CD25$^+$FoxP3$^+$, where FoxP3 is forkhead box P3) lymphocytes during NASH development in C57BL/6 WT (wild-type) and IL-17-deficient (IL-17$^{-/-}$) mice fed on the MCD (methionine choline-deficient) diet.

MATERIALS AND METHODS

Animals and experimental protocol

Male C57BL/6 mice (8 weeks of age) purchased from Harlan Nossan were fed for 4 or 8 weeks with either the MCD diet or a control diet (Laboratorio Dottori Piccioni). Mice were killed after 1, 2, 4 or 8 weeks of feeding on this diet, and livers were collected for pathological and immunological evaluation. IL-17$^{-/-}$ C57BL/6 mice were obtained from Dr Iwakura (Center for Experimental Medicine Institute of Medical Science, University of Tokyo, Tokyo, Japan) and generated and genotyped as reported in the literature [24]. Animal studies were carried out in accordance with the U.S. National Institutes of Health guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication no. (NIH) 85-23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.] and all experimental procedures were approved by the Italian Ministry of Health and by the Commission for Animal Care of the University of Torino or Piemonte Orientale.

Biochemical analysis

Plasma ALT (alanine aminotransferase) and liver TGs (triacylglycerols) were determined using spectrometric kits (Radim and Sigma Diagnostics).

Histology and histochemistry

Steatosis, lobular inflammation and other hepatic histological changes were scored blind according to the methods of Kleiner et al. [25] in haematoxylin/eosin-stained liver sections. Fibrotic changes were evaluated in liver sections stained using the Gomori silver method.

Intrahepatic lymphocyte isolation and flow cytometry analysis

HMNCs (hepatic mononuclear cells) were isolated from livers by mechanical dissection, gently passed through a 20-gauge stainless steel mesh, and then suspended in RPMI 1640 medium containing 10% (v/v) FBS. Cell suspensions were centrifuged at 1500 rpm for 10 min and pellets were resuspended in 40% Percoll solution containing 100 units/ml heparin, followed by loading on to the 70% Percoll layer and then re-centrifuged at 1500 rpm for 20 min. The HMNCs obtained from the Percoll interface were used for flow cytometry. For evaluation of T$_{reg}$ cells, 10^6

HMNCs were stained with FITC-conjugated anti-mouse CD4 and PerCP (peridinin chlorophyll)-conjugated anti-mouse CD25 (BioLegend) mAbs and washed in PBS (Sigma) with 0.1% sodium azide and 2% (w/v) BSA (Sigma). Cell pellets were fixed and permeabilized with Fix/Perm buffer (eBioscience) and then stained with APC (allophycocyanin)-conjugated anti-mouse/rat FoxP3 mAb (eBioscience). To detect intracellular cytokine production, 10^6 HMNCs were cultured in 48-well plates and activated with 50 ng/ml PMA, 500 ng/ml ionomycin and 10 ng/ml brefeldin A (Sigma) for 18 h. Cells were then harvested, washed in PBS/1% (w/v) BSA and labelled on the surface with FITC-conjugated anti-mouse CD4 monoclonal antibodies. Cells were then washed and fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature; after washing, cells were permeabilized with 5% saponin (Sigma) in PBS and labelled intracellularly with PerCP-conjugated anti-mouse IL-17, phycoerythrin-conjugated anti-mouse IFNγ and APC-conjugated anti-mouse IL-22 mAbs (BioLegend). Stained cells were acquired on a FACSCalibur instrument and analysed using CellQuest (BD Biosciences).

mRNA extraction and real-time PCR
Liver RNAs were retrotranscribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed in a Techne TC-312 thermal cycler using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNFα, TGFβ (transforming growth factor β), CCL20 (CC chemokine ligand 20), α-SMA (α-smooth muscle actin), procollagen and β-actin (Applied Biosystems). All samples were run in duplicate and the relative gene expression was calculated as $2^{-\Delta CT}$ over that of the housekeeping β-actin gene. Results were expressed as fold increases over the control samples.

Isolation and treatment of liver cells
Liver cells were isolated by liver perfusion with collagenase digestion. Hepatocytes were obtained by differential centrifugation at 50 $\boldsymbol{g}$ for 5 min at 4 °C. Hepatocyte suspensions (purity >95%) were plated on collagen-coated culture dishes and cultured for 48 h in DMEM (Dulbecco's modified Eagle's medium)/Ham's F12 containing 10% (v/v) FBS, 1% penicillin/streptomycin and 1% glutamine. For lipotoxicity assays [26], hepatocytes were incubated with fresh medium supplemented with 50 μmol/l PA (palmitic acid) (Sigma) in the presence or absence of 10 nmol/l recombinant murine IL-17 and or IL-22 (eBioscience).

Analysis of PTEN expression and the phosphorylation state of Akt and JNK
Protein extracts were separated by SDS/PAGE (10% gel), blotted on to nitrocellulose membranes and probed with antibodies against phospho-Akt (Ser^{473}), Akt, phospho-JNK (c-Jun N-terminal kinase) (Thr^{183}/Tyr^{185}) and JNK (Cell Signaling Technology) and PTEN (phospatase and tensin homologue deleted on chromosome 10) (Santa Cruz Biotechnology). The anti-actin monoclonal antibody (Sigma) was used to assess equal protein loading. Antigens were detected by Western Lightning Chemiluminescence Reagent Plus (ECL) (PerkinElmer) and VersaDoc

3000 quantitative imaging system (Bio-Rad Laboratories). Results are expressed as ratios.

Determining cell viability
Cell viability was evaluated by nuclear fluorescence staining with propidium iodide using a FACScan analyser (Becton Dickinson) and CellQuest software (Becton Dickinson) [27].

Statistical analyses
Statistical analyses were performed using one-way ANOVA with Tukey's correction for multiple comparisons or the Kruskal–Wallis test for nonparametric values. Significance was set at 5% ($P < 0.05$). Normal distribution was preliminarily assessed using the Kolmogorov–Smirnov test.

RESULTS

Th17 cells are associated with NASH initiation
Infiltration of the different populations of CD4$^+$, Th1, Th17, Th22 and T$_{reg}$ cells during NASH development was evaluated in the liver of C57BL/6 mice fed for up to 8 weeks on an MCD diet. The MCD model lacks some critical features of human NASH (such as obesity and insulin resistance), but presents the unique advantage of unifying, in a single model, all of the different phases typical of NASH development in patients [28]. This feature allowed us to characterize the kinetic and the hepatic levels of different CD4$^+$ T-cell subsets during the early phase of steatohepatitis development (first week), established steatohepatitis (second to fourth weeks) and evolution steatohepatitis/fibrosis (fourth to eighth week) as measured by ALT transaminase release, hepatic TG content, histological changes and pro-inflammatory/profibrotic cytokine content. In accordance with previous observations [26], the MCD diet induced a progressive increase in hepatic TG content (Figure 1A) that paralleled the extension of steatosis (Figure 1B). There was a significant increase in ALT release after just 1 week on the MCD diet, which peaked at the second week and remained significantly higher than that of mice receiving the ND (normal diet) at the fourth and eighth weeks (Figure 1A). Liver injury, confirmed histologically as ballooning of liver cells, microgranulomas, megamitochondria and Mallory's hyaline bodies, was associated with a progressive increase in inflammatory foci and TNFα production (Figures 1B and 1C). At the fourth week, there was an increase in pro-fibrotic gene expression (α-SMA and pro-collagen) (see Figure 3F), which was associated with the appearance of fields of fibrosis, mainly with perisinusoidal distribution (fourth to eighth weeks) (Figure 1B, and see Figure 3E).

During the 8 weeks of being fed on the MCD diet, the percentage of CD4$^+$ cells which infiltrated the liver increased significantly compared with the ND-fed mice (Figure 2A). Of interest, as the disease progressed, activation was increased, evident by the expression of CD25 molecules on their membranes (Figure 2A). To understand the role of CD4$^+$ cells during NASH, we evaluated the infiltration of T$_{reg}$ (CD4$^+$CD25$^+$FoxP3$^+$), Th1 (CD4$^+$IFNγ^+), Th17 (CD4$^+$IL-17$^+$) and Th22 (CD4$^+$IL-22$^+$) cells in HMNCs by flow cytometry (Figures 2B and 2C). There
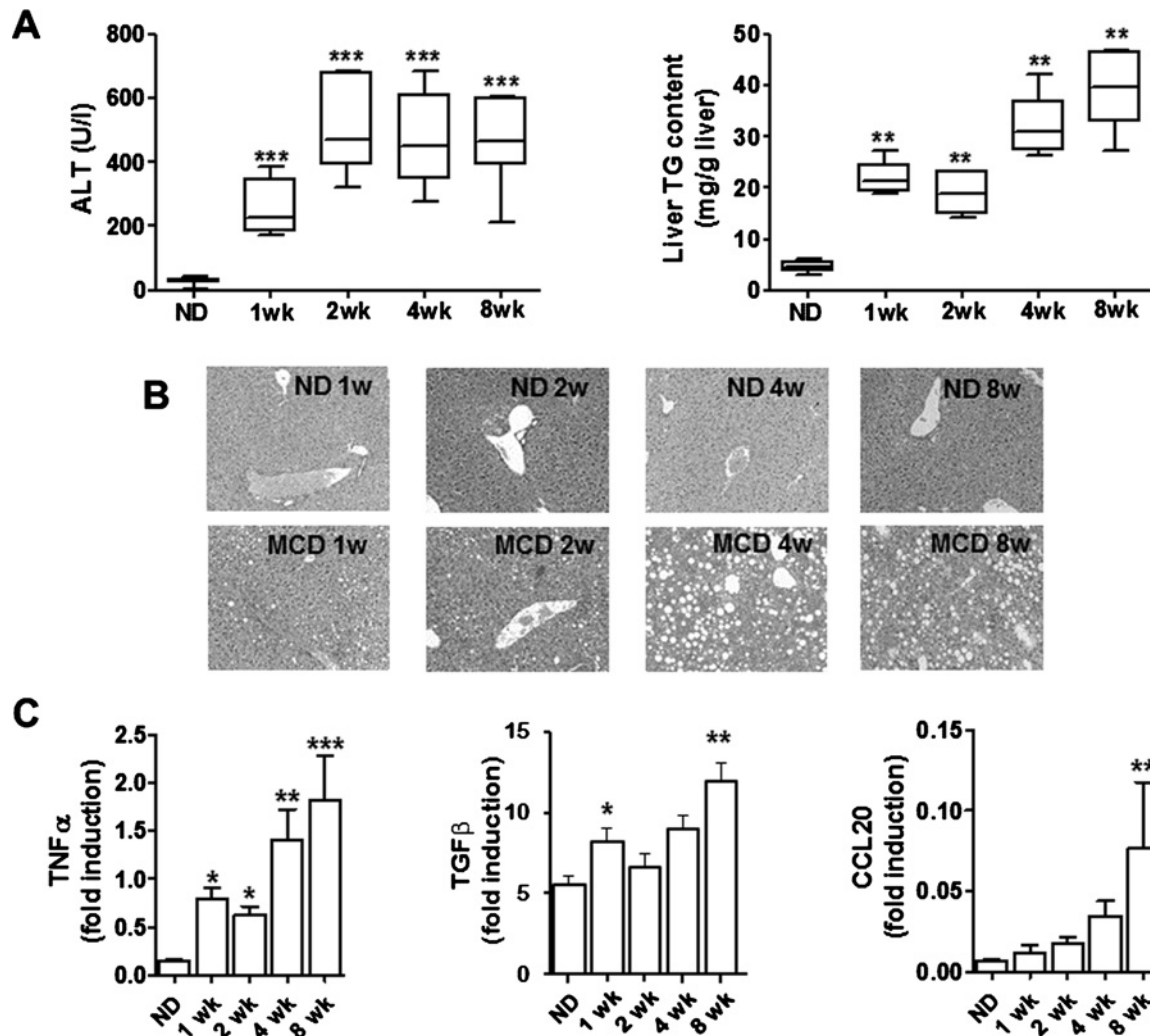
Figure 1   Hepatic damage and TNFα, TGFβ and CCl20 levels in mice fed on an MCD diet
C57BL/6 mice were fed with either an ND or an MCD diet for 8 weeks. (**A**) Serum ALT release and liver TG content. The results are for eight to ten animals in each group and the boxes include the values within the 25th and 75th percentiles, with horizontal bars representing the medians. The extremities of the vertical bars (tenth–90th percentile) comprise the 80% values. (**B**) Representative haematoxylin/eosin-stained liver section from the ND and MCD diet-fed mice. (**C**) TNFα, TGFβ and CCL20 mRNA levels in the liver. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (one-way ANOVA). w/wk, week.

was no difference in the percentage of T_{reg} cells in the liver in MCD diet-fed mice compared with ND-fed mice. Th1 cells did not significantly change during the first 4 weeks of the MCD diet, but significantly increased at the eighth week ($11.7\pm2.1\%$) when fibrosis was established in the liver of MCD diet-fed mice. On the other hand, the percentage of Th17 cells significantly increased by 4.7 fold just after 1 week of the MCD diet ($5.2\pm1.3\%$) compared with ND-fed mice ($1.1\pm0.3\%$). The percentage of Th17 cells then decreased, but remained significantly higher compared with ND-fed mice ($3.3\pm0.5\%$ at 2 weeks and $3.3\pm0.6\%$ at 4 weeks), and at 8 weeks there was a further increase in the levels compared with 1 week ($6.9\pm1.9\%$). Of interest, in MCD diet-fed mice, the kinetics of Th22 cells displayed a complete reverse modulation as they significantly increased after 2 and 4 weeks on the diet ($6.4\pm1.5\%$, and $6.1\pm1.3\%$), and decreased after 8 weeks ($4.2\pm1.2\%$). This unique IL-17 and IL-22 production pattern was

also observed in stimulated HMNCs (Figure 2D). These cytokine serum levels did not change during the time on the MCD diet (Figure 2E), indicating that perturbations of the immune response due to the MCD diet were restricted to the liver.

Several signals can drive Th17 or Th22 polarization [29]. Among them, a major role is played by IL-6, TNFα and TGFβ, which can drive naïve T-cells to differentiate into IL-17-producing cells, whereas the same cytokines, except for TGFβ, promote IL-22 expression [29]. Recent evidence has shown that IL-22 itself is able to induce recruitment of Th17 cells through a CCL20-mediated process [23]. We therefore investigated whether production of IL-6, TNFα, TGFβ and CCL20 was detectable during NASH development by stimulated HMNCs or in the liver tissue. The increase in IL-17 in activated HMNCs after 1 week of the MCD diet was accompanied by the production of IL-6 and TNFα pro-inflammatory cytokines and of CCL20 (Figure 2D).
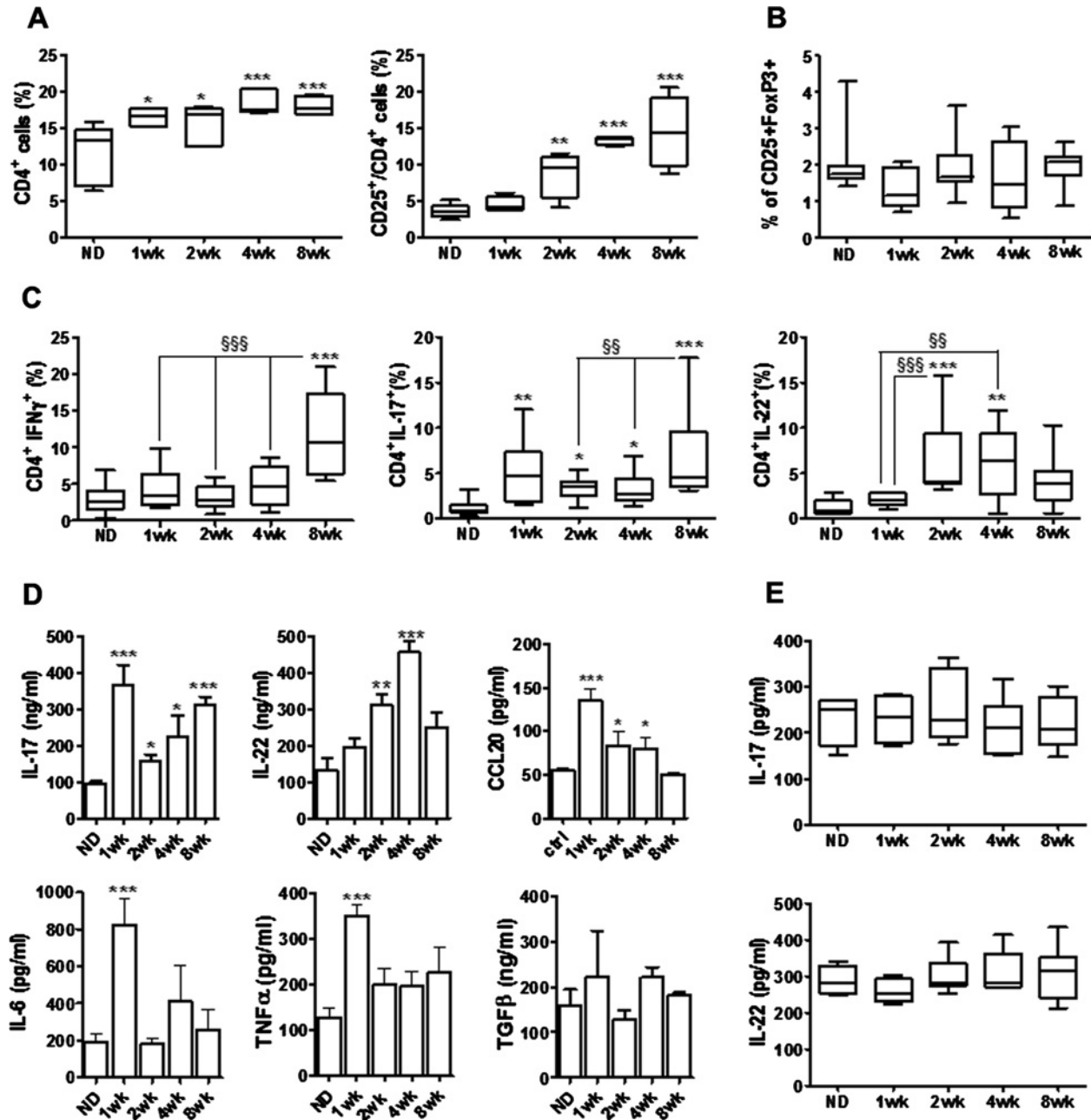
Figure 2 Hepatic infiltration of CD4+ cells and HMNC cytokine/chemokine production during NASH

HMNCs were isolated from C57BL/6 mice fed with an ND or MCD diet for 8 weeks and treated as described in the Materials and methods section for FACS analysis of CD4+, CD4+CD25+ cells (**A**), T_{reg} (**B**) and of Th1, Th17 and Th22 (**C**) cells. IL-17, IL-22, CCL20, IL-6, TNFα and TGFβ production from HMNCs activated *in vitro* with anti-CD3/CD28 and detected by ELISA (**D**). IL-17 and IL-22 serum levels (**E**). Results are for eight to 12 animals in each group and the boxes include the values within the 25th and 75th percentiles, with horizontal bars representing the medians. The extremities of the vertical bars (tenth–90th percentiles) comprise the 80% values. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ compared with ND-fed mice; §§$P < 0.001$, §§§$P < 0.0001$ for the comparisons indicated (one-way ANOVA). ctrl, control; wk, week.

CCL20 secretion by HMNCs was also evident at the second and fourth week of the MCD diet (Figure 2D), whereas its mRNA level in the liver significantly increased at the eighth week (Figure 1C). In the liver, TNFα mRNA stably increased from the first to the eighth week of the MCD diet (Figure 1C). Interestingly, hepatic RNA levels of TGFβ increased significantly at the first

week of the MCD diet, decreased at the second week and then significantly increased again at the fourth and eighth week of the MCD diet (Figure 1C). The pro-fibrogenic cytokine TGFβ classically initiates the reparative processes that follow extensive damage, and is mainly produced in the liver by Kupffer cells to begin with, and then by activated HSCs (hepatic stellate cells)

[30]. This canonical model can therefore account for the late increase in TGFβ found at the fourth and eighth weeks of the MCD diet, but not for the early increase detected at the first week. Previous observations indicate, however, that, even in the absence of major liver damage, steatosis could directly promote the production of inflammatory and pro-fibrogenic factors by hepatic cells, such as TGFβ [31]. Accordingly, employing the *in vitro* model of steatotic primary mouse hepatocytes exposed for up to 24 h to palmitate (50 μmol/l), we detected an increase in TGFβ mRNA production at the 16th and 24th hours of treatment (see Figure 4E), which was not affected by IL-17 and/or IL-22 addition (Figure 4F).

MCD-induced NASH is promoted by IL-17 and antagonized by IL-22

To clarify the role of IL-17 production in initiating and sustaining liver damage, we induced NASH in IL-17$^{-/-}$ mice. After 1 and 4 weeks on the MCD diet, IL-17$^{-/-}$ mice displayed lower serum ALT levels (Figure 3A), a lower TG content (Figure 3B) and a significant reduction in the Kleiner index, which was particularly marked at the fourth week of the diet (Figure 3C). Upon histological analysis, we observed no differences in terms of liver architecture and cellular changes in either WT and IL-17$^{-/-}$ control mice. In mice who had been fed for 4 weeks with the MCD diet, marked histological changes were found in the WT, mainly characterized by fairly extensive macro- and micro-vesicular steatosis and two to four foci of lobular inflammation per ×200 field. Moreover, in these cases, a combined pattern of perisinusoidal and periportal fibrosis was evident in histochemical staining (Figure 3D). In IL-17$^{-/-}$ mice, livers showed slight steatosis and lobular inflammation and only a small number of foci of perisinusoidal fibrosis. Gomori silver staining also demonstrated no signs of fibrosis fields (Figure 3E) in IL-17$^{-/-}$ mice and, consistently, IL-17$^{-/-}$ livers displayed a reduction in α-SMA and pro-collagen RNA expression compared with WT (Figure 3F).

Analysis of the CD4$^+$ cells showed no differences in Th1 and T$_{reg}$ (Figure 3G) cell percentages in the liver of IL-17$^{-/-}$ and WT mice. By contrast, IL-17$^{-/-}$ livers displayed a significant increase in Th22 cells compared with WT livers (Figure 3G) after 1 and 4 weeks of the MCD diet. Thus, in IL-17$^{-/-}$ mice, the reduced liver injury was associated with the absence of IL-17, and also with the increase in IL-22-producing cells.

To investigate the relative role of IL-17 and IL-22 production in the modulation of liver damage, we analysed their effects in the *in vitro* model of hepatocyte lipotoxicity induced by palmitate treatment (Figure 4). Primary mouse hepatocytes were untreated or treated with PA (50 μmol/l) in the presence of IL-17 (10 nmol/l) or IL-22 (10 nmol/l), administered either alone or together. IL-17 significantly exacerbated the lipotoxic effects of PA, evident from the increased phosphorylation of Thr^{183}/Tyr^{185} of JNK-1/JNK-2 (Figure 4A), the central mediator of hepatocyte lipoapoptosis [9,26] and by the decreased cell viability (Figure 4D). Conversely, IL-22 treatment reduced the loss of cell viability induced by PA (Figure 4D), and this protection was associated with a sharp increase in the phosphorylation of Akt (Figure 4B), the downstream mediator of the anti-apoptotic and protective effects of PI3K (phosphoinositide 3-kinase)

[32], and with a reduction in JNK phosphorylation (Figure 4A). These protective effects of IL-22 were reversed by inhibiting PI3K with wortmannin (250 nmol/l) or Ly294002 (75 μmol/l). On the other hand, JNK inhibition by SP600125 (10 μmol/l) prevented the loss of cell viability induced by PA with or without IL-17 and by PA with IL-22 plus PI3K inhibitors (Figures 4A, 4B and 4D). When PA was administered in combination with IL-17, IL-22 did not induce Akt phosphorylation and failed to prevent JNK activation and lipotoxicity (Figures 4A, 4B and 4D). Remarkably, the capacity of IL-17 to inhibit IL-22 cytoprotection was associated with the up-regulation of the PI3K inhibitor PTEN [32] (Figure 4C).

These data indicate that IL-17 exacerbated hepatocyte lipotoxicity by sustaining the activation of the JNK pathway and that IL-22 prevented the toxic effects of PA treatment, by PI3K-dependent inhibition of JNK. This protective activity of IL-22 was attenuated, however, in the presence of IL-17 which, by increasing PTEN, could inhibit the PI3K-mediated protection. Consistently, in IL-17$^{-/-}$ mice fed for 4 weeks on the MCD diet, the protection of hepatic damage was associated with decreased JNK dual phosphorylation (Figure 5A), a prevention of PTEN increase (Figure 5B) and a concomitant increase in Akt phosphorylation (Figure 5C).

DISCUSSION

The present study shows that NASH development in mice fed with the MCD diet is critically affected by the infiltration of activated CD4$^+$ cells in the liver and in particular by Th17 and Th22 cells. Several lines of evidence support these observations. (i) NASH initiation and progression to fibrosis is associated with a biphasic increase in the liver content of Th17 lymphocytes. The decrease in Th17 cells after NASH initiation and before liver fibrosis corresponds to an increase in Th22 cells. (ii) IL-17$^{-/-}$ mice are protected by NASH development and are characterized by an extensive liver infiltration of Th22 lymphocytes. (iii) IL-17 exacerbates the JNK-dependent hepatocyte lipotoxicity *in vitro* induced by palmitate, whereas IL-22 protects it via PI3K/Akt-mediated inhibition of JNK. The protective activity of IL-22 is only effective, however, in the absence of IL-17, which increases the level of the PI3K inhibitor PTEN and prevents PI3K/Akt activation. Consistently, livers of IL-17$^{-/-}$ mice fed with the MCD diet display decreased JNK activation, reduced PTEN expression and increased Akt phosphorylation compared with the livers of WT mice.

We are aware that NASH induced by the MCD diet lacks some of the key features of the human disease, such as obesity and insulin resistance [28]; however, in the present study, we exploited the capacity of the MCD diet-induced NASH to cause extensive steatohepatitis and its evolution to fibrosis [28]. This progression is typical of human NASH and cannot be reproduced by other NASH models of mice such as feeding with a high-fat diet [28]. Taking advantage of the effects of MCD diet feeding, we have shown that the two critical phases of human NASH development, namely the steatosis–steatohepatitis transition and
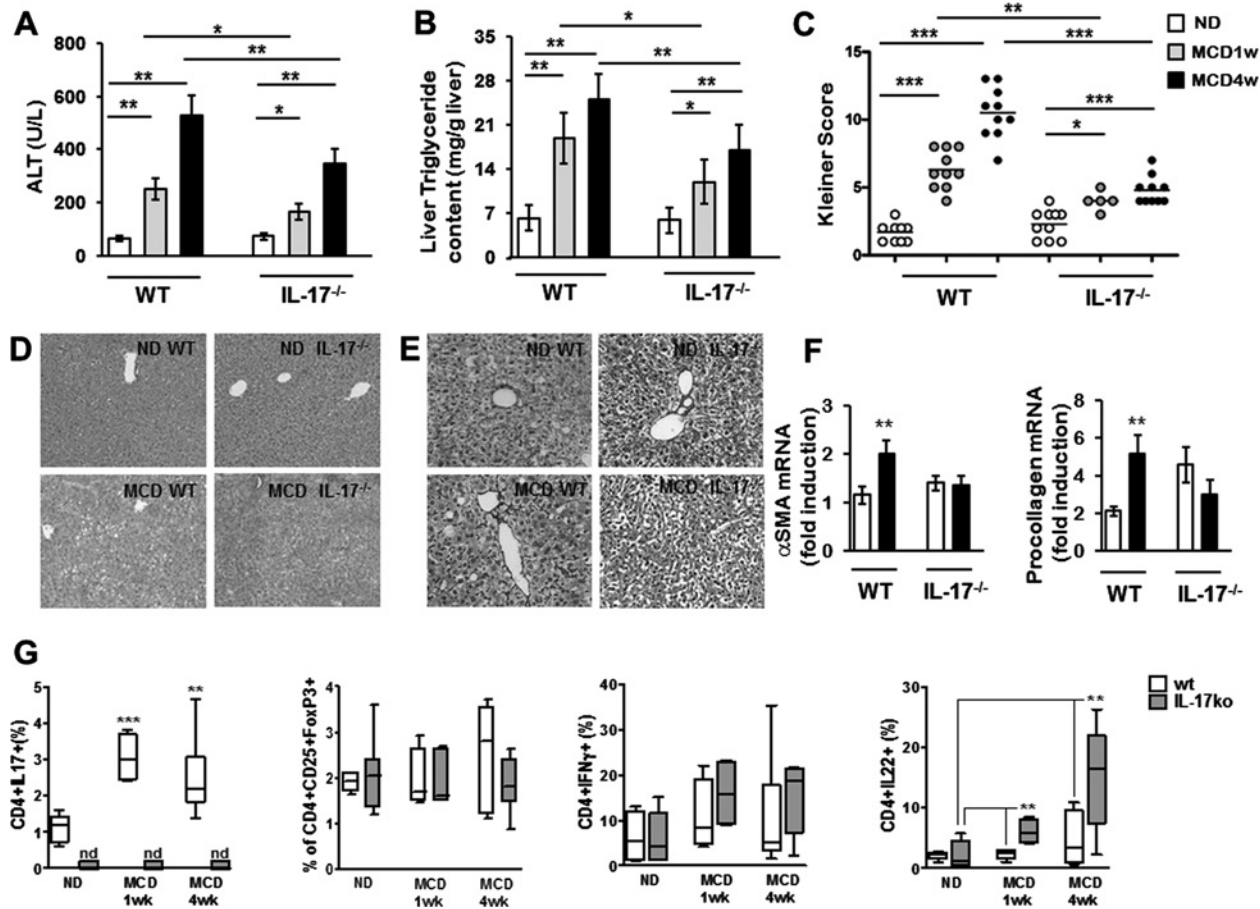
Figure 3   IL-17 absence abrogates NASH and induces Th22 cell expansion
C57BL/6 WT and IL-17$^{-/-}$ mice were fed with either an ND or an MCD diet for 1 and/or 4 weeks. (**A**) Serum ALT release. (**B**) Liver TG content. (**C**) Kleiner score (1 and 4 weeks, indicated as 1w and 4w respectively). (**D**) Haematoxylin/eosin-staining histochemical evaluation. (**E**) Gomori silver-staining histochemical evaluation. (**F**) α-SMA and procollagen mRNA (4 weeks). (**G**) *Ex vivo* FACS analysis of Th17, T$_{reg}$, Th1 and Th22 cells (1 and 4 weeks). ko, knockout; nd, not detectable. Results are for five to 12 animals in each group and the boxes include the values within the 25th and 75th percentiles, with the horizontal bars representing the medians. The extremities of the vertical bars (tenth–90th percentiles) comprise the 80% values. Results are means$\pm$S.E.M. *$P < 0.05$,**$P < 0.001$, ***$P < 0.0001$ (one-way ANOVA).

the steatohepatitis–fibrosis transition, were associated with a peak of hepatic infiltration of Th17 cells. These data correlate well with those showing that Th17 cells and IL-17 release were responsible for inducing steatohepatitis produced by LPS treatment of steatotic mice [11] and with those reporting that, in the same model, IL-17A stimulated hepatic damage independently of liver lipid accumulation, by promoting inflammatory oxidative reactions [21]. Our results show, for the first time, an additional earlier hepatotoxic effect of IL-17, which is apparently unrelated to inflammation and is, instead, associated with lipotoxicity. Infiltration of Th17 cells was already evident at the first week before the induction of extensive liver damage (second week) and the appearance of sustained inflammatory reactions. IL-17 exacerbates the lipotoxic effect of palmitate by sustaining the activation of JNK, a central marker of NASH-associated damage in patients and in mouse models [9]. Moreover, according to reports with IL17RA-deficient mice [21], total genetic ablation of IL-17 prevents NASH development. These observations indicate that the early production of IL-17 by CD4$^+$ Th cells can represent a first determinant for NASH initiation. The association of the second peak of Th17 cell infiltration and, consequently, of the production of IL-17-mediated responses with the appearance of liver fibrosis is therefore in agreement with the evidence on the role of IL-17 in inducing hepatic fibrogenesis and fibrosis [21,22]. Meng et al. [22] found that IL-17 signalling was critical for producing liver fibrosis in the carbon tetrachloride and bile duct ligation mice model and that this effect was due to an IL-17 targeting of Kupffer cells and HSCs. We have shown that an increase in Th17 cells was associated with the NASH evolution to fibrosis, and that IL-17$^{-/-}$ mice were protected from producing pro-fibrotic factors, HSC activation and fibrosis. These results indicate that in NASH and, at least in the NASH model of mice fed with the MCD diet, Th17 cells and IL-17 production critically affects fibrosis development, thus supporting the appealing idea of a role for the immune system in the regulation of liver fibrosis [33].
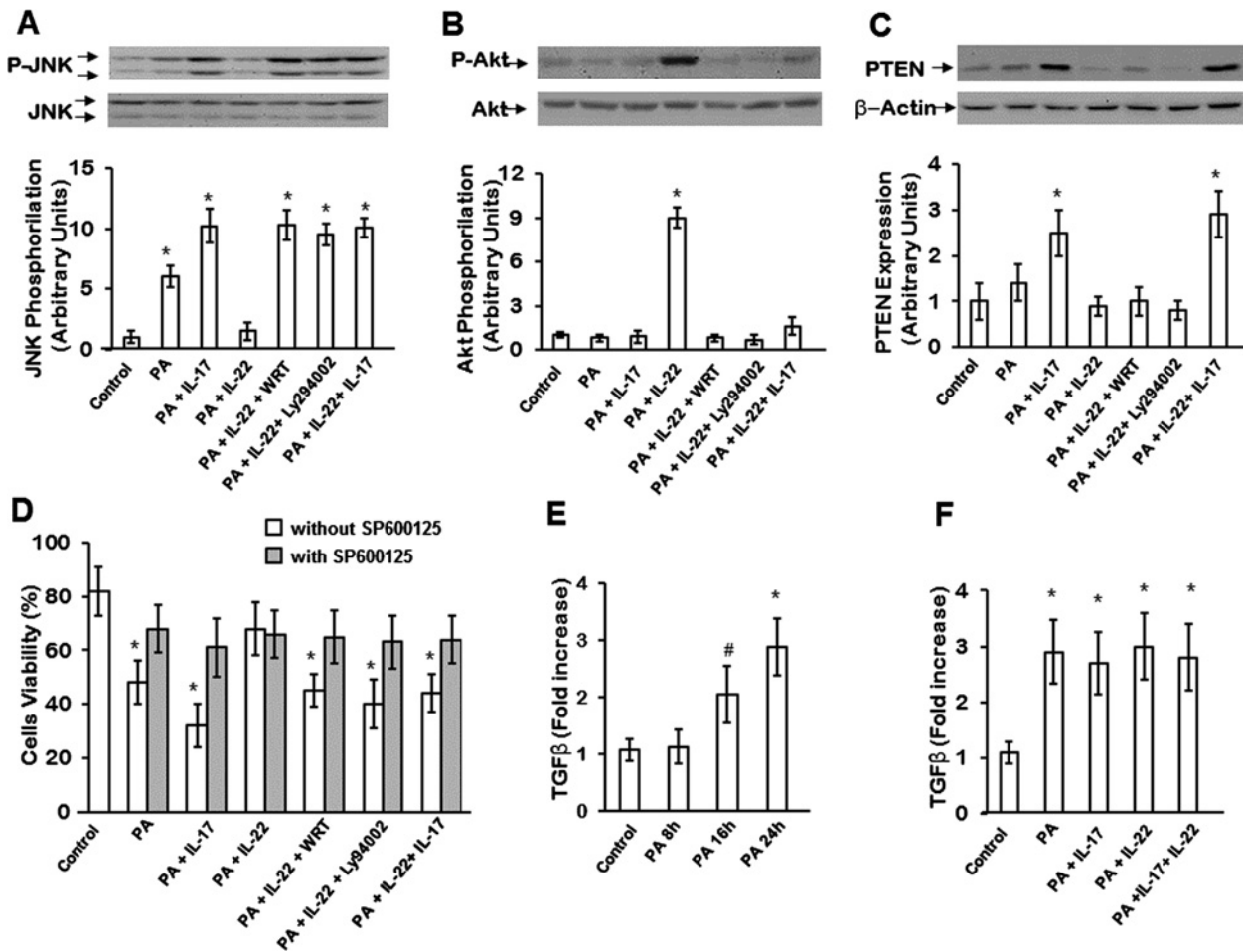
Figure 4   IL-17 and IL-22 modulate hepatocyte lipotoxicity induced by PA

Primary mouse hepatocytes were exposed to PA (50 μmol/l) in the presence or absence of IL-17 (10 nmol/l) or IL-22 (10 nmol/l) with or without the PI3K inhibitors wortmannin (WRT; 250 nmol/l) or Ly294002 (75 μmol/l) or the JNK inhibitor SP600125 (10 μmol/l). JNK-1/2 (**A**) and Akt (**B**) phosphorylation, PTEN level (**C**), cell viability (**D**) and TGFβ levels (**E**) and (**F**) evaluated respectively after 180 (**A**) or 30 min (**B** and **C**), 8, 16 and 24 (**E**) or 24 h (**D** and **F**) of PA treatment. Results are means$\pm$S.E.M. for six different experiments for cell viability assays and four different experiments for the remaining data. *$P < 0.001$, #$P < 0.05$ (one-way ANOVA).

An intriguing aspect of our findings regards the specular hepatic infiltration of Th17 and Th22 cells during NASH development, and the different effects of IL-22 production in the presence (WT mice) or absence (IL-17$^{-/-}$ mice) of IL-17. These issues meet the stimulating discussion on the protective/toxic effect of IL-22 and on the relationship between IL-17/IL-22 production [34]. We found that Th22 cells increased after the first peak of Th17 cells and that, in IL-17$^{-/-}$ mice, the prevention of NASH development was associated with an extensive increase in hepatic infiltration of Th22 cells, which occurred after just 1 week of the diet, as well as with a lower hepatic TG content, a decrease in JNK activation and an increase in Akt phosphorylation. We also observed that, *in vitro*, IL-22 reduced hepatocyte lipotoxicity by Akt-dependent inhibition of JNK. These results support the idea of the hepatoprotective function of Th22 cells and are fully consistent with the reports on the hepatoprotective effects of IL-22 delivery as well as on the detrimental effects of IL-22 genetic deletion in other models of liver toxicity, such as

concanavalin A, carbon tetrachloride or Fas ligand [34,35]. Our observations also support the findings on the ability of IL-22 to reduce liver fat content and liver injury in alcoholic steatohepatitis and NASH by inhibiting lipogenesis [36,37]. On the other hand, the Akt-mediated capacity of IL-22 to inhibit hepatocyte damage is consistent with the reported ability of IL-22 to induce the activation of cytoprotective signal pathways [34,35]. The Akt-dependent protection produced by IL-22 is also in agreement with the prevention of hepatocyte lipoapoptosis induced by PI3K-mediated inhibition of MKK4 (mitogen-activated protein kinase kinase 4)/SEK1 (stress-activated protein kinase/extracellular-signal-regulated kinase kinase-1), which is the upstream activator of JNK-1/2 [26]. In our NASH model, the increase in Th22 cells subsequent to that of Th17 cells may represent an attempt to counteract the injurious effects of IL-17, avoiding a further increase in hepatic damage. This increase in Th22 cells, however, is not enough to prevent the progression to fibrosis, and our *in vitro* experiments offer a first explanation
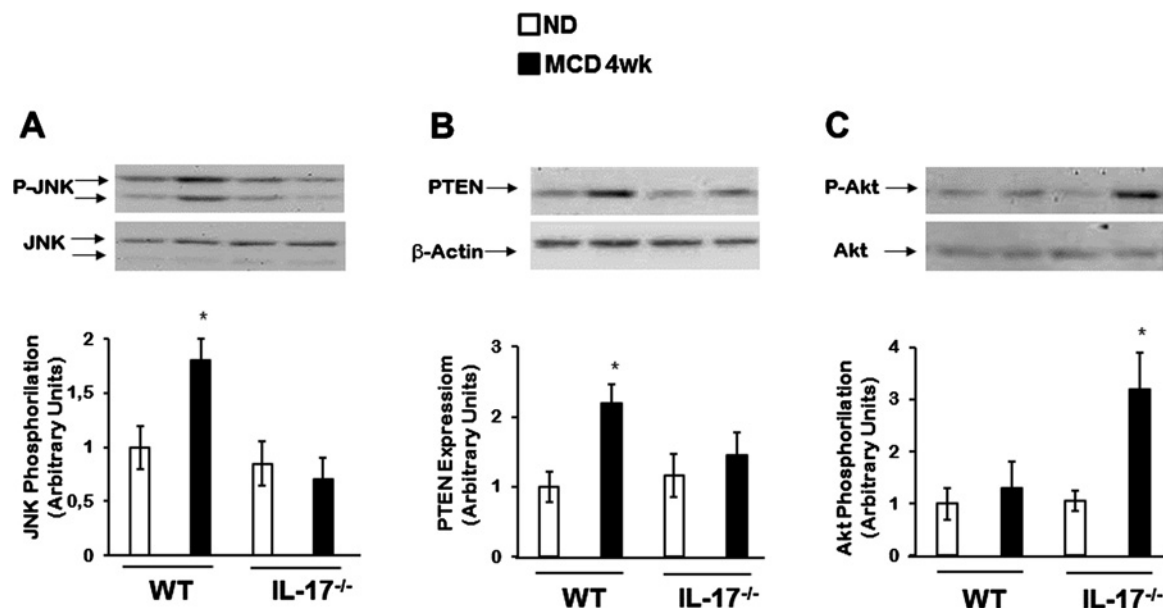
Figure 5   Increased Akt activation and decreased PTEN levels and JNK phosphorylation in IL-17$^{-/-}$ mice fed on an MCD diet
C57BL/6 WT and IL-17$^{-/-}$ mice were fed with either an ND or an MCD diet for 4 weeks (4wk). (**A**) JNK phosphorylation. (**B**) PTEN level. (**C**) Akt phosphorylation. Results are means±S.D. *$P < 0.001$ (one-way ANOVA).

of this apparent paradox. We have found, in fact, that, in the presence of IL-17, the protection of IL-22 was null, and that this effect was associated with the up-regulation of the PI3K inhibitor PTEN induced by IL-17 and to a corresponding reduced capacity of IL-22 to activate Akt. Consistently, we have observed that, *in vivo*, Th22 cells expansion only accompanied the prevention of NASH development in the complete absence of IL-17 (IL-17$^{-/-}$ mice) and that, in the liver of IL-17$^{-/-}$ mice, the increase in Akt phosphorylation corresponded to a decreased expression of PTEN that was, instead, up-regulated in the WT liver. Taken together, these results suggest that, in NASH, IL-22 is protective, but only in the absence of IL-17. This finding is in agreement with data reported by Sonnenberg et al. [38], who found that, during airway inflammation, IL-22 has pro-inflammatory effects, but only in the presence of IL-17.

A further mechanism can explain why, in the presence of IL-17, IL-22 not only avoids, but also may contribute to exacerbate liver fibrosis by enhancing Th17 cell recruitment. In an HBV (hepatitis B virus) transgenic mouse model of T-cell-mediated chronic liver inflammation and fibrosis, IL-22 is, in fact, responsible for promoting Th17 cell migration to the liver by stimulating HSCs to secrete CXCL10 (CXC chemokine ligand 10) and CCL20 [23]. The CCL20 receptor CCR6 (CC chemokine receptor 6) is expressed by Th17 cells, and these cells can produce CCL20. We observed that activated HMNCs produced high amounts of CCL20 at the first week of the MCD diet concomitantly with the first increase in Th17 cells, and to a lesser extent at the second and fourth weeks when Th22 cells enlarge. Moreover, we detected an increase in hepatic CCL20 mRNA, which reached a peak at the eighth week of the MCD diet, concomitantly with

the second peak of Th17 cells. Thus IL-22$^+$ cells appear to act as both cytoprotective (delaying NASH progression to fibrosis) and toxic (mediating the CCL20-dependent recruitment of Th17 cells with a transition to fibrosis) agents, and this unifies the contrasting theories on the 'protective' or 'pathological' role of IL-22 in a single model of NASH development [34].

Our study also analysed the liver infiltration of T$_{reg}$ and Th1 cells. We found no significant changes in the T$_{reg}$ cell subset, whereas we detected a significant increase in Th1 cells infiltration in mice after having been fed for 8 weeks with the MCD diet. This indicates that NASH initiation is not linked to changes in the immunosuppressive T$_{reg}$ cells, although steatosis was found to decrease the hepatic level of T$_{reg}$ cells [39]. On the other hand, the late infiltration of Th1 cells indicates that these cells are not critical for NASH induction even if they may contribute to the extension of hepatic damage.

In conclusion, the characterization of Th cell infiltration during NASH development has revealed a critical role for the Th17 cell subset in the initiation of NASH and development of fibrosis. The molecular mechanisms of the hepatotoxic action of IL-17 (increased JNK activation of steatotic hepatocytes) and of the protective effects of IL-22 (PI3K stimulation) support the possibility of the clinical employment of pharmacological inhibitors of JNK as well as PI3K activators. In addition, the observation of the prevention of NASH development in IL-17$^{-/-}$ mice associated with the protective increase in Th22 cells strongly supports the potentiality of the clinical application of IL-17-neutralizing agents which act through the double-protective effects to simultaneously inhibit IL-17-mediated hepatoxicity and allow IL-22-mediated hepatoprotection.

CLINICAL PERSPECTIVES

- Th17 and Th22 cells are associated with different liver responses during chronic and acute hepatic diseases, but their specific role(s) and reciprocal interactions remain to be determined. The widespread NAFLD (non-alcoholic fatty liver disease) is able to evolve, via mechanisms still unclear, from steatosis to steatohepatitis (NASH) and fibrosis summarizing the main pathological changes of hepatic tissue.
- We detected a biphasic infiltration of Th17 cells at the beginning of NASH development and the NASH–fibrosis transition. Th22 cells peaked between the first and the second expansion of Th17 cells and further increased in IL-17$^{-/-}$ mice that were also protected from NASH. *In vitro*, IL-17 exacerbated lipotoxicity and IL-22 prevented it, but in the absence of IL-17 which inhibited the cytoprotective pathways activated by IL-22.
- Our data support the clinical employment of IL-17 inhibitors, which can prevent steatohepatitis and fibrosis by both abolishing the lipotoxic action of IL-17 and allowing IL-22-mediated protection.

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