


# Synergistic effect of interleukin-17 and tumour necrosis factor- $\alpha$ on inflammatory response in hepatocytes through interleukin-6-dependent and independent pathways

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## Summary

The proinflammatory cytokines interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  are targets for treatment in many chronic inflammatory diseases. Here, we examined their role in liver inflammatory response compared to that of IL-6. Human hepatoma cells (HepaRG, Huh7.5 and HepG2 cells) and primary human hepatocytes (PHH) were cultured with IL-6, IL-17 and/or TNF- $\alpha$ . To determine the contribution of the IL-6 pathway in the IL-17/TNF- $\alpha$ -mediated effect, an anti-IL-6 receptor antibody was used. IL-17 and TNF- $\alpha$  increased in synergy IL-6 secretion by HepaRG cells and PHH but not by Huh7.5 and HepG2 cells. This IL-17/TNF- $\alpha$  synergistic cooperation enhanced the levels of C-reactive protein (CRP) and aspartate aminotransferase (ASAT) in HepaRG cell and PHH cultures through the induction of IL-6. IL-17/TNF- $\alpha$  also up-regulated IL-8, monocyte chemoattractant protein (MCP)-1 and chemokine (C-C motif) ligand 20 (CCL20) chemokines in synergy through an IL-6-independent pathway. Interestingly, first exposure to IL-17, but not to TNF- $\alpha$ , was crucial for the initiation of the IL-17/TNF- $\alpha$  synergistic effect on IL-6 and IL-8 production. In HepaRG cells, IL-17 enhanced IL-6 mRNA stability resulting in increased IL-6 protein levels. The IL-17A/TNF- $\alpha$  synergistic effect on IL-6 and IL-8 induction was mediated through the activation of extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase, nuclear factor- $\kappa$ B and/or protein kinase B (Akt)-phosphatidylinositol 3-kinase signalling pathways. Therefore, the IL-17/TNF- $\alpha$  synergistic interaction mediates systemic inflammation and cell damage in hepatocytes mainly through IL-6 for CRP and ASAT induction. Independently of IL-6, the IL-17A/TNF- $\alpha$  combination may also induce immune cell recruitment by chemokine up-regulation. IL-17 and/or TNF- $\alpha$  neutralization can be a promising therapeutic strategy to control both systemic inflammation and liver cell attraction.

**Keywords:** hepatocyte, inflammation, interleukin-6, interleukin-17, tumour necrosis factor- $\alpha$

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## Introduction

Interleukin (IL)-6 is a systemic proinflammatory cytokine playing a pivotal role in the acute-phase response to tissue injury, infection or inflammation [1,2]. This response is characterized by changes in the hepatic production of acute-phase proteins such as increased C-reactive protein (CRP) production. However, the acute-phase protein response and the production of IL-6 itself are also induced

by a long list of cytokines, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$  and IL-17A, also known as IL-17 [3,4].

Clinical inhibition of IL-6 with an anti-IL-6 receptor (anti-IL-6R) antibody has shown beneficial effects on the joint manifestations of rheumatoid arthritis, with a massive decrease of CRP levels [5–7]. However, IL-6 also has anti-inflammatory functions [8]. Indeed, IL-6-deficient mice developed liver inflammation, steatosis and insulin

resistance [9]. Moreover, IL-6 blockade has been associated with colon perforation [7,10], transaminase elevation [11] and adverse lipid changes [12,13], probably related to liver changes. Therefore, the IL-6 effects on hepatocytes remain not well known and some of the liver changes in chronic inflammation may reflect the effects of other cytokines, independently or not of IL-6.

Liver diseases are characterized by immune cell infiltrates following chemokine release [14]. Neutrophils are key players in the initiation of the inflammatory response. Because IL-17 is known to induce neutrophil-attracting chemokines such as IL-8, IL-17 contributes to liver inflammation by inducing the local recruitment of neutrophils [15]. As shown in various cells, including endothelial cells or skin and synovial fibroblasts, IL-17 can cooperate with TNF- $\alpha$  to induce *in-vitro* IL-8 and IL-6 secretion in synergy [16–19]. These two cytokines are also involved in several liver disorders [15,20–22]. In the liver, IL-17 was also able to activate hepatic stellate cells and CRP production by hepatocytes independently of IL-6 [3,23].

The objective of this study was to clarify the effects of IL-17 and TNF- $\alpha$  on the induction of the inflammatory response in hepatocytes and to determine the contribution of IL-6 in these effects. Because primary human hepatocytes (PHH) are from native liver, they are considered to be the gold standard approach to reflect the specific functionality and mediators of the human organ. Therefore, PHH and human hepatoma cell lines were used. IL-17 and TNF- $\alpha$  cooperated to increase CRP expression and aspartate aminotransferase (ASAT) level in hepatocyte cultures through the activation of the IL-6 pathway. Independently of the IL-6 pathway, IL-17 and TNF- $\alpha$  induced IL-8, monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-C motif) ligand 20 (CCL20) expression and/or production synergistically. These differences may help understanding of the liver situation in chronic inflammation.

## Materials and methods

### Cell cultures

The human hepatoma Huh7.5, HepG2 and HepaRG cells were cultured as described previously [24,25]. Proliferative HepaRG cells were used after 15 days post-plating and differentiated HepaRG cells were maintained in the same standard medium supplemented by dimethylsulphoxide (DMSO) 2% for 2 more weeks. PHH were isolated from surgical liver resections and cultured as reported [24]. The samples were collected according to the local ethical committee and the Ministry of Research, which approved the study (reference number: AC-2010-1164).

### Culture conditions

Hepatocytes were exposed to IL-6 5 ng/ml (R&D Systems, Minneapolis, MN, USA) or IL-17A 50 ng/ml (Dendritics,

Lyon, France) and/or TNF- $\alpha$  1 ng/ml (R&D systems). To block the IL-6, IL-17 or TNF- $\alpha$  pathways, tocilizumab (Roche, Welwyn, UK), anti-IL-17A (R&D Systems) and infliximab (MSD, Courbevoie, France) were used at 10  $\mu$ g/ml. A monoclonal antibody against the BetV1 allergen (Dendritics) was used as a control antibody at the same concentration. Exposures to nuclear factor-kappaB (NF- $\kappa$ B) inhibitor pyrrolidine dithiocarbamate, phosphoinositide 3-kinase (PI3K) inhibitor LY294002, protein kinase B (Akt) inhibitor A6730 (all from Sigma, St Louis, MO, USA) and mitogen-activated protein kinase (MAPK) inhibitors SP6000125 [c-Jun N-terminal kinase (JNK) inhibitor], SB203580 (p38 inhibitor), U0125 [mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) inhibitor] (all from Calbiochem, San Diego, CA, USA) at 1, 10, 20 and/or 100  $\mu$ M were added 1 h prior to cytokine addition. Cells were treated for 12 and 24 h for mRNA expression, 24 h for cytokine production and 120 h for CRP and transaminase levels.

### mRNA stability

HepaRG cells were treated with IL-17 and/or TNF- $\alpha$  for 12 h. Cells were then washed and incubated with 5  $\mu$ g/ml actinomycin D (Orphan Europe, Puteaux, France) to inhibit further transcription. Total mRNA was extracted following 0, 1, 2 and 3 h incubation with actinomycin D. Results were presented as % mRNA remaining compared with the steady-state level.

### Quantitative real time PCR

Total RNA was purified using an RNeasy<sup>®</sup> Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthesized using the iScript<sup>™</sup> kit (Bio-Rad, Hercules, CA, USA). Polymerase chain reaction (PCR) amplification was performed using the CFX96<sup>™</sup> real-time system instrument (Bio-Rad) with the iTaq<sup>™</sup> universal SYBR<sup>®</sup> green supermix (Bio-Rad) and the Qiagen QuantiTect<sup>®</sup> primers. Expression of the genes of interest was normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene.

### Enzyme-linked immunosorbent assays (ELISA)

Supernatant IL-6 and IL-8 concentrations were quantified with human ELISA kits, according to the manufacturer's instructions (R&D Systems).

### Western blotting

Proliferative HepaRG cells were exposed to high concentrations of TNF- $\alpha$  alone (10 ng/ml) or a combination of IL-17 (50 ng/ml) and TNF- $\alpha$  (1 ng/ml) for 30 min. Cells were lysed using the Halt<sup>™</sup> protease and phosphatase inhibitor cocktail kit (Thermo Scientific, Rockford, IL, USA) and the protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Equal

amounts of protein (50  $\mu$ g) were loaded and separated by 15% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred on nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% non-fat milk or 5% bovine serum albumin (BSA). Blots were incubated overnight at 4°C with specific antibodies against total and phosphorylated ERK1/2, Akt and NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) (Cell Signaling Technology, Leiden, the Netherlands). Blots were washed three times and incubated with a secondary antibody for 1 h at room temperature. Protein bands were detected with an enhanced chemiluminescence (ECL) detection kit (Bio-Rad). Blots were scanned and analysed using the Gel Doc™ XR+ Gel system (Bio-Rad) and Image Lab 5.2 software. Cyclophilin B quantification was used as loading controls. Results are shown as the ratio of the total and phosphorylated forms.

### Laboratory automated analyser

The CRP level was quantified by the automated analyser BN ProSpec® (Siemens, Erlangen, Germany) and transaminases by the Architect analyser (Abbot Diagnostics, Chicago, IL, USA).

### Statistical analysis

Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least three independent experiments  $\pm$  standard error of the mean (s.e.m.). Statistical differences were analysed using the Wilcoxon paired *t*-test. *P*-values less than 0.05 were considered significant.

## Results

### IL-17 and TNF- $\alpha$ combination increases IL-6 expression and production synergistically

IL-17 and TNF- $\alpha$  have been shown previously to induce a massive IL-6 production by various cell types in synergy [16,17]. Here, the IL-17/TNF- $\alpha$  effect on IL-6 was studied in hepatic cell lines and PHH. In HepaRG cells and PHH, the IL-17/TNF- $\alpha$  combination exposure increased the IL-6 secretion synergistically compared to IL-17 or TNF- $\alpha$  alone. However, the IL-6 production by Huh 7.5 and HepG2 cell lines was not affected by IL-17/TNF- $\alpha$  exposure (Fig. 1a). Based on these results, the HepaRG cell line was used for the following experiments. The IL-17/TNF- $\alpha$  synergistic effect was also observed on IL-6 mRNA in proliferative HepaRG cells, which was up-regulated by up to 44-fold (Fig. 1b).

Up-regulation of IL-17 and TNF- $\alpha$  receptors may contribute to this IL-17/TNF- $\alpha$  synergistic effect. IL-17 binds the heterodimer receptor complex, composed of the IL-17 receptor subunit A (IL-17RA) and the IL-17RC, whereas TNF- $\alpha$  acts through two independent receptors, the TNF receptor I (TNF-RI) and the TNF-RII. Only the TNF-RII was up-regulated in HepaRG cells following IL-17/TNF- $\alpha$

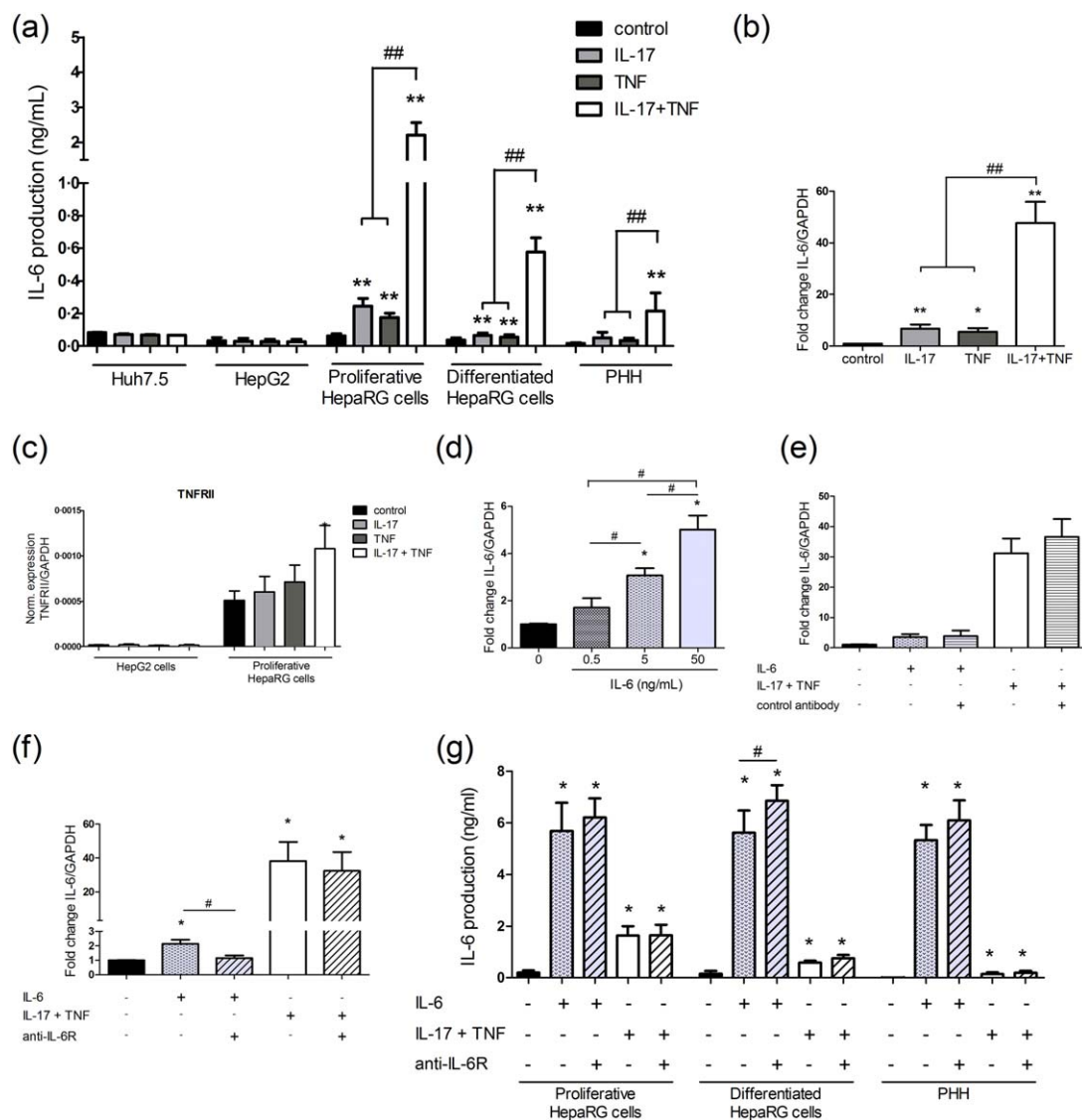
stimulation (Fig. 1c and data not shown). IL-17R and TNF-R expressions were also compared between the HepG2 and Huh7.5 cells, which did not produce IL-6 after the IL-17/TNF- $\alpha$  stimulation, and the proliferative HepaRG cells, which released IL-6 following the IL-17/TNF- $\alpha$  stimulation (Fig. 1a). The expression levels of TNF-RI, IL-17RA and IL-17RC were similar between the HepG2 and the HepaRG cell lines (data not shown). However, the TNF-RII mRNA levels were much lower in HepG2 (Fig. 1c) and Huh7.5 (data not shown) cell lines than in HepaRG cells. Therefore, TNF-RII may have a crucial role in the IL-6 production by IL-17/TNF- $\alpha$ .

To determine whether IL-6 may regulate its own mRNA, proliferative HepaRG cells were treated with different IL-6 concentrations and in the presence or not of an anti-IL-6R antibody. The specific effect of the anti-IL-6R antibody was verified with a control antibody which had no effect on the IL-6 mRNA and protein levels induced by IL-6 or IL-17/TNF- $\alpha$  (Fig. 1e and data not shown). Increasing IL-6 concentrations up-regulated IL-6 expression dose-dependently (Fig. 1d) and the IL-6R blockade inhibited this effect in proliferative HepaRG cells (Fig. 1f), indicating that IL-6 regulated its own mRNA expression positively and directly. However, the anti-IL-6R did not reduce the IL-6 up-regulation-induced IL-17/TNF- $\alpha$  significantly (Fig. 1f). The contribution of the IL-6-positive feedback loop in the IL-6 induction by IL-17/TNF- $\alpha$  was therefore very weak compared to the IL-17/TNF- $\alpha$  direct effect on IL-6. The IL-6 supernatant levels in hepatocyte cultures stimulated with IL-6 or IL-17/TNF- $\alpha$  were similar or slightly higher in the presence of the anti-IL-6R antibody (Fig. 1g). Because the anti-IL-6R blocks competitively the IL-6 binding to its receptor, the IL-6 free fraction level in supernatant increased in the presence of the anti-IL-6R antibody. This increase can be balanced by the IL-6 positive-feedback loop effect occurring in the absence of anti-IL-6R antibody.

### Induction of the IL-6-dependent CRP and ASAT level following IL-17 and/or TNF- $\alpha$ stimulation

IL-6 was shown to control CRP production [2,4]. Here, CRP expression was up-regulated significantly by IL-6 (19-fold), IL-17 (6-fold) and the synergistic IL-17/TNF- $\alpha$  combination (37-fold) *versus* control in proliferative HepaRG cells. The IL-6 pathway blockade reduced strongly the CRP mRNA level induced by IL-17 and/or TNF- $\alpha$  (Fig. 2a,b). Moreover, CRP production by PHH from different donors following IL-17/TNF- $\alpha$  stimulation correlated strongly with the PHH ability to produce IL-6 (Fig. 2c,d). Therefore, CRP up-regulation by IL-17 and/or TNF- $\alpha$  was mainly IL-6-dependent.

Liver inflammation may induce liver damage, as reflected by transaminase activity elevation in the clinic. In HepaRG cells and PHH cultures, ASAT levels increased after 5 days of IL-6 and IL-17/TNF- $\alpha$  stimulation. These



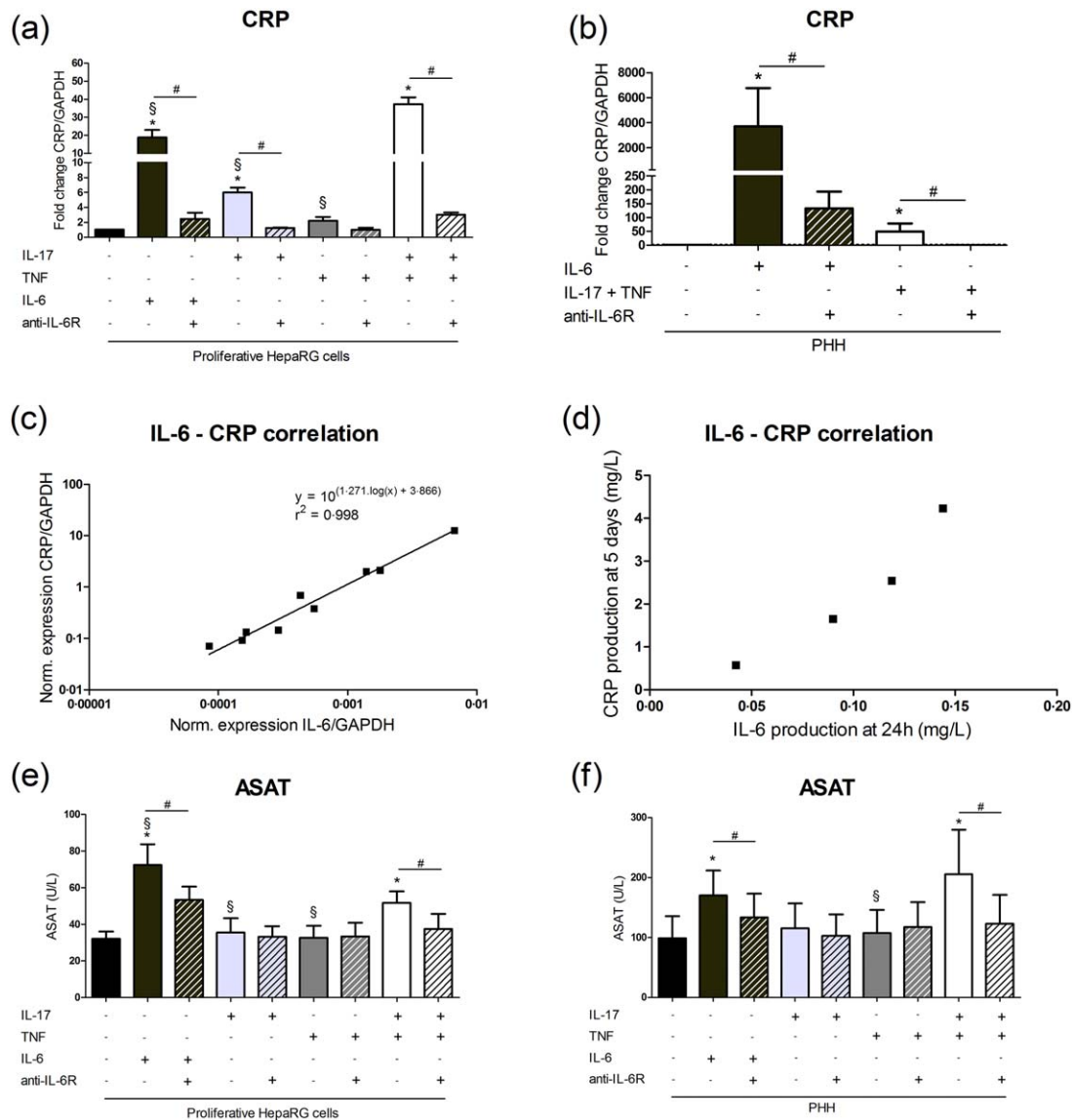
**Fig. 1.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases IL-6 expression and production synergistically. Hepatocytes were exposed to IL-17 and/or TNF- $\alpha$  or IL-6 with/without the anti-IL-6R. (a,g) IL-6 production by hepatocytes was quantified by enzyme-linked immunosorbent assay (ELISA). (b,d,e,f) IL-6 expression in proliferative human HepaRG cells at 12 h was expressed as fold changes compared to control. (c) TNF receptor II (TNF-RII) expression at 12 h was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The control antibody had no effect on IL-6 mRNA and protein levels following IL-6 or IL-17/TNF- $\alpha$  stimulation in HepaRG cells (e, data not shown). Data are the mean of three to 18 independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.

elevated ASAT levels returned to control level when an anti-IL-6R antibody was added (Fig. 2e,f). In these cultures, alanine aminotransferase (ALAT) level was lower than the detection limit (< 6 UI/l) (data not shown). The increase in ASAT level was therefore mainly IL-6-dependent.

**IL-17 and TNF- $\alpha$  increase in synergy IL-8 expression and production independently of the IL-6 pathway**

Cell recruitment is crucial for the inflammatory response. Liver biopsies in patients with active liver disease are

characterized by the presence of inflammatory infiltrates [26]. IL-8 is associated with neutrophil recruitment involved in the acute-phase inflammatory response, as in acute hepatitis [15]. The IL-17/TNF- $\alpha$  co-operation enhanced IL-8 production by HepaRG cells and PHH versus IL-17 alone, TNF- $\alpha$  alone and control (Fig. 3a). IL-8 mRNA expression in proliferative HepaRG cells was up-regulated by IL-17 alone (5-fold) and TNF- $\alpha$  alone (5-fold), with a synergistic effect of both (24-fold) (Fig. 3b). The IL-17/TNF- $\alpha$  synergistic interactions also increased IL-8 mRNA levels by up to 14-fold in PHH (Fig. 3b).

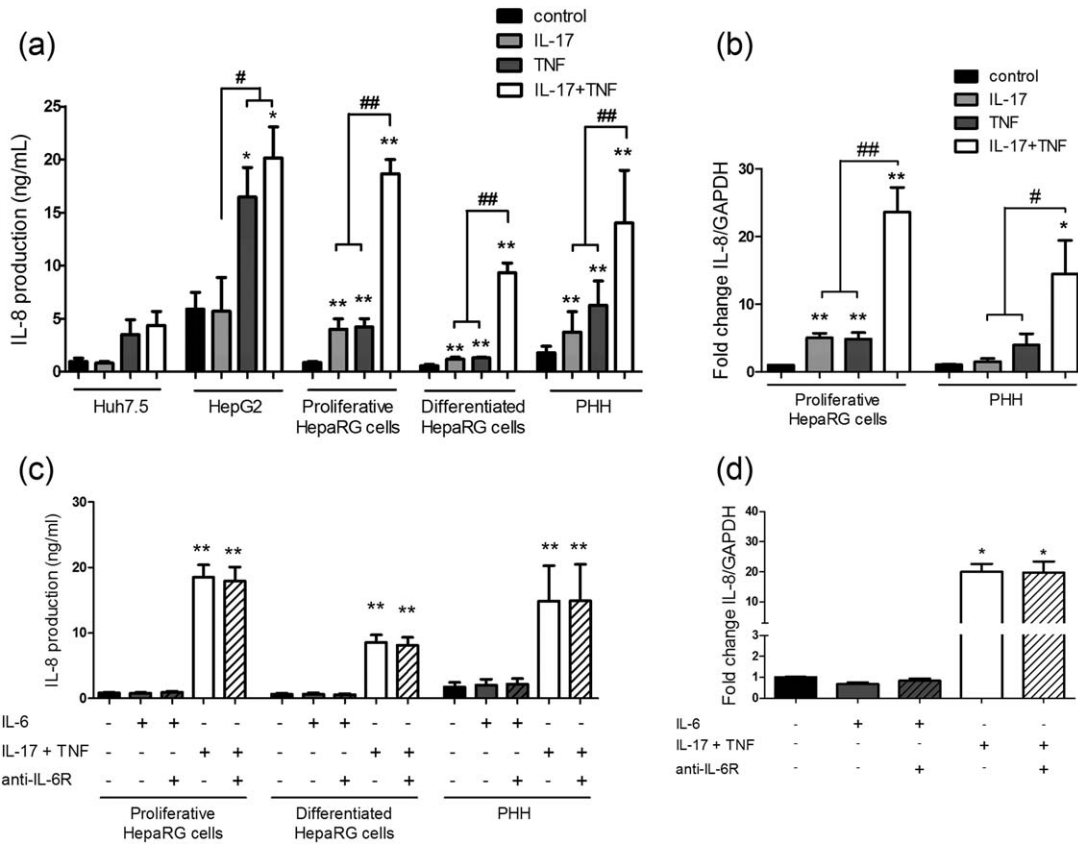


**Fig. 2.** Induction of the interleukin (IL)-6-dependent C-reactive protein (CRP) expression and aspartate aminotransferase (ASAT) activity level following IL-17 and/or tumour necrosis factor (TNF)- $\alpha$  stimulation. Hepatocytes were treated with IL-17 and/or TNF- $\alpha$  or IL-6 with/without the anti-IL-6R. (a,b) CRP expression in the human HepaRG cell line and primary human hepatocytes (PHH) at 24 h was expressed as fold changes compared to control. The control antibody had no effect on CRP mRNA levels following IL-6 or IL-17/TNF- $\alpha$  stimulation in HepaRG cells (data not shown). (c) Correlation between IL-6 and CRP mRNA levels in PHH stimulated with IL-17/TNF- $\alpha$  for 24 h. mRNA levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (d) Correlation between IL-6 and CRP production in PHH cultures treated with IL-17/TNF- $\alpha$ . (e,f) ASAT supernatant levels were quantified at 120 h. Data are the mean of four to six independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P < 0.05$  versus control, § $P < 0.05$  versus IL-17/TNF- $\alpha$  condition, # $P < 0.05$  versus other cytokine conditions.

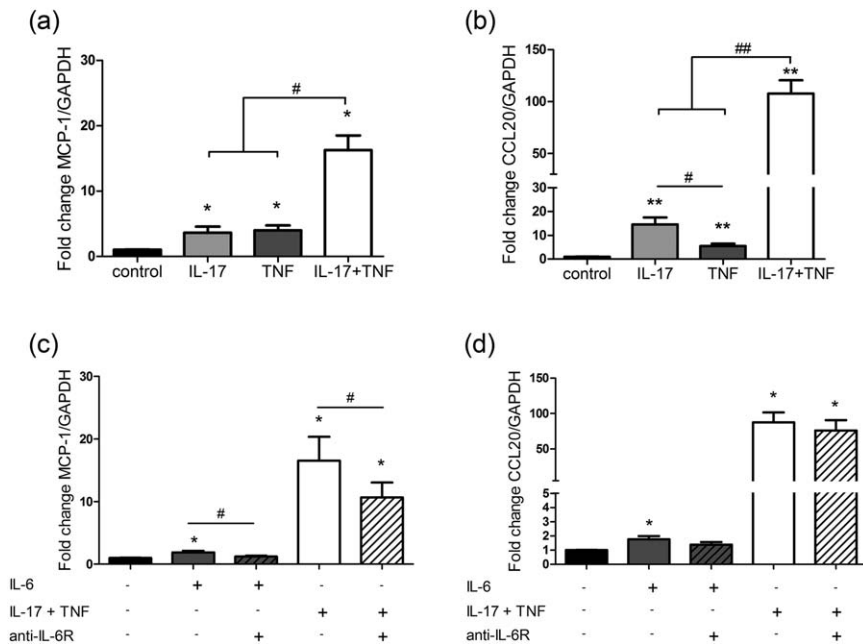
However, the IL-8 mRNA and protein level was unchanged after IL-6 exposure. Moreover, the IL-6 pathway inhibition had no effect on the induction of IL-8 expression and production by IL-17/TNF- $\alpha$  (Fig. 3c,d). Therefore, IL-8 induction by IL-17 and TNF- $\alpha$  was not mediated through IL-6. The IL-17 and TNF- $\alpha$  combination may thus have a key role in the migration of neutrophils to the liver in the context of acute hepatitis.

### IL-17 and TNF- $\alpha$ increase in synergy MCP-1 and CCL20 expression mainly through an IL-6-independent pathway

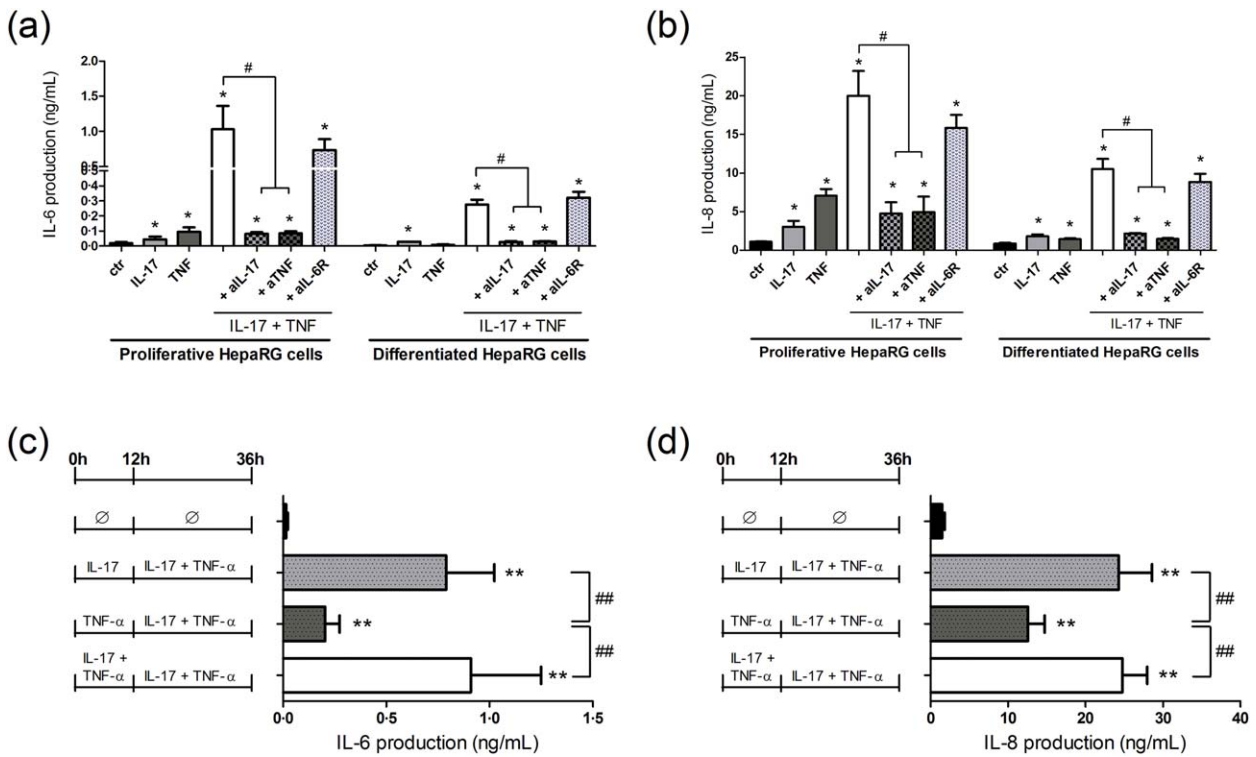
MCP-1 and CCL20 are two chemokines acting on mononuclear cells involved in the chronicity of the inflammatory response [27–29]. MCP-1 and CCL20 mRNA levels increased in the presence of IL-17 or TNF- $\alpha$ , with a clear



**Fig. 3.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases IL-8 expression and production synergistically independently of the IL-6 pathway. Hepatocytes were exposed to IL-17 and/or TNF- $\alpha$  or IL-6 with/without an anti-IL-6R antibody. (a,c). IL-8 production was quantified by enzyme-linked immunosorbent assay (ELISA). (b,d) IL-8 expression at 12 h was expressed as fold change compared to control. The control antibody had no effect on the IL-8 mRNA and protein levels following IL-6 or IL-17/TNF- $\alpha$  stimulation in human HepaRG cells (data not shown). Data are the mean of three to 18 independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.



**Fig. 4.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases monocyte chemoattractant protein (MCP)-1 and chemokine (C-C motif) ligand 20 (CCL20) chemokine expression synergistically, mainly through an IL-6-independent pathway. Proliferative human HepaRG cells were exposed to IL-17 and/or TNF- $\alpha$  or IL-6 with/without an anti-IL-6R antibody. (a-d) MCP-1 and CCL20 expression at 12 h in proliferative HepaRG cells was expressed as fold change compared to control. The control antibody had no effect on the MCP-1 and CCL20 mRNA levels following IL-6 or IL-17/TNF- $\alpha$  stimulation (data not shown). Data are the mean of seven to eight independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.



**Fig. 5.** Interleukin (IL)-17 initiates the IL-17 and tumour necrosis factor (TNF)- $\alpha$  synergistic effect on IL-6 and IL-8 production. (a,b) To evaluate the contribution of IL-17, TNF- $\alpha$  and IL-6 pathways in the IL-17/TNF- $\alpha$  synergistic effect, human hepatoma cells (HepaRG) cells were treated with IL-17 and/or TNF- $\alpha$  with/without anti-IL-17A (aIL-17), anti-TNF- $\alpha$  (aTNF) or anti-IL-6R (aIL-6R) antibody. The control antibody had no effect on the IL-6 and IL-8 protein levels following IL-17/TNF- $\alpha$  stimulation (data not shown). (c,d) Proliferative HepaRG cells were pre-exposed to IL-17 and/or TNF- $\alpha$  overnight (for 12 h) before IL-17 and/or TNF- $\alpha$  addition to have both cytokines in the culture medium (except for the control ( $\emptyset$ )). The IL-6 and IL-8 production was measured by enzyme-linked immunosorbent assay (ELISA). Data are the mean of five to seven independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.

synergistic effect of both cytokines (16- and 108-fold, respectively,  $P$  < 0.01) versus control (Fig. 4a,b).

To determine whether the IL-17/TNF- $\alpha$  effect on MCP-1 and CCL20 expression was mediated through the IL-6 pathway, IL-6 and an anti-IL-6R antibody were used. By comparison with IL-17/TNF- $\alpha$  stimulation, IL-6 had a very minimal effect on MCP-1 and CCL20 mRNA levels. Similarly, the anti-IL-6R antibody did not abrogate the MCP-1 and CCL20 expression induced by the IL-17 and TNF- $\alpha$  combination in proliferative HepaRG cells (Fig. 4c,d). Therefore, IL-17 and TNF- $\alpha$  can act independently of the IL-6 signalling pathway on the induction of MCP-1 and CCL20 expression. IL-17/TNF- $\alpha$  may thus have a key role in the migration of immune cells, including T helper type 17 (Th17) cells, to the liver in the context of chronic hepatitis.

### IL-17 initiates the IL-17 and TNF- $\alpha$ synergistic effect on IL-6 and IL-8 production

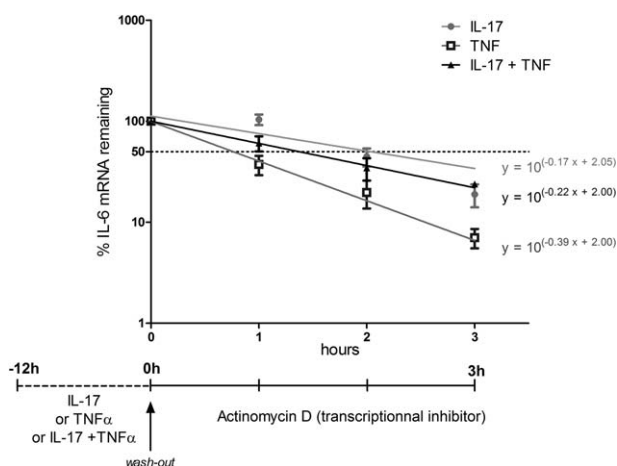
To understand more clearly the contribution of each cytokine on the IL-17 and TNF- $\alpha$  synergistic effect, antibodies

blocking the IL-17, TNF- $\alpha$  and IL-6 pathway were used. Anti-IL-17 or anti-TNF- $\alpha$  antibody exposure in conjunction of the IL-17/TNF- $\alpha$  combination reduced the synergistic effect on IL-6 and IL-8 production in HepaRG cells, whereas the IL-6R inhibition had no significant effect on IL-6 and IL-8 supernatant levels (Fig. 5a,b).

We next investigated whether first exposure to IL-17, TNF- $\alpha$  or both initiated the IL-17 and TNF- $\alpha$  synergistic effects on IL-6 and IL-8 release. Proliferative HepaRG cells were pre-exposed overnight to IL-17 and/or TNF- $\alpha$  and then IL-17 and/or TNF- $\alpha$  were added to both cytokines in the culture medium. Pre-incubation first with IL-17 followed by the addition of TNF- $\alpha$  induced two-fold higher IL-6 and IL-8 production at 24 h than pre-incubation with TNF- $\alpha$ , then with IL-17 (Fig. 5c,d). First exposure to IL-17, but not to TNF- $\alpha$ , was thus crucial for initiation of the IL-17/TNF- $\alpha$  synergistic effect.

### IL-17 enhanced the stability of IL-6 mRNA

Post-transcriptional regulations could contribute to the IL-17 and TNF- $\alpha$  synergistic effect. To determine the IL-17



**Fig. 6.** Interleukin (IL)-17 enhances the stability of IL-6 mRNA. Proliferative human HepaRG cells were incubated with IL-17 (filled circles), tumour necrosis factor (TNF)- $\alpha$  (empty squares) or the IL-17/TNF- $\alpha$  combination (filled triangles) for 12 h. Actinomycin D was added to inhibit further transcription. The IL-6 and IL-8 expression during the next 3 h were quantified. Results are presented as % of mRNA remaining over time compared with the steady-state level (at 0 h). Data are the mean of four independent experiments  $\pm$  standard error of the mean (s.e.m.).

and TNF- $\alpha$  effect on mRNA stabilization, HepaRG cells were treated with IL-17 and/or TNF- $\alpha$  for 12 h and then transcription was inhibited. TNF- $\alpha$ -induced IL-6 transcripts had a half-life of 46 min, whereas IL-17 and the IL-17/TNF- $\alpha$  combination increased the half-life up to 124 and 82 min, respectively (Fig. 6). IL-17 may thus increase IL-6 mRNA stabilization in hepatocytes.

#### IL-17 and TNF- $\alpha$ increase IL-6 and IL-8 production in synergy via the activation of ERK and/or PI3K/Akt signalling pathways and/or NF- $\kappa$ B transcription factor

The effect of the TNF- $\alpha$ /IL-17 combination on the downstream signalling pathways was then investigated, focusing on the MAPKs (JNK, p38, ERK), NF- $\kappa$ B and Akt/PI3K pathways [22,30,31]. Results are shown as the ratio of the total and phosphorylated forms (Fig. 7a). In HepaRG cells, the IL-17/TNF- $\alpha$  association induced phosphorylation of I $\kappa$ B $\alpha$  (leading to NF- $\kappa$ B release and activation) and ERK. The IL-17/TNF- $\alpha$  effect of Akt phosphorylation was weaker (Fig. 7a). Proliferative HepaRG cells were therefore treated with chemical inhibitors of these pathways. Quantification of transaminase secretion and cell viability were monitored to select the concentrations that did not induce cell death and other cytotoxic effects (data not shown).

The IL-17/TNF- $\alpha$  synergistic effect on IL-6 and IL-8 production was reduced slightly using JNK or p38 MAPK inhibitors. In contrast, ERK inhibition decreased strongly the production of IL-6 (29 and 70% of inhibition for 1 and 10  $\mu$ M, respectively) and IL-8 (31 and 69% of inhibition for 1 and 10  $\mu$ M, respectively) in a dose-dependent manner

induced by the IL-17/TNF- $\alpha$  combination (Fig. 7b,c). IL-6 production induced by the IL-17/TNF- $\alpha$  combination was also inhibited in the presence of the NF- $\kappa$ B inhibitor (60% of inhibition), the Akt inhibitor (more than 70% of inhibition for 10 and 20  $\mu$ M) and the PI3K inhibitor (40 and 65% of inhibition for 1 and 10  $\mu$ M, respectively), whereas IL-8 production was not impacted significantly (Fig. 7d-g). Activation of ERK, NF- $\kappa$ B and/or PI3K/Akt signalling pathways was therefore involved in the IL-17/TNF- $\alpha$  synergistic effect on IL-6 and/or IL-8 production.

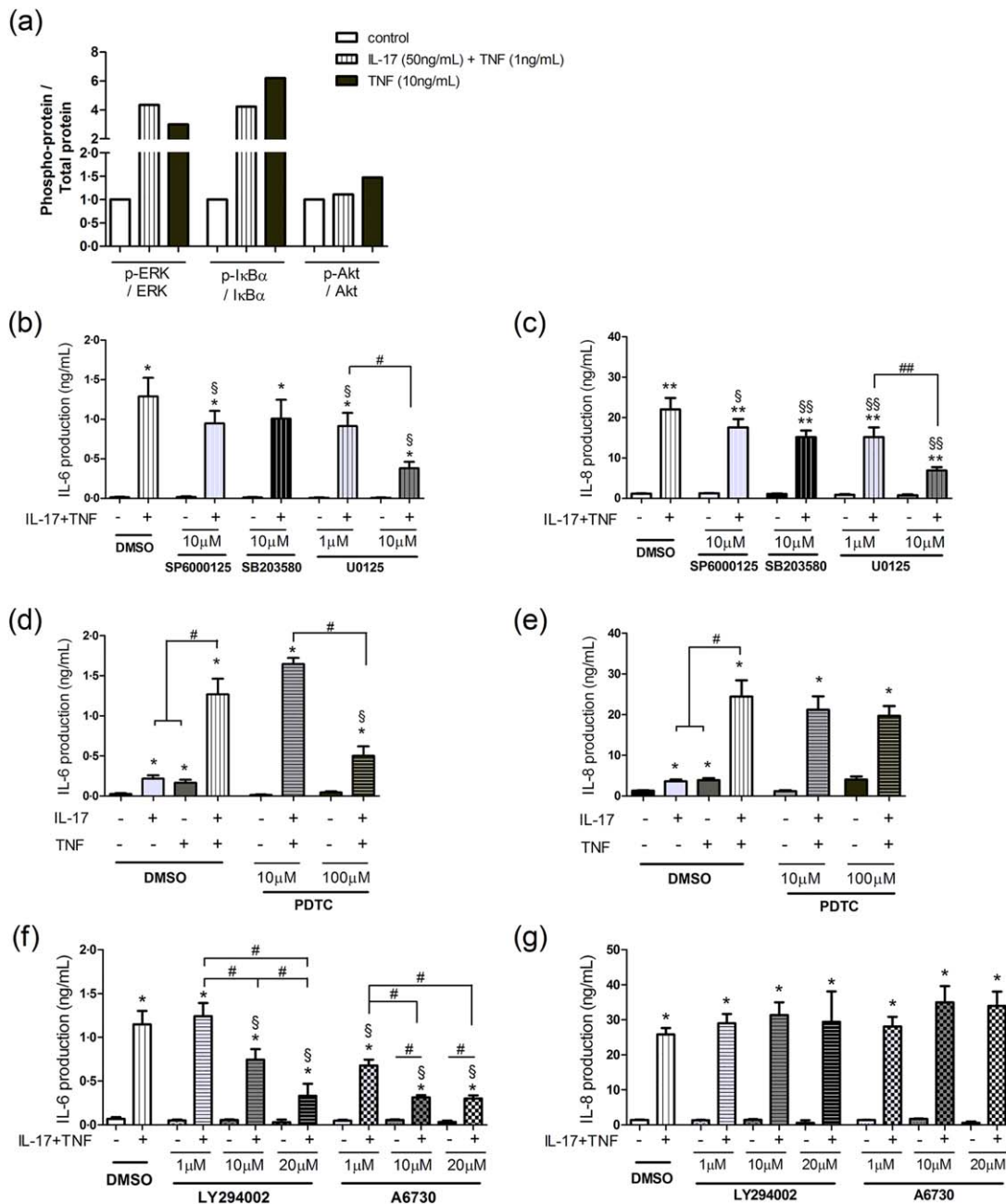
## Discussion

This study shows how the IL-17/TNF- $\alpha$  synergistic interactions mediate a hepatic inflammatory response mainly through IL-6 for CRP and ASAT induction, and independently of IL-6 for IL-8, MCP-1 and CCL20 chemokine up-regulation.

IL-6 is a systemic inflammatory mediator, which plays a key role in triggering the acute-phase response to injury or inflammation. IL-17 was shown previously to induce IL-6 production by the human hepatoma Huh7, HepG2 and Hep3B cell lines [3,32,33]. Here, IL-17 and TNF- $\alpha$  increased the production of IL-6 in HepaRG cells and PHH in synergy. Because IL-6 promotes the generation and differentiation of Th17 cells [28], the main IL-17-producing cells, the increase of IL-6 production by the IL-17/TNF- $\alpha$  synergistic effect and IL-6 autoinduction could exacerbate the inflammatory IL-6/Th17/IL-17 amplification. Interestingly, IL-6 was also able to up-regulate directly its own expression *in vitro* leading to a positive feedback loop of IL-6. However, the contribution of this positive autoregulation of IL-6 in the IL-6 induction following the IL-17/TNF- $\alpha$  synergistic interaction was very weak at 12 h (Fig. 1g) and 24 h (data not shown) compared to the direct IL-17/TNF- $\alpha$  effect. Because an anti-IL-6R antibody blocks the IL-6 binding to its receptor competitively, a higher IL-6 protein level was expected in supernatants of hepatic cultures exposed to the anti-IL-6R antibody. For this reason, the IL-6-positive feedback loop after IL-6 treatment was observed only on IL-6 mRNA levels and not on IL-6 protein levels in supernatants.

High CRP levels are associated with an increased risk of cardiovascular events [34]. *In-vitro* and *in-vivo* studies have demonstrated the potent role of IL-6 on CRP production [3,7]. Here, IL-17 and/or TNF- $\alpha$  enhanced CRP expression in HepaRG cells, showing a synergistic effect with the combination. The IL-6 pathway blockade inhibited CRP induction strongly by IL-17 and/or TNF- $\alpha$ . In PHH treated with the IL-17/TNF- $\alpha$  combination, CRP levels correlated perfectly with those of IL-6. Moreover, CRP mRNA level was lower at 12 than 24 h (data not shown), indicating that IL-17 and TNF- $\alpha$  act first on the induction of IL-6 production, which leads in turn to CRP up-regulation. Therefore, the induction of CRP appears mainly IL-6-dependent.





**Fig. 7.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases IL-6 and IL-8 production synergistically through the activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathways and nuclear factor kappa B (NF- $\kappa$ B) transcription factor. (a) Proliferative human HepaRG cells were incubated with IL-17 and/or TNF- $\alpha$  for 30 min. The total and phosphorylated forms of ERK, NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) and Akt were quantified by Western blotting and their densitometry values were normalized to the cyclophilin content. Data are presented as the fold induction change of the ratio of phosphorylated to total protein in control samples. One representative experiment is shown. (b–g) Proliferative HepaRG cells were pre-exposed to MAPKs [Janus kinase (JNK), p38, ERK], NF- $\kappa$ B, Akt and PI3K pathway inhibitors for 1 h followed by the IL-17 and/or TNF- $\alpha$  addition. IL-6 and IL-8 production at 24 h was measured by enzyme-linked immunosorbent assay (ELISA). SP6000125: JNK inhibitor, SB203580: p38 MAPK inhibitor, U0125: MEK/ERK inhibitor; pyrrolidine dithiocarbamate (PDTC): NF- $\kappa$ B inhibitor; LY294002: PI3K inhibitor and A6730: Akt inhibitor. Data are the mean of at least four to eight independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05, \*\* $P$  < 0.01 versus control in dimethylsulphoxide (DMSO) condition, § $P$  < 0.05 and §§ $P$  < 0.01 versus IL-17/TNF- $\alpha$  condition in DMSO medium; # $P$  < 0.05 and ## $P$  < 0.01 versus other inhibitor conditions.

However, one study has demonstrated that IL-17 can stimulate CRP expression independently of IL-6 in Hep3B cells [3], suggesting that a possible minor pathway independent of IL-6 could exist.

In chronic inflammatory diseases, liver changes are common and may lead to transaminase elevation [35]. The IL-17/TNF- $\alpha$  combination increased the ASAT activity level through the IL-6 pathway. These results appear consistent with our *in-vivo* studies, which showed that TNF- $\alpha$  neutralization decreased serum levels of transaminase in mice and patients with autoimmune hepatitis [35,36]. Moreover, IL-17 deficiency or IL-17 neutralization reduced transaminase levels in various mouse models of liver injury [37,38]. Therefore, controlling IL-17 and TNF- $\alpha$  levels and functions may be protective and reduce liver damage.

Hepatic infiltration of neutrophils is an early response to systemic inflammation crucial to initiate liver injury [39,40]. The link between IL-17, neutrophil recruitment and hepatic necrosis was demonstrated in several mouse models [37,41]. IL-8 has a key role in the neutrophil mobilization and activation [15]. IL-17 may stimulate IL-8 production through IL-6 up-regulation in Huh7 cells [33]. In our study, IL-8 production was increased by the IL-17/TNF- $\alpha$  synergistic interaction independently of the IL-6 pathway, as the exposure to an anti-IL-6R antibody had no effect (Fig. 3c,d). IL-6 could nevertheless act *in vivo* on the IL-8 level by promoting the Th17–IL-17 cells axis leading to an increase of IL-17 production and, in turn, of IL-8.

Chronic hepatitis is characterized by the liver infiltration by various immune cells attracted by chemokines, such as CCL20 and MCP-1. Here, IL-17 and TNF- $\alpha$  were able to up-regulate CCL20 and MCP-1 expression synergistically through an IL-6-independent pathway. In HepG2 culture and an autoimmune hepatitis mouse model, TNF- $\alpha$  induced CCL20 expression and this up-regulation was associated with the progression of fatal inflammation [27,36]. MCP-1 was described as a central co-ordinator of hepatocyte-mediated inflammation [42,43]. MCP-1 expression was up-regulated in primary hepatocytes by activation of the IL-6-mediated signalling cascade [44]. In turn, MCP-1 induced IL-6 production in mouse hepatocytes, suggesting a possible positive feedback loop between IL-6 and MCP-1 [42]. Taken together, CCL20 and MCP-1 induction by IL-17/TNF- $\alpha$  can increase the accumulation of a Th17-driven response and lead to a chronic inflammatory state.

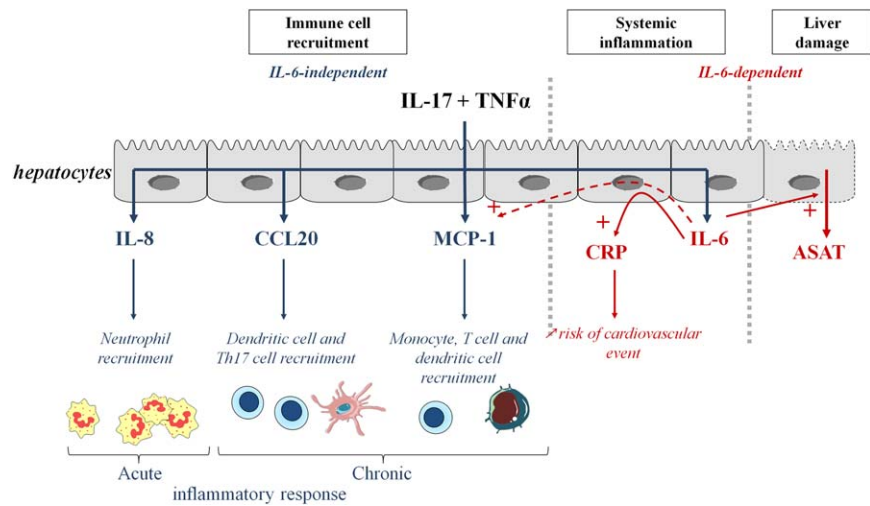
Although the IL-17/TNF- $\alpha$  synergistic effect has been well described in several cell types [16–19,45,46], the mechanism of this effect on IL-6 and IL-8 production has not yet been studied in hepatocytes. Various mechanisms may act at several levels: at a receptor level, at a post-transcriptional level and at a promoter level. In synovioocytes, the TNF receptor II (TNF-RII) contributed to the IL-17 and TNF- $\alpha$  synergistic effect on CCL20 production, and IL-17 treatment alone up-regulated its expression [47].

Here, in HepaRG cells, stimulation with the IL-17/TNF- $\alpha$  combination but not with IL-17 alone increased TNF-RII expression. Regulation of the TNF-RII expression may be different between synovioocytes and HepaRG cells. However, the TNF-RII mRNA level was much lower in HepG2 and Huh7.5 cells than in HepaRG cells (data not shown). This could explain the lack of response of the HepG2 and Huh7.5 cells to the IL-17/TNF- $\alpha$  synergistic effect on IL-6 and IL-8 production.

Post-transcriptional regulation is important to control cellular transcript abundance and, in turn, the levels of the secreted proteins. Several studies have reported that the IL-17 and TNF- $\alpha$  co-operation modulates mRNA stability [45,46,48–50]. In HepaRG cells, IL-17 could enhance IL-6 mRNA stability (Fig. 6). As the 3'-untranslated region of the IL-6 mRNA contains adenylate and uridylylate (AU)-rich elements, IL-17 may promote the binding of stabilizing AU-binding proteins over that of destabilizing AU-binding proteins, prolonging IL-6 mRNA half-life [51]. Activation of the ERK MAPK pathway may contribute to this IL-6 mRNA stabilization [52].

To investigate the potential signalling pathways involved in IL-17 and TNF- $\alpha$  synergistic stimulation on hepatic IL-6 and IL-8 production, several chemical pathway inhibitors were used. MAPK, PI3K and its downstream mediator Akt were involved in the IL-6 signalling, and also in the IL-17-induced production of IL-6, IL-8 and MCP-1 in several cell types, including human hepatocellular carcinoma cell lines [32,33,53–55]. In HepaRG cells, activation of the ERK MAPK pathway in the IL-17–TNF- $\alpha$  interaction appears crucial, as the ERK pathway inhibition reduced both IL-6 and IL-8 production. The PI3K/Akt signalling pathway contributes to the synergistic effect of IL-17/TNF- $\alpha$  on the induction of IL-6 but not of IL-8. Because the MAPK and PI3K/Akt pathways can be activated by IL-6, their contribution on the IL-6 production can be related to the positive autoregulation of IL-6 (Fig. 1c). However, the anti-IL-6R antibody failed to reduce IL-6 expression induced by IL-17/TNF- $\alpha$  (Fig. 1f) in HepaRG cells. Therefore, the IL-17/TNF- $\alpha$  combination could activate the ERK MAPK and PI3K/Akt pathways directly to induce IL-6 and/or IL-8 production.

Transcription factor-binding sites for NF- $\kappa$ B and C/EBP (CCAAT/enhancer-binding protein) in the IL-6 promoter were both involved in the IL-17 and TNF- $\alpha$  synergistic effect on IL-6 in an osteoblastic cell line [56]. Part of the IL-17/TNF- $\alpha$  synergistic effect on IL-6 may occur at the gene transcription level through the up-regulation of C/EBP $\delta$  by IL-17/TNF- $\alpha$  and the increase of C/EBP $\delta$  recruitment to the promoter by TNF- $\alpha$  [31,56,57]. In primary murine hepatocytes, NF- $\kappa$ B contributed to the activation of many IL-17 target genes related to inflammation [58]. Here, in HepaRG cells, the NF- $\kappa$ B pathway appears to be involved in IL-6 release induced by the IL-17/TNF- $\alpha$  combination (Fig. 7d). Both IL-17 and TNF- $\alpha$  signalling



**Fig. 8.** Summary of the effects of interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  on hepatocytes. IL-17 and TNF- $\alpha$  have synergistic interactions on cytokine production by hepatocytes, with IL-17 acting first to increase the effect of TNF. IL-17 and TNF- $\alpha$  induce C-reactive protein (CRP) through the induction of IL-6. IL-17 and TNF- $\alpha$  induce chemokine production independently of IL-6.

pathways promote NF- $\kappa$ B activation. However, the IL-17/TNF- $\alpha$  combination may induce a further increase of NF- $\kappa$ B activation through I $\kappa$ B $\zeta$ , which acts as a NF- $\kappa$ B co-activator [57,59]. In primary murine hepatocytes, IL-17 and TNF- $\alpha$  cooperate to enhance the I $\kappa$ B $\zeta$  mRNA expression in synergy [58].

In conclusion, targeting IL-17 and/or TNF- $\alpha$  could be a promising therapeutic strategy to control systemic inflammation, as seen with IL-6 inhibition, but also the local cell recruitment and associated liver cell injury. These results are summarized in Fig. 8. Furthermore, control of the CRP level could be critical to reduce the cardiovascular risks that represent a major cause of death in patients with chronic inflammatory diseases.

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## Disclosure

None.

## Author contributions

A. B. conducted the experiments and writing the manuscript; N. T., A. C. and B. B. developed the assays and experiments; and P. M. was responsible for the concept and writing the manuscript.

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