Gene expression of interleukin 18 in unstimulated peripheral blood mononuclear cells of patients with alcoholic cirrhosis

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Abstract
Background—Most patients with alcohol induced cirrhosis (AC) and chronic endotoxinaemia are not suffering from clinically evident systemic inflammatory reactions. This may be due to altered gene expression of cytokines, possibly related to endotoxin (for example, tolerance and sensitisation). Interleukin 18 (IL-18; interleukin γ inducing factor) modulates local cytokine production in response to endotoxin (lipopolysaccharide (LPS)).
Aim—To investigate the systemic immune response of patients with AC and to see if unstimulated peripheral blood mononuclear cells (PBMC) from patients with AC are activated and contribute to gene expression of IL-18.
Methods—Plasma levels of endotoxin (LPS) and serum levels of IL-18 were measured by enzyme linked immunoassay and the amoebocyte lysate test in 74 abstinent patients with different stages of AC (Child-Pugh stage A, n=18; B, n=22; C, n=34) and compared with healthy controls (n=43). Gene expression of IL-18 was assessed by semiquantitative reverse transcription–polymerase chain reaction in freshly isolated unstimulated PBMC of a subgroup of 14 patients with AC compared with five healthy controls.
Results—Gene expression of IL-18 specific mRNA in unstimulated PBMC was significantly enhanced in patients with advanced AC (Child-Pugh stage C) and correlated with plasma LPS and serum CD14 levels (Spearman rank correlation factors $r=0.76$ and $r=0.72$). Serum concentrations of IL-18 were also elevated compared with healthy controls (p<0.001) but correlation with serum levels of CD14 and plasma levels of LPS was much weaker compared with mRNA data (Spearman rank correlation factors $r=0.47$ and $r=0.26$).
Conclusions—Our in vivo data suggest a presensitisation of "unstimulated" PBMC in the circulation of patients with AC by endotoxin. The term "unstimulated" may be inadequate in patients with AC. Further investigations are needed to define the exact mechanisms and localisation of sensitisation of PBMC in vivo.
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Keywords: liver cirrhosis; peripheral blood mononuclear cells; interleukin 18; lipopolysaccharide

Endotoxins are physiologically present in portal venous blood.[1][2] If hepatic clearance is impaired, systemic endotoxinaemia may result (caused by portosystemic shunts, functional defects in the phagocytic capacity of the hepatic reticuloendothelial system, and decreased endotoxin binding capacity of whole blood[2–5]).

In spite of elevated serum cytokine and plasma endotoxin levels,[2][3][6–9] most patients with alcohol induced cirrhosis (AC) and chronic endotoxinaemia are not suffering from clinically evident systemic inflammatory reactions. This may be due to altered gene expression of cytokines, perhaps relating to endotoxin (for example, tolerance and sensitisation).[10]

We recently reported enhanced mRNA expression of the proinflammatory cytokine tumour necrosis factor α (TNF-α) in unstimulated peripheral blood mononuclear cells (PBMC) from patients with advanced AC but did not elucidate the mechanisms which potentially (down) regulate the immune response in AC.[11]

Interleukin 18 (IL-18; interleukin γ inducing factor) is a recently described peptide regulatory factor in the early host response to bacterial endotoxin (lipopolysaccharide (LPS)) which induces TNF-α production in PBMC.[12][13] In vitro, IL-18 gene expression was originally thought to be regulated constitutively with no increase or decrease on stimulation with LPS.[14][15] However, recent data showed that IL-18 expression was clearly detectable after LPS treatment.[16][17] Moreover, IL-18 modulates local cytokine production in response to LPS.[18][19] IL-18 is a key factor in the systemic inflammatory reactions in acute (*Escherichia coli* induced) endotoxaemia-inflammatory reactions, which may be down-regulated if IL-18 is neutralised.[20] This suggests that IL-18 may not only play a role as a marker of disease activity in patients with AC but may also have potential implications for therapeutic strategies in AC.

Hence the aim of this study was to investigate if IL-18 is involved in the immune response in patients with AC (who show no clinically evident signs of systemic inflammatory reactions) and to test the hypothesis that unstimulated PBMC from patients with AC

Table 1 Profile of 74 patients with alcohol induced cirrhosis and 43 controls

	Controls	Child-Pugh stage A	Child-Pugh stage B	Child-Pugh stage C
Ascites	0/43	0/18	7/22	28/34
Hepatic encephalopathy	0/43	0/18	11/22	30/34
INR (normal: 1.0–1.5)	1.05 (0.02)	1.08 (0.02)	1.1 (0.04)	1.38 (0.06)
Albumin (normal: >32 g/l)	48.84 (0.4)	42.8 (0.8)	34.6 (1.0)	25.6 (1.1)
Bilirubin (normal: 5–17 μmol/l)	3.4 (13.9)	24.4 (39.33)	65.3 (11.6)	167.4 (34.2)
Child-Pugh score	5.0 (0.0)	5.3 (0.1)	7.8 (0.1)	12.2 (0.3)

Values are numbers of patients or mean (SEM).
INR, international normalised ratio.

are activated and contribute to gene expression of IL-18.

Patients and methods

PATIENTS

The study group consisted of 74 patients with confirmed AC (Child-Pugh stage A, n=18; Child-Pugh stage B, n=22; Child-Pugh stage C, n=34).[21] Forty three age and sex matched healthy individuals with no history of alcohol abuse and normal liver function tests served as controls (table 1).

A diagnosis of AC was confirmed either histologically (n=55) or clinically (n=19) if biopsy was not available (for example, due to coagulopathy). A clinical diagnosis was established in patients demonstrating a Child-Pugh index >6 and signs of portal hypertension (endoscopically proved oesophageal varices; transjugularportocaval shunt). A previous history of daily ethanol intake of ≥80 g/day in men and ≥30 g/day in women for more than five years was requested.

Exclusion criteria were the presence of alcoholic hepatitis, autoimmune hepatitis, renal insufficiency (serum creatinine >115 μmol/l), or malignancy. Patients with an abstinence period of less than six months (that is, longer than in some comparable studies with abstinence periods of 1–2 months[8]) were excluded (for example, patients with markers of recent alcohol abuse—namely, the presence of elevated carbohydrate deficient transferrin serum levels and/or early decrease in gamma glutamyl transpeptidase, as previously described[22 23]) to avoid inclusion of patients with alcoholic hepatitis[24] if biopsy was not available. No patient suffered from active gastrointestinal bleeding or had overt signs of infection (fever, elevated white blood cell count).

mRNA expression of IL-18 was assessed in unstimulated PBMC in a subgroup of 14 (of 74) patients with histologically confirmed stable AC (Child-Pugh stage A, n=4; Child-Pugh stage B, n=5; Child-Pugh stage C, n=5).[21] The study was approved by the university hospital ethics committee.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Peripheral venous blood (10–20 ml) was obtained from patients and healthy human volunteers by venepuncture and collected into heparinised, sterile, pyrogen free disposable syringes with endotoxin free heparin (10 U/ml).

PBMC were separated by standard density gradient centrifugation (Ficoll-Paque method; Biochrom, Berlin[25]) and adjusted to 3×10^6

cells/ml in buffered RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum. For RNA extraction, cells were spun down at 1200 rpm and stored in 4 M GTC extraction buffer at −80°C.

IMMUNOASSAY FOR IL-18

Serum concentrations of IL-18 were determined in duplicate by a specific sandwich enzyme linked immunoassay (ELISA; Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) with minor modifications.[26 27] Briefly, 96 well microtitre plates (Nunc Maxisorp, Denmark) were coated with 100 μl of the primary monoclonal antibody against IL-18 (2.5 μg/ml in phosphate buffered saline (PBS)) and incubated for three hours at room temperature. The coated plates were washed in PBS/Tween 20 buffer and blocked with 250 μl of 1% bovine serum albumin in PBS overnight at 4°C. Samples, diluted 1:5, and serial dilutions of the recombinant IL-18 standard, were added to the plates and incubated for two hours at 20°C.

After washing with PBS/Tween 20 buffer, 100 μl of peroxidase conjugated monoclonal detection antibody (working concentration 0.05 μg/ml) were added for three hours at room temperature. The enzyme reaction was developed using 100 μl of 10 mmol/l tetramethylbenzidine, 80 mmol/l H_2O_2, and 30 mmol/l potassium citrate (pH 4.1). After 15–20 minutes of incubation at room temperature, the reaction was terminated with 50 μl of 2 N H_2SO_4 and optical density read at 450 nm (Emax Reader, MWG, Ebersberg, Germany; reference at 650 nm). Values were determined from a standard curve (range 8–500 pg/ml), constructed using serial dilutions of recombinant IL-18 standard, based on purified rabbit polyclonal anti-IL-18 conjugated with horseradish peroxidase. 4-Amino-antipyrine in potassium phosphate buffer was used as a substrate, and colour intensity was measured at 492 nm. The detection limit for this ELISA was 7.8 pg/ml with intra- and interassay variances of 5.8 (2.1) and 8.2 (2.4) respectively. The IL-18 specific ELISA showed no cross reactivity (for example, with other recombinant cytokines or cytokine receptors (IL-12, IL-2, interferon γ, IL-4, TNF-α, and recombinant TNF receptors)) over a wide range of concentrations (0.1 pg/ml to 1 μg/ml).

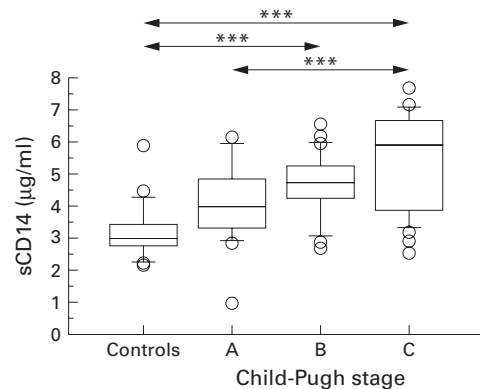### RNA ISOLATION AND IL-18 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Unstimulated PBMC from 15 abstinent patients with liver cirrhosis were freshly isolated directly after Ficoll gradient separation. Total cellular RNA was extracted from isolated PBMC using an acid phenol-chloroform extraction method, as previously described.[28] RNA concentration was quantitated spectrophotometrically, and RNA degradation and purity were assessed by electrophoresis on a 1.5% agarose gel containing formaldehyde.

cDNA of PBMC was obtained by RT using 1 μg of RNA and oligo d (T) primers. PCR for
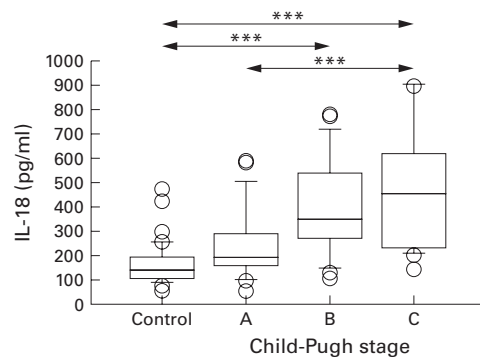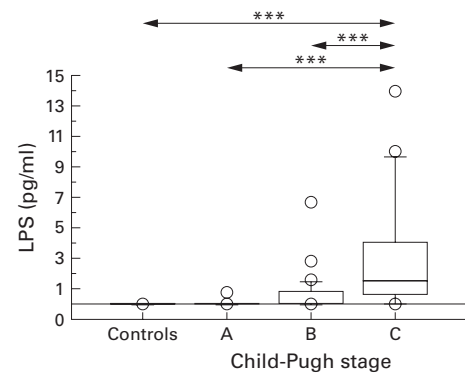
*Figure 1   Interleukin 18 (IL-18) serum levels (ELISA) were significantly increased in patients with alcohol induced cirrhosis with Child-Pugh stage C disease (n=34; median 456.15 pg/ml; range 163.1–1643.0) compared with healthy controls (n=43; median 135.5 pg/ml; range 62.9–476.1) and patients with Child-Pugh stage A (n=18; median 195.25 pg/ml; range 59.8–598.9). Moreover, significant differences were found between healthy controls and Child-Pugh stage B (n=22; median 355.15 pg/ml; range 119.8–792.9). Box plots indicate the median and 95% confidence intervals (CI). Individual data points are mean serum levels above or below the 95% CI. ***p<0.001.*

*Figure 3   Lipopolysaccharide (LPS) plasma levels were significantly increased in patients with alcohol induced cirrhosis with Child-Pugh stage C disease (n=34; median 1.4 pg/ml; range 0–63) compared with healthy controls (n=43; median 0 pg/ml; range 0–0) and patients with Child-Pugh stage A (n=18; median 0 pg/ml; range 0–0.7). Moreover, significant differences were found between healthy controls and Child-Pugh stage B (n=22; median 0 pg/ml; range 0–6.7). Box plots show median and 95% confidence intervals (CI). Individual data points are mean serum levels above or below the 95% CI. ***p<0.001.*

Palo Alto, USA) up 5'TGA AGG TCG GAG TCA ACG GAT TTG GT, down 3'CAT GTG GGC CAT GAG GTC CAC CAC.[15]

The amplified products were separated by electrophoresis on a 2% agarose gel and visualised by UV light illumination using ethidium bromide staining.

A 100 bp DNA ladder was run in parallel as a molecular size marker. Densitometric assessment of PCR products was performed using EASY Plus 3.2 software (Herolab, Wiesloch, Germany).

Semiquantitative PCR results were generated by grading a ratio between the densitometry results of the target cytokine and the housekeeping enzyme (for example, the ratio of IL-18 mRNA divided by G3PDH).

*Figure 2   CD14 serum levels (ELISA) were significantly increased in patients with alcohol induced cirrhosis with Child-Pugh stage C disease (n=34; median 5.95 µg/ml; range 2.62–7.71) compared with healthy controls (n=43; median 2.98 µg/ml; range 2.19–5.9) and patients with Child-Pugh stage A (n=18; median 4.0 µg/ml; range 1–6.18). Moreover, significant differences were found between healthy controls and Child-Pugh stage B (n=22; median 4.8 µg/ml; range 2.86–6.54). Box plots show median and 95% confidence intervals (CI). Individual data points are mean serum levels above or below the 95% CI. ***p<0.001.*

IL-18 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was performed in a final volume of 25 µl using 5 µl of cDNA on a thermal cycler (Perkin Elmer Cetus) for 35 cycles, each comprising 94°C for one minute, 55°C for one minute, with a further extension at 72°C for one minute.[25] Components of the PCR reaction were 10× PCR buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl) and 10 mM dNTP mix (10 mM of each dATP, dCTP, dGTP, dTTP; Boehringer-Mannheim, Mannheim, Germany). Cytokine primer pairs were designed spanning exon-exon conjunctions and are therefore mRNA/cDNA specific and non-reactive with genomic DNA.

The following oligonucleotide primer sequences were used: IL-18 (amplification product 342 bp) up 5'GCT TGA ATC TAA ATT ATC AGT C, down 3'GAA GAT TCA AAT TGC ATC TTA T; G3PDH (amplification product 983 bp; Clontech Laboratories Inc.,

ENDOTOXIN ASSAY

Plasma endotoxin levels were determined using an automated kinetic turbodimetric limulus amoebocyte lysate microtitre test with individual internal standardisation as previously described.[29 30] An endotoxin reference curve was established in each sample by spiking 50 µl of each of the samples (diluted 1:5 and heated at 80°C) with 25 µl of LPS to obtain 2500, 500, 50, 5, 0.5, 0.05, and 0 pg/ml of LPS. LPS NP 3 (Novo Pyrexal, Weidner, Waldorf, Germany; 100 pg NP3=1 EU EC-5, FDA) was used. Then 25 µl of lysate were added (Weidner). The increase in optical density was measured at 37°C at 30 second intervals for 100 minutes at 405 nm. The sensitivity of the assay was 0.1 pg/ml.[30]

SOLUBLE CD14 ASSAY

Serum concentrations of soluble CD14 were determined using a commercially available ELISA according to the manufacturer's specifications (IBL, Hamburg, Germany).

STATISTICAL ANALYSIS

Non-parametric tests were used to analyse the results of IL-18. In particular, the Mann-Whitney U test was used to compare data between the two groups and the Kruskal-Wallis
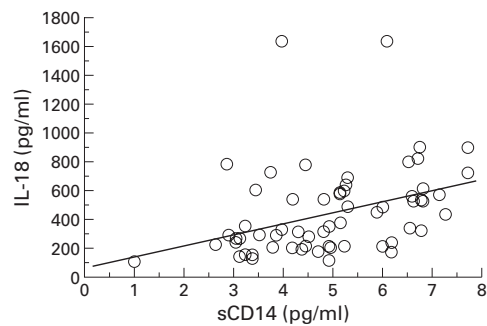
Figure 4 Spearman rank analysis reveals a significant correlation between serum levels of interleukin 18 (IL-18) and CD14 (r= 0.47, p<0.001).

test to compare data between three and more groups. All values are expressed as median (range). Box plots are provided with median values (95% confidence intervals). A p value < 0.05 was considered significant.

Results

SERUM IL-18 LEVELS (ELISA) IN AC

Serum levels of IL-18 in patients with AC of Child-Pugh stage B (n=22) and C (n=34) were significantly elevated compared with patients suffering from Child-Pugh stage A (n=18) or healthy controls (n=43) (p<0.001) (fig 1).
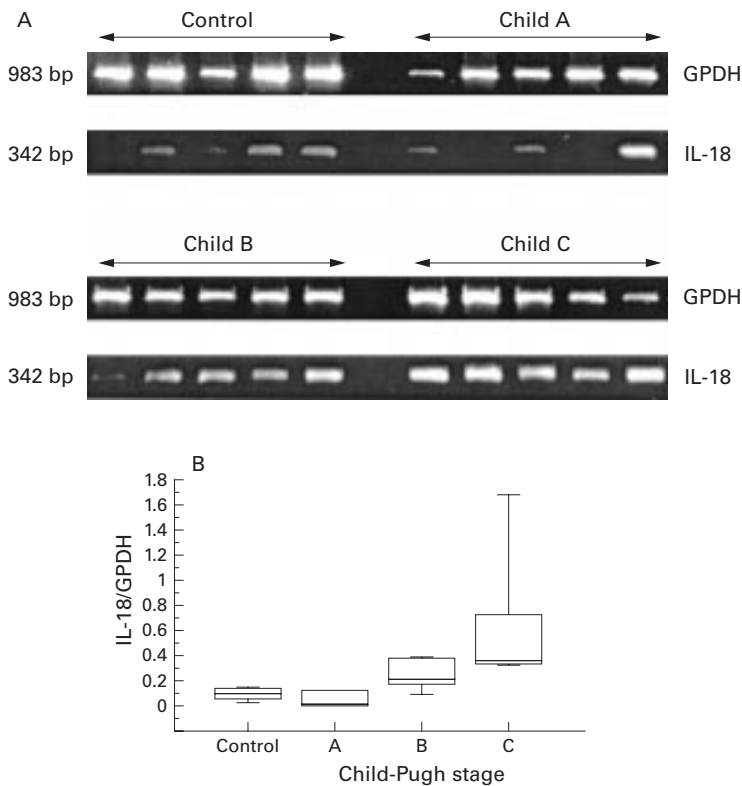

*Figure 5 (A) mRNA expression of human interleukin 18 (IL-18) in unstimulated freshly isolated peripheral blood mononuclear cells of patients with alcohol induced cirrhosis (Child's stage A–C; n=14) compared with healthy controls (n=5). One patient with Child-Pugh stage A was retrospectively excluded as this patient did not fulfill the inclusion criteria (for example, a single drink/alcohol intake shortly before liver biopsy and venous puncture; histologically no alcoholic hepatitis; no blood parameters of chronic alcohol consumption either at the time of venous puncture or at previous examinations in the outpatient department for more than two years). GPDH, glyceraldehyde 3-phosphate dehydrogenase. (B) Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) (ratio of mRNA of IL-18/GPDH) revealed that significantly more RT mRNA was expressed in Child's stages B/C than in stage A. Median (range) for Child's stage A, 0.02 (0.01–0.15); for Child's stage B, 0.21 (0.09–0.38); and for Child's stage C, 0.39 (0.32–1.69). Box plots show median and 95% confidence intervals (CI). Individual data points are mean serum levels above or below the 95% CI. *p<0.05.*

Moreover, both serum levels of CD14 (fig 2) and plasma levels of LPS (fig 3) in patients with AC were significantly elevated in Child-Pugh stages B and C (p<0.001) compared with healthy controls (p<0.001).

Analysis by Spearman rank correlation showed a significant correlation between serum levels of IL-18 and CD14 (r=0.47, p<0.001) (fig 4) and a weak correlation with corresponding plasma levels of LPS (r=0.26; p<0.05; data not shown).

EXPRESSION OF HUMAN IL-18 DETECTED BY RT-PCR

Semiquantitative RT-PCR showed no difference between mRNA expression of IL-18 in unstimulated PBMC of patients with AC (n=14) of Child-Pugh stages A (n=4) and B (n=5) compared with five healthy controls. In contrast, enhanced mRNA expression of IL-18 was found in unstimulated PBMC of patients with AC of Child-Pugh stage C (n=5) compared with both healthy controls (n=5; p<0.01) and Child-Pugh stage A (n=4; p<0.01) (fig 5A, B).

Results of Spearman rank correlation analysis demonstrated a significant correlation between mRNA expression of IL-18 in unstimulated blood mononuclear cells of patients with AC (n=14) and both sCD14 (r=0.72; p<0.01) (fig 6A) and plasma LPS (r=0.76; p<0.01) (fig 6B).
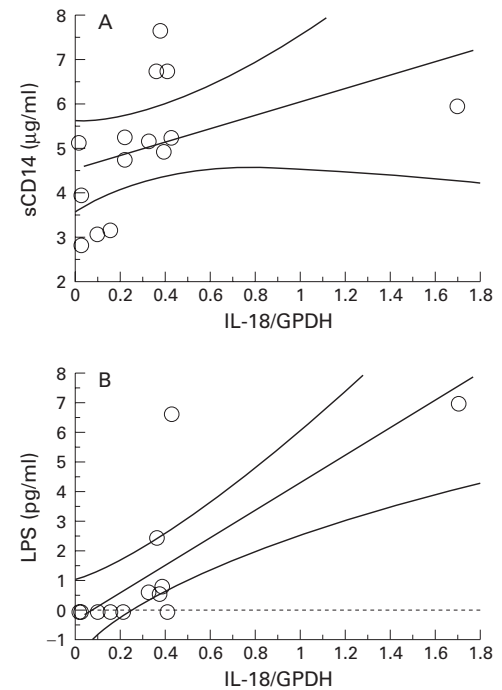

Figure 6 (A) Spearman rank analysis demonstrated a significant correlation between mRNA expression of interleukin 18 (IL-18) in unstimulated peripheral blood mononuclear cells of patients with alcohol induced cirrhosis and sCD14 (r= 0.72; p < 0.01). (B) Spearman rank analysis demonstrated a significant correlation between mRNA expression of IL-18 and lipopolysaccharide (LPS) (r=0.76; p<0.01). GPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Our results demonstrate enhanced gene expression of IL-18 in freshly isolated unstimulated PBMC of patients with severe AC (Child-Pugh stage C) compared with PBMC from healthy controls (with constitutive expression of IL-18 specific mRNA).

Recent in vitro data show that endotoxin (LPS) stimulates IL-18 mRNA expression of PBMC.[16 20 31 32] Our in vivo data demonstrate a significant correlation between endotoxin (LPS) plasma (and CD14 serum) levels and IL-18 mRNA expression of unstimulated PBMC. These strongly positive correlations suggest presensitisation of PBMC in the circulation of patients with AC by endotoxin. The term "unstimulated" may therefore be misleading in patients with AC.

Recent in vitro data showed that 1 µg/ml of pure LPS (in the absence of CD14) stimulated IL-18 mRNA expression in mouse macrophage-like cells.[17] CD14 dependent in vitro stimulation of mononuclear blood cells by LPS was shown to occur with even lower LPS concentrations (1 ng/ml LPS).[33] These endotoxin concentrations may also occur in portal venous blood (endotoxin concentrations of 100 pg/ml to 1 ng/ml[1]) and sensitise PBMC in vivo. Further investigations are needed to define the exact mechanisms and localisation of sensitisation of PBMC in vivo (for example, portal blood *v* low endotoxin concentrations encountered in peripheral blood from patients with AC of Child-Pugh stage C (5.0 (1.8) pg/ml; range 0–63)) and the possible additional enhancing or inhibiting factors.[34]

Preliminary data based on mRNA expression of IL-18 in unstimulated PBMC of six patients with (hepatitis C) virus induced cirrhosis (n=6; Child-Pugh stage A=3; C=3) suggest that endotoxin dependent sensitisation of PBMC may be more specific to cirrhosis per se and reduced hepatic clearance than to the aetiology of the disease (unpublished data).

Ethanol affects the production of proinflammatory cytokines such as TNF-α.[35 36] To avoid direct ethanol effects, we excluded patients with an abstinence period of less than six months and histological or biological markers of alcoholic hepatitis (thus longer than in comparable studies with abstinence periods of 1–2 months).[37]

Previous studies repeatedly reported a close relationship between elevated circulating plasma levels of proinflammatory cytokines (for example, TNF-α) and the severity of AC.[7 38] We have demonstrated for the first time a close relationship between elevated plasma levels of IL-18 and Child-Pugh stage of the disease. Even if IL-18 serum levels of cirrhotics may be supposed to reflect impaired hepatic clearance (as previously discussed for other cytokines[6 39]) increased cytokine serum levels encountered in peripheral venous blood are believed to be derived at least partially from PBMC. However, preliminary ELISA data based on 22 patients with hepatitis C virus induced cirrhosis (Child-Pugh stage A, n=11; Child-Pugh stage B, n=4; Child-Pugh stage C, n=7) led us to the conclusion that elevated IL-18 serum levels in severe AC are more specific to cirrhosis per se than to the aetiology of the disease.

Concentrations of LPS measured in patients with AC were comparable with cell free plasma endotoxin levels reported in the literature.[7 9 29 30 40] The rather weak correlations between plasma LPS and serum IL-18 protein levels are not surprising for at least two reasons: firstly, portosystemic shunting is known to result in repetitive peaks of endotoxinaemia and not in a constant high endotoxinaemia. Secondly, IL-18 protein production is mostly regulated constitutively (not inducibly).

In conclusion, enhanced expression of IL-18 in unstimulated PBMC supports the assumption that IL-18 is involved in the inflammatory response of PBMC in patients with AC. Together with recent in vitro data in human PBMC from healthy individuals, it may be suggested that gut derived bacterial endotoxin is involved in enhanced gene expression of IL-18.[16 31] Although semiquantitative RT-PCR is thought to be appropriate for analysing cytokine mRNA,[13 41–44] data should be confirmed by quantitative PCR.

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