Interleukin-18 (IL-18), originally named interferon-γ (IFN-γ) induc- ing factor, is a 18-kD cytokine synthesized by Kupffer cells and by activated macrophages [1–3]. IL-18 is a coinducer of IFN-γ while decreasing the production of IL-10 [4], and is structurally related to IL-1β [4]. Similarly to the IL-1β precursor, the IL-18 precursor (pro-IL-18) also lacks a signal peptide and requires caspase-1 (also known as IL-1β-converting enzyme, ICE) for cleavage and release of the mature, bioactive molecule from the intracellular compartment [5–7]. IL-12 and IL-18 are currently regarded as the primary inducers of IFN-γ in inflammatory reactions [8].

Previous studies provide significant evidence indicating that IL-18 plays a prominent role in liver injury [9]. Experimental liver failure in mice can be induced by sequential administration of Propionibacterium acnes and lipopolysaccharide (LPS) [10]. Neutralization of IL-18 by administration of anti-IL-18 mononclonal antibodies (mAb) results in total prevention of liver injury [10]. Accordingly, IL-18 deficient mice are resistant to LPS-induced liver injury [11]. Furthermore, Fas ligand (FasL)-induced liver injury is dependent on caspase-1-independent IL-18 secretion from macrophages [12]. In chronic hepatitis C virus (HCV) infection, a significant up-regulation of IL-18 in the inflammatory infiltrate has been demonstrated, suggesting a role of this cytokine in the chronic cellular immune response toward hepatocytes in the course of the disease [13,14].

Recently, a circulating IL-18 binding protein (IL-18BP) was purified, characterized and cloned [15]. Administration of IL-18BP to mice abrogates circulating IFN-γ following LPS treatment, demonstrating in vivo that IL-18BP functions as an inhibitor of the early Th1 response [15]. The human IL-18BP gene encodes for at least four distinct isoforms derived from mRNA splice variants, with isoform IL-18BPa being the most abundant and most active as inhibitor of IL-18 [15,16].

Interferon-alpha (IFN-α) has proven its clinical efficacy in the treatment of chronic HCV infection [17,18]. Currently, the detailed molecular mechanisms of action of IFN-α are not completely understood, and interactions with the cytokine cascade as well as direct antiviral action might account for these properties [19,20]. Since IL-18 plays a significant role in liver injury, we evaluated whether IFN-α affects IL-18 activity in HCV patients.
MATERIALS AND METHODS

Clinical study
Thirteen patients with chronic HCV infection were assigned to receive 1 × 10^7 IU IFN-α (Intron A, AESCA GmbH, Traiskirchen, Austria) s.c. daily. During the treatment period concomitant medication remained stable in all patients studied. Peripheral venous blood was drawn immediately before and 2, 6, 12, 24, 48 and 72 hours and 7, 10, 14, 17, 21, 24, and 28 days after initiation of therapy. The blood was centrifuged and heparinized plasma stored at –80°C until determination of IL-18 and IL-18BP. The study was approved by the institutional ethics committee, and written informed consent was obtained from all patients.

IL-18, IL-18BP and IFN-γ enzyme-linked immunosorbent assay (ELISA)
IL-18 was measured by a commercial ELISA according to manufacturer’s (R & D Systems, Minneapolis, MN, USA) instructions. The lower detection limit of the assay was 15 pg/ml. No significant cross-reactivity with a multitude of recombinant cytokines has been observed. IL-18BP as high as 160 ng/ml has no effect on the IL-18 ELISA. The predominant, neutralizing isoform a of IL-18BP was determined with a recently developed ELISA [21]. The limit of detection was 62 pg/ml. No significant cross-reactivity with human serum, related cytokines and IL-18BP isoforms (b, c and d) has been observed [21]. Free IL-18 was calculated according to the law of mass action as recently described [21]. In brief, the calculation was based on a 1:1 stoichiometry in the complex of IL-18 and IL-18BP, a molecular weight of 18kD and 24kD, respectively, and a dissociation constant (Kd) of 0.4 nM [21]. IFN-γ was determined using a commercially available antibody pair (Becton Dickinson, Heidelberg, Germany) according to manufacturer’s instructions. The lower detection limit of this assay was 10 pg/ml.

Media, reagents, and antibodies
The culture medium used in this study was RPMI 1640 (Schoeller Pharma, Vienna, Austria) supplemented with 10% heat-inactivated (30 min, 56°C) fetal calf serum (FCS, Gibco, Vienna, Austria), 100 U/ml penicillin G and 100 μg/ml streptomycin (Schoeller Pharma). Recombinant human (rhu) GM-CSF (Molgramostim, Leucomax®) was purchased from Novartis (Vienna, Austria), and IFN-α (Intron A®) from Schering-Plough (AESCA, Traiskirchen, Austria). CD1a and CD14 antibodies were purchased from Pharmingen (Hamburg, Germany), HLA-DR antibody was from Serotec (Oxford, UK), and CD83 from Instrumentation Laboratory (Vienna, Austria).

Cell culture
Macrophages were generated as described [22]. In brief, peripheral blood mononuclear cells (PBMC) were obtained from leucocyte-enriched buffy coats from healthy volunteers (provided by the local blood bank, n = 11) or HCV+ patients (n = 12) by centrifugation over Ficoll-Hypaque (Lymphoprep®, Nycomed, Oslo, Norway). After washing in PBS, 5 × 10^7 PBMC were resuspended in culture medium, and monocytes were allowed to adhere for 45 min in 75 cm² tissue culture flasks. After extensive washing in PBS, 10 ml culture medium supplemented with 1000 U/ml rhuGM-CSF was added to the monocyte cultures, and differentiation into macrophages allowed for 14 days. The medium was replaced every 3 days. Purity of the macrophages was confirmed by flow cytometry.

Day-14 macrophages were harvested and seeded at a density of 2.5 × 10^5 per ml in culture medium. IFN-α was added at concentrations of 1000 IU/ml as indicated.

Northern blot analysis
Total RNA was prepared from 2 × 10^7 macrophages stimulated with or without 1000 U/ml IFN-α for 24 h. Ten μg of each was gel-electrophoresed and blotted onto nylon membranes as recently described [23]. The IL-18 probe was a Bgl II/Spe I insert of a cDNA kindly provided by Dr T. Ghayur, BASF Bioresearch Corp, Worcester, MA, USA. The IL-18BP probe was an EcoR I insert of a cDNA recently described [15]. The probes were radioactively labelled with 32P dCTP employing the random primed labelling method according to manufacturer’s (Boehringer Mannheim, Vienna, Austria) instructions and hybridized as described [23]. Control-hybridizations were performed with the rat cDNA of the housekeeping gene glyceraldehyde-3-phosphatehydrogenase (GAPDH) to ensure equal loading of RNA.

Flow cytometric analysis
Macrophages (2.5 × 10^7) were washed in PBS/2% FCS, resuspended in PBS containing 250 μg/ml HlgG and 2% FCS and incubated for 20 min at 4°C. Cells were then washed and incubated with the indicated primary antibodies at 10 μg/ml for 30 min at 4°C. After washing in PBS/2% FCS, a 1:40 dilution of FITC-anti-mouse IgG in PBS/2% FCS was incubated for 30 min at 4°C. After washing in PBS/2% FCS, cells were resuspended and immediately analysed on a FACScan (Becton Dickinson, San Diego, CA). Data analysis was performed with CellQuest software (Becton Dickinson).

Statistical analysis
Data are presented as mean ± SEM. Statistical significance was evaluated by paired Student’s t-test for the clinical study and the in vitro experiments. Differences between HCV+ patients and healthy controls in in vitro experiments were tested with the unpaired Student’s t-test.

RESULTS

IFN-α therapy enhances circulating IL-18BP plasma levels
IL-18BP plasma levels were determined at various time points in 13 patients with chronic hepatitis C virus infection receiving IFN-α, at a daily dose of 1 × 10^7 IU subcutaneously. The mean level of IL-18BP in healthy donors was shown to be 2.15 ± 0.15 ng/ml [21]. In HCV patients prior to IFN-α treatment (time 0) a higher plasma level (8.48 ± 1.75 ng/ml) was found (Fig. 1a). The levels rose already at 6 h (11.7 ± 2.2 ng/ml) reaching more than a three-fold increase 24 h after the first administration of 1 × 10^7 IU IFN-α (8.48 ± 1.75 versus 27.48 ± 2.55 ng/ml, P < 1 × 10^-7, at times 0 and 24 h, respectively). IL-18BP levels then gradually declined, though remained substantially elevated throughout the 28 days of IFN treatment compared to time 0 (e.g. 12.48 ± 1.63 ng/ml at day 10 versus 8.48 ± 1.75 ng/ml at time 0, P < 0.05).

Presumably as a negative feedback loop, IFN-γ induces IL-18BP mRNA expression and protein release, as demonstrated in epithelial cell lines [24,25]. To exclude the possibility that induction of IL-18BP is secondary to IFN-α-induced IFN-γ release, we determined IFN-γ plasma levels during the course of IFN-α treatment. During the entire treatment period, IFN-γ levels were just
at the detection limit of the assay, and IFN-γ was neither induced at early nor at late time-points (data not shown).

**Free plasma IL-18 is reduced by IFN-α treatment**

The mean level of IL-18 in healthy donors was shown to be 64 ± 17 pg/ml [21]. The mean total plasma level of IL-18 in the HCV patients, as measured by the IL-18 ELISA, was 113 ± 25 pg/ml at time 0 (Fig. 1b) and no significant change was observed throughout the IFN treatment except for day +24 and +28 (53 ± 15 pg/ml, P < 0.05; Fig. 1b).

Since IFN-α induced plasma IL-18BP, which tightly binds IL-18 and blocks its activity, we calculated the level of free IL-18 as recently described [21]. Figure 2a shows a time-course of free plasma IL-18 during daily s.c. administration of 1 × 10^7 IU IFN-α. Free IL-18 reached a minimum 24 h after the first injection of IFN-α (3.41 ± 0.9 pg/ml versus 1.11 ± 0.3 pg/ml, P < 0.01). Figure 2b compares total IL-18 to free IL-18 24 h after the first injection of IFN-α, and demonstrates that most of the IL-18 is bound by IL-18BP.

**IL-18 synthesis is diminished by IFN-α in human macrophages**

To study the effect of IFN-α on IL-18 production, peripheral blood was obtained from healthy volunteers and macrophages were derived by culturing of plastic-adherent monocytes with rhuGM-CSF for 14 days as previously described [22]. On day 14, the macrophage population was virtually pure as confirmed by flow cytometry and staining for T, B, and NK cell markers (data not shown). Stimulation of macrophages with IFN-α was performed over a period of seven days. IL-18 in the supernatants of these macrophage cultures was determined by ELISA. As shown in Fig. 3a, unstimulated macrophages secreted 1891 ± 688 pg/ml IL-18 into the supernatant. Addition of IFN-α resulted in a 50% decrease (937 ± 390, P < 0.05). Similar results were obtained in cultures of macrophages derived from chronic hepatitis C patients, where IFN-α decreased IL-18 secretion from 2419 ± 761 pg/ml to 657 ± 108 pg/ml (P < 0.05, Fig. 3b). No statistically significant difference was noted between HCV patients and healthy controls. To exclude the possibility that IFN-α led to a phenotypic alteration of the macrophage population, flow cytometry was performed. As indicated in Fig. 4, surface markers presented equally in untreated and IFN-α-treated macrophages.

In contrast with its effect on IL-18, IFN-α had no significant effect on secretion of IL-18BP from healthy macrophage cultures (2.65 ± 0.259 versus 2.37 ± 0.31 ng/ml, n.s., Fig. 5a). As depicted in Fig. 5b, in cultures of macrophages derived from HCV patients, IFN-α had a significant but rather small inhibitory effect (2.166 ± 0.259 versus 1.547 ± 0.157 pg/ml, P < 0.01).

**IFN-α increases IL-18BP mRNA steady-state levels in macrophages**

Day-14 macrophages were stimulated with or without IFN-α for 24 h and subsequently RNA extracted and analysed for IL-18 and

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IL-18BP mRNA steady-state levels by Northern hybridization. As depicted in Fig. 6, IFN-α increased IL-18BP mRNA levels. However, stimulation with IFN-α did also result in a slight increase in IL-18 mRNA levels (Fig. 6).

Fig. 3. IFN-α down-regulates IL-18 release from macrophages. Macrophages derived from healthy volunteers (a) or chronic hepatitis C patients (b) were cultured for seven days either unstimulated, or stimulated with IFN-α (1000 IU/ml). IL-18 release into supernatant was assayed by ELISA. *indicates $P < 0.05$.

IL-18 was originally identified as IFN-γ-inducing factor in the serum and the liver of mice that had been infected with Propionibacterium acnes and after 8 days, injected with LPS [1]. This sequential treatment induces lethal shock in mice, and the surviving mice show evidence of acute liver injury. Administration of neutralizing anti-IL-18 mAbs prevents this acute liver injury, pointing toward a prominent role of this cytokine in liver pathology [9]. Injection of LPS-challenged mice with recombinant IL-18BP results in abrogation of IFN-γ induction [15]. There exists direct evidence for a prominent role of IL-18 in chronic HCV infection. McGuinness et al. [14] reported significantly increased IL-18 (and IFN-γ) in liver specimen of chronic HCV patients compared to specimen derived from HCV control subjects. We herein demonstrate that IFN-α exerts anti-inflammatory properties by induction of IL-18BP and late suppression of IL-18. Administration of IFN-α to chronic HCV patients results in elevation of IL-18BP plasma levels within 6 h, peaking 24 h post administration with a more than three-fold increase. Sequential treatment with IFN-α over a 28-day period results in partial adaptation, with a still 47% increase at the end of the study period compared to pretreatment levels. In biological accordance with this marked augmentation of the antagonist, IL-18 plasma levels steadily declined over the study period, resulting in a 53% decrease at day +28 compared to pretreatment values. As demonstrated, the unexpected net effect of IFN-α administration on IL-18 and IL-18BP plasma levels consists in a substantial reduction of free IL-18, with a minimum at 24 h post IFN-α administration.

We have previously suggested that anti-inflammatory properties of IFN-α might partly account for its clinical efficacy in chronic HCV infection [19,26]. It is important to note that IFN-α promotes anti-inflammatory effects – among others – via two cytokine families prominently involved in liver pathology, namely the TNF-α family [27,28] and the IL-1 family [29,30]. Our demonstration of anti-inflammatory interaction of IFN-α with IL-18 and IL-18BP expands this view to the currently appreciated central cytokine pathway in experimental liver failure.

Discussion

Fig. 4. IFN-α does not alter macrophage phenotype. Macrophages cultured with (b) or without (a) IFN-α (1000 IU/ml) for seven days were stained for indicated surface markers and analysed by flow cytometry. The flow cytometric analysis shown is representative for 3 independent experiments.

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Evidence exists showing that the clinical efficacy of IFN-α in chronic HCV infection depends on the induction of HCV-specific Th1 and Tc1 cells [31–33]. From this point of view, it might be argued that suppression of the Th1 cytokine IL-18 and induction of IL-18BP by IFN-α is not favourable for the resolution of disease. However, another study demonstrated that the expression of IFN-γ by activated T cells was significantly lower in patients who favourably responded to a combined treatment of IFN-α and ribavirin [34]. Induction of IL-18BP and suppression of IL-18 by IFN-α as shown here, might contribute to reduction of the antigen nonspecific pro-inflammatory attack toward hepatocytes, while IFN-α might promote the generation of HCV antigen-specific T cells [31]. However, it should be noted that definitive demonstration of a reduction of hepatic inflammation by IFN-α through the IL-18/IL-18BP pathway warrants further investigation.

Viral infections are known to suppress the immune system. As an example, infection of dendritic cells with measles virus blocks their allostimulatory properties for CD4+ T cells, although the exact molecular mechanism is currently not understood [35]. Induction of IL-18BP and inhibition of IL-18 by endogenous IFN-α might constitute another contribution to the immunosuppressive state during viral infections. This effect of IFN-α on IL-18 is in accordance with its effect on IL-1, since it has previously been demonstrated that IFN-α suppresses IL-1-induced IL-1 expression and augments LPS-induced IL-1 [36,37]. Since IL-18 is constitutively expressed [38] in contrast to IL-1β [39,40], IFN-α even suppresses constitutive production of IL-18.

In this study we have also demonstrated that IFN-α downregulates IL-18 release into the supernatant of cultured macrophages. Interestingly, this effect is evident after seven days of culture, while no effect is observed after 24 h (data not shown). Retention of the surface phenotype of the in vitro differentiated macrophages stimulated for 7 days with IFN-α was confirmed by flow cytometric analysis, and excluded the possibility that the macrophage population itself was altered by incubation with IFN-α. These in vitro data correspond to the presented in vivo data and might outline the cellular basis of IFN-α effects on IL-18.

Mühl et al. and Paulukat et al. [24,25] recently reported induction of IL-18BP mRNA by IFN-γ in nonleucocytic cells, suggesting a negative feedback regulation of IL-18. IFN-γ plasma levels were not elevated during the course of IFN-α treatment, excluding the possibility that induction of IL-18BP is secondary to IFN-γ. We did not observe induction of IL-18BP by IFN-α-treated macrophages on the protein level in vivo, although an increase in IL-18BP mRNA steady-state levels was demonstrated. However, other cells might account for the induction of IL-18BP by IFN-α treatment in vivo. It might be speculated that the IFN-α-inducible transcription factor interferon regulatory factor-1 (IRF-1) might have some role in the up-regulation of IL-18BP by IFN-α [41], since reduced constitutive and IL-12-induced IL-18BP mRNA expression has recently been demonstrated in IRF-1 knock-out mice [42].

In conclusion, we show that in HCV patients IFN-α upregulates IL-18BP expression, thereby reducing free IL-18. We propose that this anti-inflammatory mechanism contributes to the clinical efficacy of IFN-α in HCV related liver failure on top of its direct antiviral activity.
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