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Involvement of interleukin 18 in Crohn's disease: evidence from *in vitro* analysis of human gut inflammatory cells and from experimental colitis models

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SUMMARY

An imbalance of immunoregulatory factors and/or cells contributes to uncontrolled mucosal T cell activation and inflammation in Crohn's disease (CD). Bioactive interleukin (IL)-18 has been shown to be produced by macrophages in CD lesions. We report here that T cells freshly isolated from inflamed tissue of CD patients (and not T cells from control intestinal tissue) were responsive to IL-18. In the presence of IL-18, these T cells produced more interferon (IFN)-γ and less IL-10. To analyse further the role of IL-18 in this disease, an acute and a chronic model of murine colitis were used. IL-18 mRNA was significantly enhanced in trinitrobenzene sulphonic acid (TNBS) induced colitis, and treatment with IL-18 binding protein (IL-18BPa), which neutralizes IL-18 bioactivity, significantly reduced the severity of colitis. However, IL-18BPa did not affect the course of chronic colitis in CD45RB^{high}CD4$^+$ T cell reconstituted SCID mice. Production of IFN-γ in lamina propria mononuclear cell cultures from IL-18BPa-treated SCID mice was decreased, but at the same time fewer lamina propria CD4$^+$ T cells harvested from IL-18BPa-treated mice compared to non-treated mice were in apoptosis. We conclude that IL-18 clearly has a modulatory role in the inflammatory cascade of CD and experimental colitis by affecting IFN-γ and IL-10 production, and apoptosis. In view of the divergent effects of IL-18 neutralization in the two different murine colitis models, it is unlikely that IL-18 is at the top of this cascade.

Keywords apoptosis Crohn's disease IL-18 IFN-γ IL-10

INTRODUCTION

Crohn's disease (CD) is a major form of inflammatory bowel disease. In genetically susceptible individuals, CD probably arises because of a breakdown in the regulatory constraints on mucosal immune responses to enteric bacteria [1]. Once the balance is broken, a T helper (Th) 1 cell-mediated immune response arises, in which cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-12 are involved [2–5]. Recently, up-regulation of IL-18 mRNA levels in ileal and colonic intestinal epithelial cells (IEC) and lamina propria mononuclear cells (LPMC) from CD tissue has been reported [6,7]. The bioactive form of IL-18 could be found only in CD and not in controls [6,7]. IL-18-expressing cells in inflamed CD tissue are mainly macrophages, while T cells express IL-18R [8,9]. In the serum of CD patients, IL-18 concentrations measured by enzyme-linked immunosorbent assay (ELISA) are significantly higher compared to the concentrations in control subjects and correlate with disease activity [10]. A significant positive correlation between IL-18 and IL-18 binding protein (BP), a natural inhibitor of IL-18, in colon specimens from active CD patients has been reported [11]. Recently several groups demonstrated a beneficial effect of IL-18 blocking agents in animal models of colitis [12–16].

IL-18 is produced mainly by macrophages and Kuppfer cells as a 24 kDa precursor protein that is cleaved by IL-1β converting enzyme into a bioactive 18·3 kDa glycoprotein [17,18]. Its receptor consists of two chains: the ligand binding IL-18Rα chain and the signal transducing IL-18Rβ chain, expressed on CD4$^+$ (Th0 and Th1), CD8$^+$ cells, natural killer (NK) cells, B cells and macrophages [17,19,20]. The signalling proceeds through IL-1R associated kinase (IRAK) resulting in nuclear translocation of the p65/p50 NF-κB complex [17]. The immunomodulatory actions of IL-18 are complex. It synergizes in IL-12-driven Th1 differentiation [21–23] and activates anti-CD3-stimulated Th1 cells, CD8$^+$ cells, NK cells and macrophages to produce IFN-γ, particularly in the presence of IL-12 [18–20,24]. Part of this effect is due to IL-18-

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induced up-regulation of the IL-12Rβ2 subunit [22]. However IL-18 is also able to promote Th2 cytokine production (IL-4, IL-13) and to aggravate Th2-mediated diseases [17]. IL-18 induces Fas-mediated apoptosis of T cells and tumours [25,26] and is involved in chemoattraction of IL-18R$^+$ CD4$^+$ T cells [27].

In the present paper we explored the functional effects of IL-18 in LPMC cultures from CD patients and controls. Moreover, we report our study on the role of IL-18 in both an acute and chronic model of colitis in mice.

MATERIALS AND METHODS

Reagents and mice

Recombinant human (rh) IL-18 was a kind gift from Dr H. Shiratsuchi (Case Western Reserve University School of Medicine, Cleveland, OH, USA) and Dr H. Okamura (Hyogo College of Medicine, Nishinomiya, Japan). rhIL-12 was obtained as a gift from Genetics Institute (Cambridge, MA, USA). IL–18 BP isoform-a is a polypeptide that belongs to the immunoglobulin superfamily and was shown to neutralize IL-18 activity by preventing its interaction with the IL-18R [28]. IL-18BPa cross-reacts with murine IL-18 [29]. rhIL-18BPa used in this study was a gift from Serono (Geneva, Switserland). The monoclonal antibodies (MoAbs) used in this study included anti-human CD3 MoAb UCHT-1 (a gift from Dr P. Beverley, Edward Jenner Institute for Vaccine Research, Compton, UK). Anti-mouse CD3ε MoAb (clone 500A2, hamster IgG), PE-conjugated anti-mouse CD4 MoAb (clone GK1·5, rat IgG2b) and FITC-conjugated anti-mouse CD45RB MoAb (clone 16 A, rat IgG2a) were purchased from PharMingen (San Diego, CA, USA). 7-Amino actinomycin-D (7AAD) was obtained from Novabiochem (La Jolla, CA, USA).

Specific pathogen-free female BALB/c mice and male C57BL/6 mice were obtained from Taconic M&B (Ry, Denmark). Specific pathogen-free female C.B-17 SCID mice were obtained from the REGA institute (University of Leuven, Belgium). All mice were maintained in the Animal Care Facility of Gasthuisberg, Catholic University of Leuven, in microisolator cages with filtered air and free access to autoclaved food and water. All studies were approved by the local ethical committee on animal experimentation of the Catholic University of Leuven.

Patient samples

Mucosal samples were obtained from surgical specimens of 11 CD patients. This group included five men and six women, aged 20–60 years. All patients had pure ileal involvement. Of all patients, five were receiving an oral aminosalicylic acid preparation at the time of operation, five patients received corticosteroids, one methotrexate and three received antibiotics. The preoperative diagnosis of CD was based upon classical clinical, radiological and endoscopic features and was confirmed by histological examination of the resection specimens. Indications for resection in CD were the presence of fistulae ($n = 1$), abscess formation ($n = 2$) and stenosis with clinical signs of obstruction ($n = 8$). Samples of normal intestinal ileal tissue were obtained as control (CO) from surgical specimens of eight patients (four men and four women, aged 64–87 years) undergoing right hemicolectomy for colonic carcinoma (< T3N0M0). These control samples were macroscopically and microscopically normal. All surgical specimens were immediately transferred to the laboratory of pathology in a container filled with ice-chilled RPMI-1640 solution (Bio-Whittaker, Verviers, Belgium). Samples from macroscopically non-lesional and lesional tissue from CD and normal tissue from controls (2×3 cm mucosal area each) were used for isolating ileal LPMC. Informed consent was obtained from all subjects.

TNBS colitis model

TNBS colitis was induced as described previously [14]. On day 0, acute colitis was induced in 6-week-old C57Bl/6 mice by intrarectal administration of 4 mg of 2, 4, 6-trinitrobenzene sulphonic acid (TNBS; Sigma Chemical Co., St Louis, MO, USA) in 0·1 ml 50% ethanol through a trochar needle approximately 3·5–4 cm proximal to the anus. Control mice received 0·1 ml 50% ethanol alone. During instillation, the mice were anaesthetized with nembutal (Sanofi, Brussels, Belgium) and after instillation, mice were kept vertically for 60 s to avoid reflux. On day 7 all mice were again manipulated as described for day 0; however, only 2 mg of TNBS was used. On day 9 all mice were sacrificed. Mice were treated by daily intraperitoneal (i.p.) injection with 200 μl of phosphate buffered saline (PBS) or with 200 μg of rhIL-18BPa, suspended in 200 μl PBS. Some mice were killed 2 days after intrarectal injection with TNBS or ethanol. Disease activity was monitored daily on the basis of body weight. At the end of the experiment colons were harvested and scored for macroscopic damage using a scoring system with a maximal score of 13, described previously by Morris *et al*. [30].

SCID colitis model

Spleen cells from BALB/c mice were used as a source of CD4$^+$ cells and separated further into a CD45RB^{high} and CD45RB^{low} fraction under sterile conditions by two-colour sorting on a FACS Vantage (Becton Dickinson, CA, USA), as described previously [31]. All populations were >98% pure on re-analysis. To induce colitis, C.B-17 SCID mice were injected i.p. with sorted syngeneic CD45RB^{high}CD4$^+$ T cells (4×10^5 cells/mouse). Some mice were injected i.p. with total CD4$^+$ T cells (5×10^5 cells/mouse) as a control. Reconstituted C.B-17 SCID mice were treated by i.p. injection with PBS alone (200 μl) or with rhIL-18BPa, suspended in 200 μl PBS. In two independent experiments, different doses of rhIL-18BPa were used: 10 mg/kg/2d from day 10 after T cell reconstitution over a period of 7 weeks (low dose) or 10 mg/kg/2d from days 1–10 after T cell reconstitution and 10 mg/kg/day from days 11–56 (high dose). Disease activity was monitored weekly on the basis of body weight and soft stool or diarrhoea.

Isolation of LPMC and culture conditions

Human LPMC were isolated from both CD and control mucosa, as described previously [32]. Cells were washed three times, counted with a haemocytometer and stained with trypan blue 0·4% (Bio-Whittaker, Verviers, Belgium) in order to correct for viability. The isolated LPMC were diluted into culture medium at a final concentration of 5×10^5 viable cells in 1 ml (24-well plates) and incubated at 37°C in 5% CO_2 humidified air. The culture medium was RPMI-1640 solution supplemented with 0·3 mg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Bio-Whittaker, Verviers, Belgium), 4 U/ml polymyxin, 5 μg/ml amphotericin B (Sigma Chemicals, St Louis, MO, USA) and 10% iron-supplemented bovine calf serum (HyClone, Logan, UT, USA). Cells were stimulated with anti-human CD3 MoAb (5 μg/ml) and human CD80-transfected mitomycin C-treated (50 μg/ml) P815 cells (5×10^5 in 1 ml) in the absence or presence of

rhIL-18 (40 ng/ml) and rhIL-12 (1 ng/ml). Supernatants were collected after 48 h.

Mice LPMC were harvested from the colon, as reported previously [31]. The isolated LPMC were diluted into culture medium at a final concentration of 5×10^5 cells in 1 ml (24-well plates). Cells were stimulated with soluble anti-mouse CD3ε (5 μg/ml) and mouse CD80-transfected mitomycin C-treated (50 μg/ml) P815 cells (5×10^5 in 1 ml) and supernatants were collected after 48 h.

Cytokine assays

Measurements of IFN-γ, IL-4, IL-10 and TNF-α production levels were performed by sandwich ELISA, using matched MoAb pairs according to the manufacturers' instructions (BioSource International, Fleurus, Belgium for human IFN-γ, human IL-4, human TNF-α and mouse IFN-γ, PharMingen, San Diego, CA, USA for human IL-10).

Histological examination

Murine colon tissue samples were collected at the end of the experiment and fixed in PBS containing 6% formalin. Paraffin-embedded sections (5 μm) were stained with haematoxylin and eosin. The sections were analysed without prior knowledge of the type of T cell reconstitution or treatment. In the T cell transfer model, mucosal and submucosal lesions were scored, using a previously described score system [31]. In the TNBS model a scoring system with a maximal score of 14, adapted with minor modification from McCafferty *et al.* was used [33].

Flow cytofluorometric analysis of apoptosis

LPMC were isolated from the colon of mice as previously described [31]. Cells were stained with PE-conjugated anti-CD4 MoAb and incubated in a small volume of PBS for 30 min at 4°C. Cells were washed twice and stained with 7AAD solution [34]. 7AAD was dissolved in acetone and diluted in PBS at a concentration of 20 mg/ml. Cells were incubated for 10 min at 4°C protected from light. Data were acquired using a FacSort (Becton Dickinson, CA, USA) without wash or fixation of the sample. Data on 10 000 events were analysed using cellquest software.

RNA extraction and quantitative PCR

Colonic tissue samples (two samples from 0·5 to 1 cm length each, from diseased areas in case of colitis or randomly in the absence of colitis) were obtained from SCID mice, either IL–18 BP- or PBS-treated, 8 weeks after $CD45RB^{high}CD4^+$ or total $CD4^+$ T cell reconstitution as well as from C57Bl/6 mice, 2 days after intrarectal injection with TNBS or ethanol. All samples were frozen immediately in liquid nitrogen after dissection and stored at –80°C until use. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. A constant amount of 2 μg target RNA was used for cDNA synthesis (Ready-to-go-kit; Pharmacia, Uppsala, Sweden). After 90 min at 37°C, the reverse transcriptase was inactivated by incubating the cDNA samples for 5 min at 95°C. The cDNA samples were then subjected to real-time quantitative PCR, performed in the ABI prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) as described previously [35]. The sequences of the primers and probes for IFN-γ, TNF-α, IL-12p40, IL-18, IL-10 and β-actin have been reported previously [35]. All primers and probes were designed with the assistance of the computer program Primer Express (AB) and purchased from Eurogentec (Seraing, Belgium). The 5′-nuclease activity of the *Taq* polymerase was used to cleave a non-extendable dual-labelled fluorogenic probe. Fluorescent emission was measured continuously during the PCR reaction. PCR amplifications were performed in a total volume of 25 μl containing 5 μl cDNA, 12·5 μl Universal PCR Master Mix, no AmpErase® UNG (AB), 100–300 nM concentrations of each primer and 200 nM concentrations of the corresponding detection probe. Each PCR amplification was performed in triplicate wells using the following conditions: 94°C for 10 min, followed by 40 or 45 cycles at 94°C for 15 s and 60°C for 1 min cDNA plasmid standards, consisting of purified plasmid DNA specific for each individual target, were used to quantify the target gene in the unknown samples, as described [35]. All results were normalized to β-actin to compensate for differences in the amount of cDNA in all samples.

Statistics

Statistical analysis was performed with Graph Pad Prism, version 3 (San Diego, CA, USA). The Wilcoxon matched-pairs testing was used for paired data and the Mann–Whitney *U*-test for unpaired data. For the weight curves, a one-way analysis of variance with Bonferroni post-testing was used. Statistical significance was established at $P < 0{\cdot}05$.

RESULTS

Effect of IL-18 on IFN-γ and TNF-α production in LPMC cultures from CD tissue

Up-regulation of IL-18 mRNA levels and the presence of bioactive IL-18 in CD tissue have been reported recently [6,7]. To evaluate the potential role of IL-18 on cytokine production in inflamed gut tissue from CD patients we studied the effects of rhIL-18, alone or together with rhIL-12, on the production of the proinflammatory cytokines TNF-α and IFN-γ by LPMC cultured *in vitro*. On the basis of preliminary dose–response experiments with CD LPMC cultures, the dose of 40 ng rhIL-18 was chosen for *in vitro* experimentation.

Data in Fig. 1 show that IFN-γ secretion was significantly higher in CD LPMC cultures than in control LPMC cultures ($P = 0{\cdot}005$ for non-lesional and $P = 0{\cdot}0009$ for lesional tissue). In LPMC cultures from controls ($n = 8$), rhIL-18 did not affect IFN-γ production, while rhIL-12 significantly up-regulated IFN-γ production ($P < 0{\cdot}05$). In contrast, rhIL-18 was found to induce an independent and significant up-regulation of IFN-γ production in LPMC cultures from non-lesional ($P = 0{\cdot}01$) and lesional ($P = 0{\cdot}02$) CD tissue ($n = 11$) (Fig. 1). Comparable effects were found when rhIL-12 was added into the cultures.

Baseline TNF-α levels were significantly higher in LPMC cultures from CD patients *versus* controls ($P = 0{\cdot}0012$ for non-lesional and $P = 0{\cdot}009$ for lesional tissue *versus* control tissue). rhIL-18 added either alone or together with rhIL-12 did not affect TNF-α production, neither in CD nor control LPMC cultures (data not shown). We thus conclude that T cells from CD tissue (but not from control intestinal tissue) are responsive to IL-18, and that IL-18 enhances IFN-γ production by these cells.

Effect of IL-18 on IL-4 and IL-10 production in LPMC cultures from CD tissue

IL-18 has been shown to be involved in both Th1 and Th2 cell differentiation and/or activity [17]. We therefore evaluated whether rhIL-18 could modify IL-4 and IL-10 production in LPMC

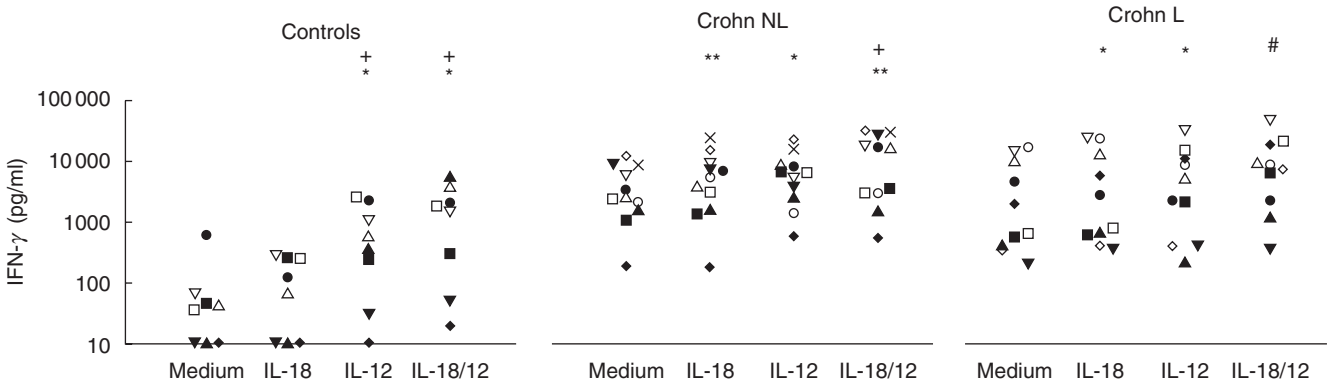

Fig. 1. Effect of rhIL-18 and rhIL-12 on IFN-γ production in human lamina propria mononuclear cell (LPMC) cultures. LPMC were isolated from ileum of controls ($n = 8$) and of Crohn's disease patients, both from non-lesional (NL) ($n = 11$) and lesional (L) ($n = 10$) tissue. Cells were cultured with anti-human CD3 MoAb (5 μg/ml) and with hCD80-expressing P815 cells (5×10^5/ml) in the absence or presence of rhIL-18 (40 ng/ml) and/or rhIL-12 (1 ng/ml), as indicated. After 48 h, supernatants were assayed for IFN-γ by ELISA. Each symbol represents the mean IFN-γ in duplicate cultures of LPMC from an individual patient. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ *versus* medium; #$P < 0{\cdot}05$ *versus* IL-12; +$P < 0{\cdot}05$ *versus* IL-18.

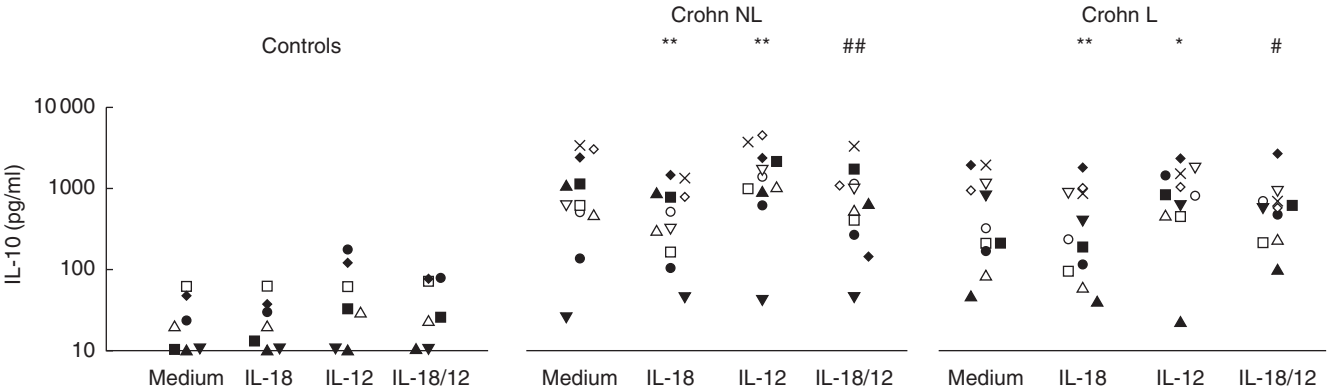

Fig. 2. Effect of rhIL-18 and rhIL-12 on IL-10 production in human lamina propria mononuclear cell (LPMC) cultures. LPMC were isolated from ileum of controls ($n = 8$) and Crohn's disease patients, both from non-lesional (NL) ($n = 11$) or lesional (L) ($n = 10$) tissue. Cells were cultured with anti-human CD3 MoAb (5 μg/ml) and with hCD80 expressing P815 cells (5×10^5/ml) with or without rhIL-18 (40 ng/ml) and/or rhIL-12 (1 ng/ml) as indicated. After 48 h, supernatants were assayed for IL-10 by ELISA. Each symbol represents the mean IL-10 in duplicate cultures of LPMC from an individual patient. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ *versus* medium; #$P < 0{\cdot}05$ and ##$P < 0{\cdot}01$ *versus* IL-12.

cultures. First, IL-10 levels were significantly higher in culture supernatants from non-lesional and lesional CD LPMC *versus* controls ($P = 0{\cdot}0013$ and $P = 0{\cdot}0015$, respectively) (Fig. 2). rhIL-18 did not affect IL-10 production in control LPMC cultures, but significantly reduced IL-10 production in non-lesional ($P = 0{\cdot}003$) and lesional ($P = 0{\cdot}005$) CD tissue LPMC cultures. rhIL-12 significantly enhanced the IL-10 production ($P = 0{\cdot}005$ for non-lesional and $P = 0{\cdot}03$ for lesional). Thus, IL-18 and IL-12 have opposite effects on IL-10 production. When we analysed the combined effects of both rhIL-18 and rhIL-12, rhIL-18 significantly reversed the effect of rhIL-12 ($P < 0{\cdot}01$ for non-lesional and $P < 0{\cdot}05$ for lesional samples).

IL-4 levels were higher in culture supernatants of non lesional *versus* lesional LPMC from CD tissue and in both *versus* control LPMC cultures. rhIL-18 alone or together with rhIL-12 induced no changes in IL-4 secretion in LPMC cultures (from either controls or CD) (data not shown). IL-4 levels were decreased by rhIL-12. None of these differences reached statistical significance.

Effect of IL-18 BP on TNBS-induced colitis

To analyse further the potential role of IL-18 in inflammatory bowel disease, we have induced colitis in mice and evaluated the effect of treatment with IL-18BPa, which neutralizes IL-18 activity [29]. To induce acute colitis, mice were injected intrarectally with TNBS in ethanol on days 0 and 7 and were treated by daily i.p. injection with 200 μg of rhIL-18BPa, suspended in 200 μl PBS or with 200 μl PBS alone. All mice were sacrificed on day 9. Two independent experiments were performed, and data were pooled. Weight decrease over time was significantly lower in rhIL-18BPa-treated mice compared to non-treated mice ($P < 0{\cdot}05$) (Fig. 3a). Two of 15 mice in the non-treated group died on days 7 and 8, suggesting severe colitis (no histological analysis, however), while no deaths were observed in the rhIL-18BPa-treated group. The macroscopic disease severity score was significantly lower in the rhIL-18BPa-treated compared to the non-treated mice (median score: 4 *versus* 9, $P = 0{\cdot}0008$). On microscopic analysis, a marked decrease in the cellular infiltration and presence of red blood cells

was observed in the rhIL-18BPa-treated mice (median histological score: 4·5 *versus* 7·0, $P = 0{\cdot}0083$). No colitis was observed in mice injected intrarectally with ethanol alone (median score: 2) (Fig. 3b). In an independent experiment we measured mRNA levels for IL-18 2 days after induction of colitis. As shown in Fig. 4, mRNA levels for IL-18 were increased in tissue from mice injected with TNBS compared with non-diseased (ethanol injected) controls ($P = 0{\cdot}026$).

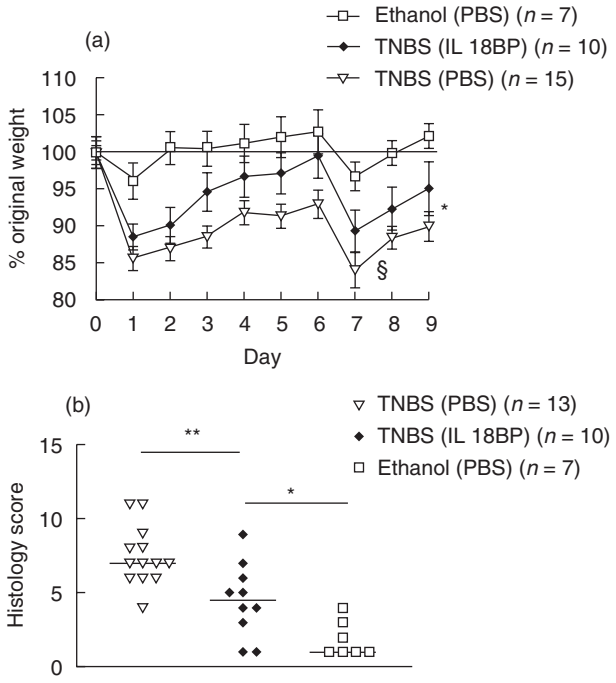

Fig. 3. Effect of rhIL-18BPa treatment on TNBS-induced colitis. C57Bl/6 mice were injected intrarectally (on days 0 and 7) with TNBS in ethanol or with ethanol alone as a control, and treated by daily i.p. injection of 200 μg rhIL-18BPa or PBS as indicated. (a) The change of weight over a period of 9 days is expressed as a percentage of the original weight at the start of the experiment. Data are pooled from two independent experiments and represent the mean ± s.e.m. (b) Histological scores of colon sections, scored according to the system described by McCafferty *et al.* [33]. The individual score for each mouse and the median for each group are shown. §Two mice of the TNBS/PBS group died on day 7 and on day 8, respectively, $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$.

Effect of IL-18 BP in a chronic colitis model

CD45RB^{high}CD4$^+$ T cell reconstituted SCID mice developed clinical features of colitis with progressive decrease in body weight, as reported previously [36] (Fig. 5a). Macroscopically, the colon was enlarged and had a greatly thickened wall. These changes were due to inflammation as shown by microscopy (Fig. 5b). SCID mice reconstituted with total CD4$^+$ T cells did not develop colitis. In these latter mice, weight increased over time and microscopic findings were normal (Figs 5a,b). mRNA levels [determined by real-time reverse transcription-polymerase chain rection (RT-PCR)] for IL-12p40, IFN-γ and TNF-α were significantly higher in CD45RB^{high}CD4$^+$ T cell reconstituted mice compared to total CD4$^+$ T cell reconstituted mice ($P < 0{\cdot}05$ for IL-12p40 and TNF-α; $P < 0{\cdot}01$ for IFN-γ) (Table 1), but mRNA levels of IL-18 were not significantly different between both groups (not shown).

In two independent experiments, CD45RB^{high}CD4$^+$ T cell reconstituted C.B-17 SCID mice were treated with IL-18BPa (at a low or high dose of rhIL-18BPa). Weight decrease and microscopic findings in these treated mice were not significantly different from that observed in sham-treated mice (Fig. 5). mRNA levels for IL-12p40, IFN-γ and TNF-α were not significantly different in the IL-18BPa-treated compared to the non-treated CD45RB^{high}CD4$^+$ T cell reconstituted mice (Table 1). To evaluate further the effect of IL-18BPa treatment on intestinal IFN-γ production, colonic LPMC were isolated from CD45RB^{high}CD4$^+$ T

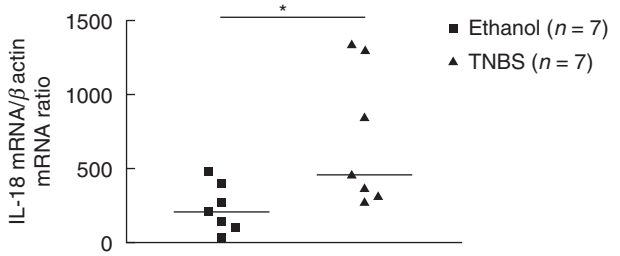

Fig. 4. C57Bl/6 mice were injected intrarectally with TNBS in ethanol to induce colitis or with ethanol alone as a control ($n = 7$ for each group), and mRNA levels of IL-18 in colonic samples were analysed using real-time quantitative RT-PCR. The data represent the ratio of cDNA copy number for IL-18 divided by the cDNA copy number for β-actin and multiplied by 10^3 and for each sample one median for each group is shown. $*P < 0{\cdot}05$.

Table 1. Effect of T cell reconstitution and rhIL-18BPa therapy[a] on cytokine mRNA levels in SCID mice

	Cytokine/β actin ratio[b]			
Type of T cell reconstruction and treatment	IFN-γ	IL-10	IL-12	TNF-α
CD45RB^{high} T cells + rhIL-18BPa[c] ($n = 12$)	110·0	77·5	7·28	689.·0
CD45RB^{high} T cells + PBS[c] ($n = 8$)	95·5	56·0	6·9	616·5
Total CD4+ T cells + rhIL-18BPa ($n = 4$)	13·5	57·5	2·72	141·5
Total CD4$^+$ T cells = PBS ($n = 4$)	20·5	89·5	1·79	294·5

[a]C.B-17 SCID mice were reconstituted with CD45RB^{high}CD4$^+$ or total CD4$^+$ T cells and treated with either rhIL-18BPa or phosphate buffered saline (PBS) as indicated. [b]mRNA levels of cytokines in colonic samples from all groups were analysed using real-time quantitative RT-PCR. The numbers represent the ratio of cDNA copy number for the cytokine divided by the cDNA copy number for β-actin and were multiplied by 10^6. Data represent the median for each group. [c]For CD45RB^{high} T cell reconstituted mice, data from two independent experiments (with low and high dose of rhIL-18BPa treatment) were pooled.

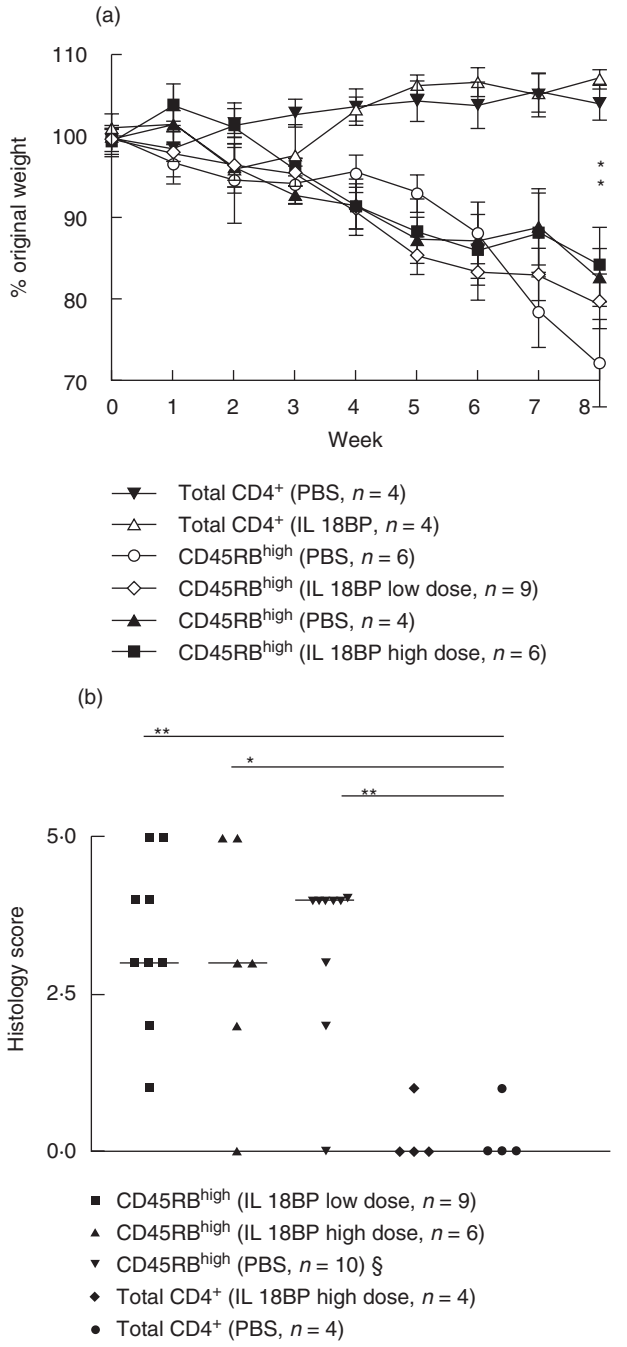

Fig. 5. Effect of rhIL-18BPa on colitis induced by CD45RB^{high}CD4$^+$ T cell transfer to SCID mice. C.B-17 SCID mice were reconstituted with CD45RB^{high}CD4$^+$ (4×10^5/mouse) or total CD4$^+$ T cells (5×10^5/mouse) and treated in two independent experiments with low or high dose of IL-18BPa or with PBS as indicated. (a) The change of weight over an 8-week period of observation is expressed as percentage of the original weight at the start of the experiment. Data represent the mean ± s.e.m. (b) Histological scores of colonic sections, scored according to Liu *et al*. [31]. Data represent the individual score for each mouse and the median for each group. §Data pooled from the two independent experiments; $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

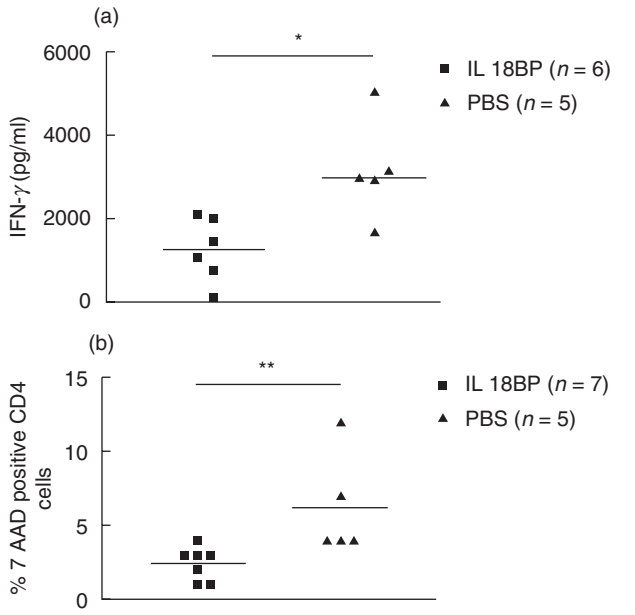

Fig. 6. Effect of IL-18BPa treatment on IFN-γ production and lamina propria mononuclear cells (LPMC) apoptosis. LPMC were isolated from colon of C.B-17 SCID mice, 8 weeks after reconstitution with CD45RB^{high}CD4$^+$ T cells and treated *in vivo* with IL-18BPa or with PBS, as indicated. (a) LPMC were stimulated *in vitro* and supernatants were assayed for IFN-γ by ELISA. Results from individual mice and the median for each group are shown. (b) LPMC were stained with PE-conjugated anti-CD4 and 7AAD. Data represent the percentage of apoptotic CD4$^+$ T cells, identified as 7AAD-positive CD4$^+$ T cells in a colon sample from each individual mouse and the median for each group is shown. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

cell reconstituted mice treated either with PBS or rhIL-18BPa, and kept in culture for 48 h under stimulatory conditions with anti-mouse CD3ε and mouse CD80-transfected P815 cells. The number of CD4$^+$ T cells in the cultures was equal in both groups (data not shown). IFN-γ production (measured by ELISA) was significantly lower in the LPMC cultures obtained from the rhIL-18BPa-treated mice compared to the sham-treated mice ($P = 0{\cdot}017$) (Fig. 6a). In the same culture supernatants, TNF-α levels were not significantly different in rhIL-18BPa-treated or non-treated mice.

It has been reported that IL-18 induces apoptosis [25,26] and apoptosis is thought to play a role in protection from IBD [37]. To analyse whether IL-18BPa treatment could reduce T cell apoptosis, LPMC were harvested from colons of mice reconstituted previously with CD45RB^{high}CD4$^+$ T cells and treated with rhIL-18BPa or PBS. The number of CD4$^+$ T cells harvested from the colons was not different in both groups (not shown). Apoptosis of CD4$^+$ T cells was analysed by flow cytofluorometry using 7AAD, which bind to the DNA of apoptotic cells [34]. As shown in Fig. 6b, CD4$^+$ T cells harvested from rhIL-18BPa-treated mice had a significantly lower proportion of apoptotic cells compared to PBS-treated mice ($P = 0{\cdot}005$).

DISCUSSION

The current study explored the functional effects of rhIL-18 on cytokine production in lamina propria cell cultures from CD tissue and evaluated the potential efficacy of IL-18 targeting with IL-18BPa in two different models of murine colitis.

In the first part of this study, we analysed the effect of IL-18 in human LPMC cultures. In agreement with earlier reports [38,39], we observed enhanced production of TNF-α and IFN-γ in LPMC cultures from CD patients compared to controls. It is thought that IFN-γ contributes to inflammation in CD tissue [5], and we found that addition of rhIL-18 into LPMC cultures from CD patients but not from controls enhanced the production of IFN-γ. No effect of rhIL-18 on TNF-α production was observed and IL-10 production was decreased significantly. Because IL-10 is thought to be an important anti-inflammatory cytokine in CD [40–42], this effect on IL-10 production might contribute further to the proinflammatory properties of IL-18 in CD. Neutralizing IL-18 activity in CD might thus be beneficial through decreasing IFN-γ and increasing IL-10 production in gut tissue. One of the possible reasons why IL-10 therapy is not very effective in CD is a paradoxically enhanced IFN-γ production when high amounts of rhIL-10 were used [43]. Targeting IL-18 will not have this disadvantage, because this treatment will directly decrease IFN-γ production. The effects of rhIL-18 alone (in the absence of rhIL-12) on cytokine production were restricted to cell cultures from CD patients, and were not found in LPMC cultures from controls. The fact that T cells from CD patients are responsive to rhIL-18 is in accordance with data from Kanai *et al*. [8], and is probably the result of their activated state, or of previous exposure *in vivo* to high amounts of IL-12 [44], resulting in up-regulation of IL-18R expression [20,22]. Increased production of IL-18 in CD tissue together with enhanced T cell responses to IL-18 both point to the involvement of IL-18 in the inflammatory cascade of CD.

This potential involvement was then studied further in experimental colitis. rhIL-18BPa therapy significantly ameliorated TNBS-induced acute colitis. Colon IL-18 mRNA was increased significantly in TNBS-injected mice. A beneficial effect of therapy blocking IL-18 was previously reported in this and in the dextran sulphate sodium model of colitis [12,14–16]. In the T cell transfer model of chronic colitis, however, we observed significantly reduced IFN-γ (but not TNF-α) production in LPMC cultures from rhIL-18BPa-treated mice, but no beneficial effect of rhIL-18BPa treatment was observed on colitis severity. It is unlikely that rhIL-18BPa itself was inactive and/or unable to neutralize mouse IL-18. Indeed, in the TNBS colitis model the same IL-18BPa was used and found to be efficient. Moreover, others have demonstrated the efficacy of this construct in murine arthritis and colitis [14,45]. SCID mice also lack functional B cells and are unable to produce antibodies against foreign proteins, which exclude IL-18BPa neutralization in these long-term experiments. It is therefore not clear why efficacy was different in these two models, but the most likely explanation is that the importance of IL-18 differs in the two models. Indeed, IL-18 mRNA levels were not significantly different in CD45RB^{high}CD4$^+$ and total CD4$^+$ T cell reconstituted mice, supporting the conclusion that IL-18 has a limited role in the T cell transfer model of colitis. Our results are in contrast to those of Wirtz *et al*. [13], who reported amelioration of colitis in this model when IL-18 production was blocked using intrarectal instillation of an IL-18 antisense mRNA-expressing adenovirus. We have no real explanation for this discrepancy, but should take into consideration that the manner of interference with IL-18 activity was obviously totally different in the study by Wirtz *et al*. and in our study, and efficacy of local IL-18 antagonism might be different from that obtained by systemic IL–18 BP administration.

We observed further that rhIL-18BPa-treated mice compared to non-treated mice had a reduced amount of apoptotic lamina propria CD4$^+$ T cells. The latter is consistent with findings that IL-18 induces apoptosis [25,26]. Importantly, this effect on apoptosis might, in part, be the reason why rhIL-18BPa was not effective as longer T cell survival will promote inflammation. Moreover, it has been reported that lamina propria T cells from CD patients are relatively resistant to apoptosis, and that this might contribute to perpetuation of inflammation [37].

In summary, our data suggest that IL-18 does play a modulatory role in the inflammatory cascade in CD. The fact that IFN-γ but not TNF-α production was affected by IL-18 addition to CD tissue-derived LPMC cultures, and the divergent effects of IL-18BPa in two different murine colitis models make it unlikely that IL-18 is at the top of the inflammatory cascade.

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