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IL-22 Induces Lipopolysaccharide-Binding Protein in Hepatocytes: A Potential Systemic Role of IL-22 in Crohn's Disease

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Crohn′s disease (CD) is a common, chronic, inflammatory bowel disease characterized by intestinal infiltration of activated immune cells and distortion of the intestinal architecture. In this study, we demonstrate that IL-22, a cytokine that is mainly produced by activated Th1 and Th17 cells, was present in high quantities in the blood of CD patients in contrast to IFN-γ and IL-17. In a mouse colitis model, IL-22 mRNA expression was elevated predominantly in the inflamed intestine but also in the mesenteric lymph nodes. IL-22BP, the soluble receptor for IL-22, demonstrated an affinity to IL-22 that was at least 4-fold higher than its membrane-bound receptor, and its strong constitutive expression in the intestine and lymph nodes was decreased in the inflamed intestine. To investigate the possible role of systemic IL-22 in CD, we then administered IL-22 to healthy mice and found an up-regulation of LPS-binding protein (LBP) blood levels reaching concentrations known to neutralize LPS. This systemic up-regulation was associated with increased hepatic but not renal or pulmonary LBP mRNA levels. IL-22 also enhanced the secretion of LBP in human primary hepatocytes and HepG2 hepatoma cells in vitro. This increase was mainly transcriptionally regulated and synergistic with that of other LBP inducers. Finally, elevated LBP levels were detected in CD patients and the mouse colitis model. These data suggest that systemic IL-22 may contribute to the prevention of systemic inflammation provoked by LPS present in the blood of CD patients through its induction of hepatic LBP. ***The Journal of Immunology,*** **2007, 178: 5973–5981.**

Interleukin-22 is an α-helical cytokine that was discovered in 2000 (1). Because of its structure and receptor characteristics, it was grouped with IL-10, with type I and type II IFNs, and with other recently described cytokines, IL-19, IL-20, IL-24, IL-26, IL-28$\alpha\beta$, and IL-29, in the so-called IFN-IL-10 cytokine family (2).

IL-22 is produced by activated T cells and NK cells (3, 4). No expression has been found in any other immune cells, resting or stimulated, or in nonimmune tissue cells (3, 4). Among the T cells, Th1 cells and, at least in mice, Th17 cells, are the main producers (3, 5–7). Th1 cells have been well established for years as the pathogenetically relevant cell population in organ-specific autoimmune disorders (8, 9). More recently, a causal role of Th17 cells in murine organ-specific autoimmunity models such as colitis, experimental autoimmune encephalomyelitis, and collagen-induced arthritis has also been proposed (10–13).

IL-22 acts via engagement of a membrane-bound, heterodimeric receptor complex consisting of the primary IL-22-binding receptor chain IL-22R1 and the accessory chain IL-10R2 (2). Both receptor chains are each also part of receptor complexes for other members of the IFN-IL-10 family: IL-22R1 additionally functions as the R1 chain of the IL-20 and IL-24 receptor complexes; and IL-10R2 also functions as the R2 chain of the IL-10, IL-26, IL-28$\alpha\beta$, and IL-29 receptor complexes (2). This sharing, however, does not appear to be associated with binding competition or mutual limitation of the biological effects of the different mediators (14). Binding of IL-22 to its receptor complex induces signal transduction particularly via the JAK-STAT pathway (1, 4). In addition to the cell surface-associated IL-22 receptor complex, there is a soluble, single-chain IL-22-binding receptor named IL-22-binding protein (IL-22BP)[3] (3), IL-22Rα2, or CRF2s1-short (15–18). This receptor has been shown to antagonize IL-22 cellular binding and signaling in vitro (15–17). In adult mice, IL-22BP is constitutively expressed in lymph nodes (19).

Although classified as an IL, IL-22 does not serve as the means of communication between leukocytes given that it does not seem to directly influence immune cells (20). However, a range of nonimmune cell types from tissues such as the skin, and tissues of the digestive and the respiratory systems appear to be targets of this cytokine as deduced from their IL-22 receptor chain expression (20). Effects of IL-22 have been described on different cell populations, including hepatocytes and keratinocytes (20–22). In hepatocytes, IL-22 stimulation up-regulates the expression of the acute

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[3] Abbreviations used in this paper: IL-22BP, IL-22-binding protein; CD, Crohn's disease; LBP, LPS-binding protein; DSS, dextran sulfate sodium; HPRT, hypoxanthine phosphoribosyltransferase 1; RU, resonance units; CDAI, Crohn's disease activity index; SAPK, stress-activated protein kinase.

phase reactants serum amyloid A, α_1-antichymotrypsin, and haptoglobin (20, 21). Additionally, IL-22 has a hepatoprotective role during liver injury (22). Regarding keratinocytes, we demonstrated that IL-22 induces the expression of antimicrobial proteins that may promote the pathogen defense in the skin (4, 20). Therefore, IL-22 mediates the interaction between the adaptive immune system and the innate immunity of the epithelium. Moreover, IL-22 also down-regulates the expression of genes that are involved in the terminal differentiation of keratinocytes and are therefore important for the formation of the stratum corneum of the skin (4, 23). Lastly, IL-22 also increases the mobility of keratinocytes (4, 23). These three functions are also altered in keratinocytes from patients suffering from psoriasis vulgaris. These facts in connection with the high IL-22 expression in the inflamed skin of such patients suggest a pathogenetic role of this cytokine in this autoimmune disease (4, 20).

This study describes a potential role of IL-22 in another organ-specific autoimmune disease, Crohn's disease (CD), through its induction of the hepatocyte product LPS-binding protein (LBP).

Materials and Methods

Patients and control participants

Blood samples for the recovery of plasma and serum were obtained from patients with CD who were being treated at the University Hospital Charité Berlin, Medical Clinic 1 (Berlin, Germany), with approval from the institutional human research ethics committee. Informed consent was obtained from all patients. All patients had an established diagnosis of CD based on standard clinical, endoscopic, and histological criteria. Additionally, blood samples from healthy persons were obtained from staff members of the University Hospital Charité Berlin.

Mice

For the induction of acute colitis in mice, 6- to 8-wk-old female BALB/c mice were provided with dextran sulfate sodium (DSS; molecular mass 36,000; MP Biomedicals) dissolved in drinking water (2.5%) given ad libitum for 5 days. Control mice were provided with water without DSS. On day 5, mice were killed and weighed before the blood, the mesenteric lymph nodes, and the intestine were recovered. Severity of colitis was evaluated by weight loss, loose or bloody stool, and colon length as previously described (24), as well as by colon histopathological score (see below). In addition, lymph nodes and samples from the inflamed colon were taken for mRNA analysis. To study the effect of IL-22 on LBP expression in vivo, 14-wk-old male BALB/c mice were injected i.p. with 1 μg of murine IL-22 (R&D Systems) or a corresponding volume of PBS (controls). Mice were sacrificed before or 1, 3, 6, 24, 48, or 72 h after injection. At these time points, blood was drawn for plasma recovery. Additionally, liver, lung, and kidney samples were harvested at 48 h. All animal studies were performed in accordance with the institutional, state, and federal guidelines.

Cell culture

Primary human hepatocytes were obtained from Cambrex and BD Biosciences. Cells were cultured on thin-layer Biocoat Matrigel (BD Biosciences) in culture medium according to the manufacturer's instructions. After a 24-h preculture period and subsequent medium change, cells were or were not (control) exposed to IL-22 for 24, 48, and 72 h (kinetic study) or 48 h only. HepG2 cells were purchased from the European Collection of Cell Cultures and maintained according to the supplier's instructions. For experimental procedure, HepG2 cells were precultured for 48 h. After subsequent medium change, cells were exposed or not (control) to IL-22 for 48 h, unless indicated otherwise. To test the IL-22 effect in combination with other stimuli, IL-22 exposure was performed in the presence of IL-1β, IL-6, TNF-α, IL-17A, or combinations of these cytokines. To confirm the specificity of the IL-22 effect, IL-22 exposure of resting or IL-1β/TNF-α-costimulated HepG2 cells was performed in the presence of increasing concentrations of 1) anti-IL-10R2 polyclonal Abs or the respective control Abs (polyclonal mouse IgG; 0, 0.1, 0.32, 1, 3.2, or 10 μg/ml each) or 2) increasing concentrations (0, 74, 148, 296, or 592 ng/ml) of IL-22BP that had been preincubated for 2 h with IL-22. All cytokines and Abs, as well as IL-22BP, were purchased from R&D Systems. If not indicated otherwise, all cytokines were applied at 10 ng/ml.

Real-time RT-PCR

Murine tissue samples, snap frozen in Invisorb lysing solution (Invitek) were homogenized during thawing by means of Ultraturrax tissue homogenizer (Jahnke and Kunkel) and then treated with 4 mg/ml proteinase K for 1 h (Clontech Laboratories). Isolation of total cellular RNA from murine tissues and human cells was done by use of the Invisorb RNA kit II (Invitek). mRNA was reverse transcribed and analyzed in triplicate assays by TaqMan PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described previously (3, 14). For detection of human LBP as well as murine IFN-γ, IL-17A, IL-17F, IL-22, IL-22BP, and LBP, appropriate assays including double-fluorescent probes in combination with assays for the murine and human housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were developed by ourselves (IL-22) or purchased from Applied Biosystems. Expression levels were calculated relative to the data for HPRT obtained with the every matching assay.

ELISA and Immulite

Human IL-22, IFN-γ, and IL-17A were quantified by ELISAs from R&D Systems. Human LBP, C-reactive protein, IL-1β, and IL-6 were quantified by Immulite (DBC Biermann). Mouse LBP and bacterial LPS were quantified by an ELISA and chromogenic *Limulus* amebocyte lysate assay, respectively, both from HyCult biotechnology. Regarding the LPS measurement, only free LPS was assessed.

Western blot analysis

Cell lysing, protein electrophoresis, and Western blot were performed as described previously (20, 25). Blotted samples were incubated with polyclonal Abs against phospho-STAT1 (Tyr^{701}), phospho-STAT3 (Tyr^{705}), phospho-STAT3 (Ser^{727}), acetyl-STAT3 (Lys^{685}), phospho-STAT5 (Tyr^{694}), phospho-p38 (Thr^{180}/Tyr^{182}), phospho-stress-activated protein kinase (SAPK)/JNK (Thr^{183}/Tyr^{185}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), phospho-IκB-α (Ser^{32}), total IκB-α, or total STAT3 (all from Cell Signaling Technology) and peroxidase-conjugated AffiniPure $F(ab')_2$ goat anti-rabbit IgG (H and L; Dianova) before undergoing ECL detection (Amersham Biosciences).

Histology

Serial 2- to 3-μm sections of murine colonic tissue were obtained as described previously (24) and stained with H&E. Histological scoring was performed in a blinded manner by a pathologist based on the assessment of epithelial loss and infiltration as previously described (26): 1) epithelium: 0 = normal morphology, 1 = loss of goblet cells, 2 = loss of goblet cells in large areas, 3 = loss of crypts, 4 = loss of crypts in large areas; 2) infiltration: 0 = no infiltrate, 1 = infiltrate around crypt basis, 2 = infiltrate reaching to lamina muscularis mucosae, 3 = extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema, 4 = infiltration of the lamina submucosa. The total histological score represented the sum of the epithelium and infiltration scores and thus ranged from 0 to 8. The DSS-treated group involved in this study had a score of 4.7 $\pm$ 0.33 compared with 0 $\pm$ 0.0 in the control group, which received only water.

Determination of binding data for IL-22-IL-22BP interaction

For the determination of the affinity between IL-22 and IL-22BP, two kinds of surface plasmon resonance analyses were conducted, equilibrium binding experiments and kinetic binding experiments. All measurements were performed using the Biacore X instrument, associated Biacore X2.2 control software, CM5 sensor chips, and running buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 (all from Biacore). On the sensor chip surface of the second flow cell, the protein of interest (IL-22 or IL-22BP) was immobilized, whereas a control protein (IL-29 or IL-20R1) was immobilized on the sensor chip surface of the first flow cell (reference cell). Ligand immobilization was achieved by an amine coupling chemistry standard procedure (Biacore) at a flow rate of 5 μl/min and in 10 mM sodium acetate buffer (pH 4.5) as follows: IL-22 at 25 μg/ml for 20 min (equilibrium binding experiments) or at 5 μg/ml for 12 min (kinetic binding experiments); IL-29 at 25 μg/ml for 14 min (equilibrium binding experiments) or at 5 μg/ml for 4 min (kinetic binding experiments); IL-22BP and IL-20R1 at 5 μg/ml for 37 min (IL-22BP) or 9 min (IL-20R1; kinetic binding experiments). The achieved ligand density after immobilization corresponded to 2257 resonance units (RU) for IL-22, 2954 RU for IL-29 (equilibrium binding experiments), 200 RU for IL-22, 426 RU for IL-29, 3222 RU for IL-22BP, and 3448 RU for IL-20R1 (kinetic binding experiments). IL-22, IL-29, IL-22BP, and IL-20R1 were purchased from R&D Systems. Recombinant IL-22BP and IL-20R1 constituted disulfide-linked homodimeric proteins, each with two

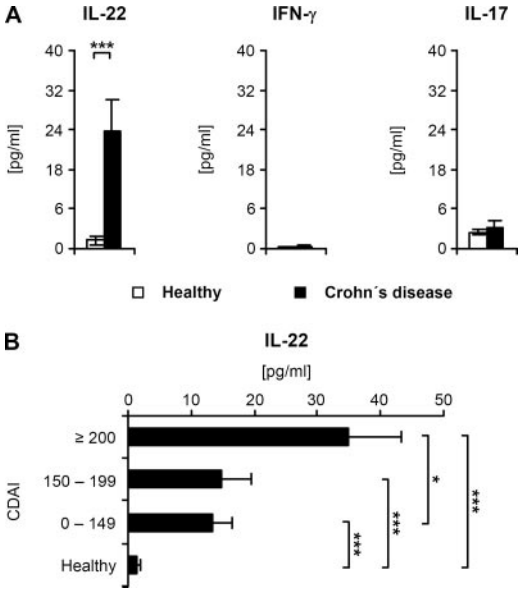

FIGURE 1. CD patients show elevated systemic IL-22 levels. *A*, Blood plasma from CD patients (mean ± SEM; CDAI 161 ± 17) and healthy participants was analyzed for the concentrations of IL-22, IFN-γ, and IL-17A by ELISA. Data from 10 participants per group are given as the mean ± SEM. *B*, Blood plasma from CD patients with the indicated CDAIs and from healthy participants was analyzed for the concentration of IL-22 by ELISA. Data from 15 (healthy), 14 (CDAI 0–149), 7 (CDAI ≥ 150–199), and 10 (CDAI ≥ 200) participants per group are given as the mean ± SEM. Statistical significance of deviations was tested using the Mann-Whitney *U* test. ∗, $p < 0.05$; ∗∗∗, $p < 0.001$.

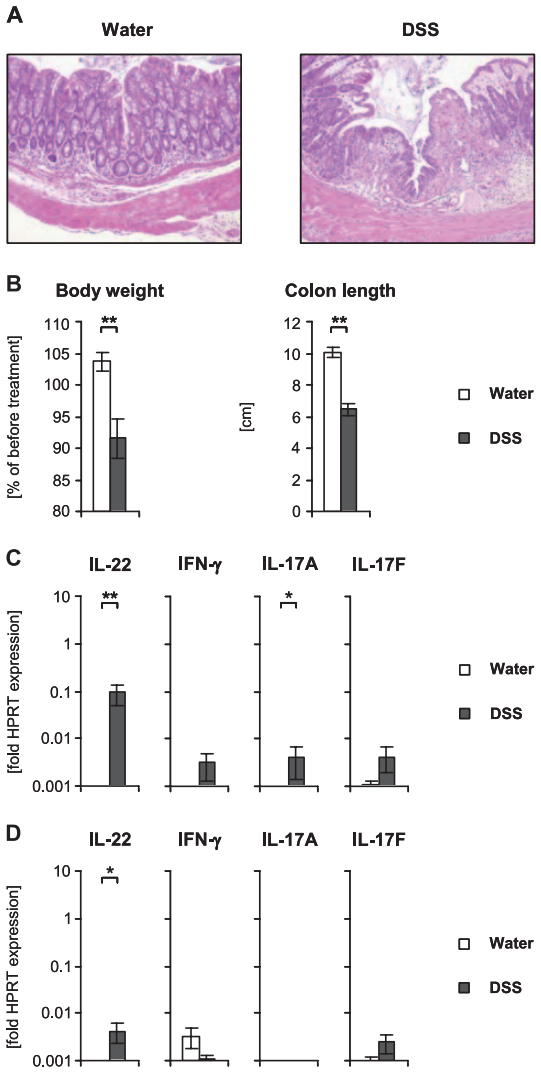

FIGURE 2. In experimental colitis in mice, IL-22 expression is increased particularly in the inflamed intestine. Colitis was induced in BALB/c mice by DSS diluted in the drinking water over 5 d. Control mice received water only. *A*, Disease severity was assessed by histological analysis of colonic sections stained with H&E on day 5. *B*, Body weight and colon length, both indicators of colitis, were assessed on day 5. *C* and *D*, m*RNA* expression of IL-22, IFN-γ, IL-17A, and IL-17F in inflamed colon (*C*) and mesenteric lymph node (*D*) was analyzed by real-time RT-PCR on day 5 as relative to HPRT expression. Values are representative of data from one of six (*A*) or mean (±SEM) from six mice (*B–D*) per group. Statistical significance of deviations was tested using the Mann-Whitney *U* test. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

molecules fused to the Fc region of human IgG1 via a linker peptide. For equilibrium binding measurements, different concentrations of IL-22 (20, 15, 10, 7.5, 5, 4, 3.25, 2.5, 1.75, 1, and 0 nM) were incubated with 5.85 nM IL-22BP for 2 h at room temperature. Response data of free IL-22BP were collected for 5 min at a flow rate of 10 μl/min, and concentrations were calculated using a calibration curve that was made from response data at different IL-22BP concentrations (7.31, 5.85, 4.39, 2.92, 1.95, 0.98, 0.49, 0.24, and 0 nM) in duplicate assays. For kinetic binding experiments, different concentrations of IL-22BP (800, 400, 200, 100, 50, 25, 12.5, and 0 nM) or IL-22 (400, 200, 100, 50, 25, 12.5, and 0 nM) were run over the sensor chip surface bearing immobilized IL-22 or IL-22BP, respectively, at 50 μl/min for 2 min. Kinetic rate constants were obtained by fitting the data into a bivalent analyte (binding of IL-22BP to immobilized IL-22) or a heterogenous ligand (binding of IL-22 to immobilized IL-22BP) binding model using BiaEvaluation 3.2 software (Biacore). In all experiments, response data of IL-22BP binding to immobilized IL-29 and of IL-22 binding to immobilized IL-20R1 were subtracted from the response data obtained from the binding of IL-22BP to immobilized IL-22 and of IL-22 to immobilized IL-22BP, respectively. Regeneration of sensor chips was performed after each measurement using 10 mM glycine-HCl (pH 2.0) for 12 s.

Results

CD is an inflammatory bowel disease characterized by a chronic, uncontrolled inflammation in the intestinal mucosa with transmural infiltration of activated immune cells, including Th1 and Th17 cells, and patchy granulomatous lesions (27, 28). Recently, the presence of IL-22 was demonstrated in the inflamed intestine of CD patients (29, 30). In line with in vitro results regarding the cellular sources of this cytokine (3, 4), IL-22 was expressed in the inflamed intestine by Th cells (29). On the basis of these findings, we aimed to further elucidate the role of IL-22 and thus investigated whether this cytokine is also present in the circulation of CD patients. For this purpose, blood plasma of patients with established CD and of healthy control participants was analyzed for the concentration of IL-22 by ELISA. Additionally, we characterized the plasma levels of IFN-γ and IL-17A, the lead cytokines of Th1 and Th17 cells, respectively. As shown in Fig. 1*A*, elevated IL-22 plasma levels were indeed found in CD with an average of 24 pg/ml, compared with an average of ~2 pg/ml in the control group. Surprisingly, in contrast to IL-22, no elevation of IFN-γ or IL-17A could be systemically detected in CD patients (Fig. 1*A*). Those CD patients who were in remission (CD disease activity index (CDAI), 0–149) also had elevated IL-22 plasma levels compared with healthy controls, which, however, were significantly lower than levels in patients with a moderate to severe case (CDAI ≥200) (Fig. 1*B*).

Next, we were interested in locating the production site of the systemic IL-22 in CD. To answer this question, we used the experimental colitis mouse model induced by oral application of the chemical agent DSS (31). The colitis was accompanied by a

A

Immobilized Protein	Soluble Phase	K_a [$M^{-1}s^{-1}$]	K_d [s^{-1}]	K_D [nM]
IL-22	IL-22BP	$5.2 \pm 0.2 \times 10^4$	$7.0 \pm 0.3 \times 10^{-5}$	1.4 ± 0.6 nM
IL-22BP	IL-22	$2.9 \pm 0.1 \times 10^5$	$2.0 \pm 0.2 \times 10^{-4}$	0.7 ± 0.1 nM
(IL-22BP)*	IL-22, IL-22BP	-	-	< 3 nM

* measurement of free IL-22 by IL-22BP-coated sensor chip

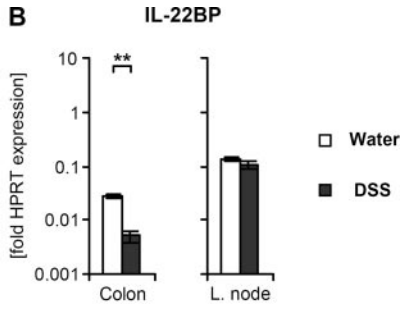

FIGURE 3. IL-22BP shows high affinity to IL-22 and is decreased in its expression in the inflamed intestine in DSS-treated mice. *A*, Interaction between IL-22 and IL-22BP was analyzed by surface plasmon resonance analysis. For kinetic binding experiments, IL-22BP was run over the IL-22-immobilized sensor chip surface or vice versa at different concentrations, and kinetic (on, off) rate constants (K_a, K_d) were obtained by fitting the recorded response data to a bivalent binding model (IL-22 immobilized) or a heterogeneous ligand model (IL-22BP immobilized), respectively. For equilibrium binding experiments, IL-22 was allowed to interact with IL-22BP at different concentrations in solution, and remaining IL-22 was detected by its binding to the IL-22BP-coated sensor chip. Data are given as the mean ± SEM from three (kinetic binding experiments with immobilized IL-22BP), four (kinetic binding experiments with immobilized IL-22), or five (solution affinity experiments) independent experiments. *B*, Colitis was induced in BALB/c mice by DSS diluted in the drinking water during 5 days. Control mice received water only. mRNA expression of IL-22BP in inflamed colon and mesenteric lymph node was analyzed by real-time RT-PCR on day 5 as relative to HPRT expression. Data (mean ± SEM) from six mice per group are shown. Statistical significance of deviations was tested using the Mann-Whitney *U* test. **, $p < 0.01$.

marked mucosal loss and an inflammatory cell infiltrate, as analyzed by colon histology (Fig. 2*A*), as well as a significant reduction of the body weight and colon length in these mice (Fig. 2*B*). Samples from the inflamed colon and mesenteric lymph nodes of DSS-treated and control mice were analyzed for IL-22, and for comparison, for IFN-γ, IL-17A, and IL-17F mRNA expression by real-time RT-PCR. As demonstrated in Fig. 2*C*, IL-22 mRNA expression was highly increased in the inflamed intestine of DSS-treated mice compared with the intestine of control mice where no IL-22 expression was detected at all. Some elevated IL-22 mRNA expression was also found in the mesenteric lymph nodes in that model with levels being more than twenty times lower than those in the inflamed intestine (Fig. 2*D*). Regarding IFN-γ, IL-17A, and IL-17F, significantly increased expression was found only for colonic IL-17A reaching levels that were ~20 times lower than colonic IL-22 levels (Fig. 2, *C* and *D*).

IL-22 activity may be regulated by its specific soluble receptor, IL-22BP. In fact, IL-22BP at high concentrations has previously been shown to inhibit the IL-22 action on cells in vitro by hindering this cytokine from binding to its membrane-associated receptor (14–17, 32). However, soluble receptors may systemically have a positive regulatory role by increasing the half-life of the cytokine (33, 34). The kind of regulation depends on the affinity of the soluble receptor to its ligand as well as its actual concentration. To address these points, we first measured the affinity of IL-22BP to IL-22 using surface plasmon resonance techniques. Equilibrium

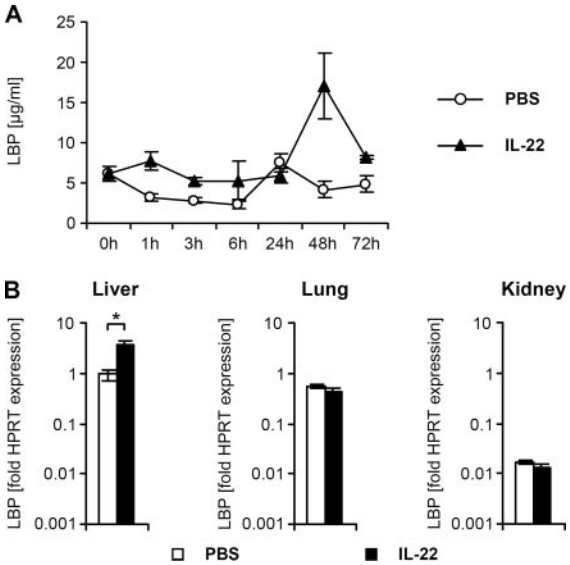

FIGURE 4. IL-22 increases LBP production in mice. BALB/c mice were injected i.p. with PBS (control) or murine IL-22. *A*, Directly before (0 h) and 1, 3, 6, 24, 48, and 72 h after injection, blood was collected for plasma recovery and plasma LBP was quantified by ELISA. Data from three mice per time point are given as the mean ± SEM with the exception of 0 h (six mice), and 6 h (two mice) for the IL-22 group. *B*, 48 h after injection, liver, lung, and kidney samples were harvested and expression of LBP mRNA was analyzed by real-time RT-PCR. Data from four (PBS group) or three (IL-22 group) mice are given as relative to HPRT expression (mean ±SEM). Statistical significance of deviations was tested using the Mann-Whitney *U* test. ∗, $p < 0.05$.

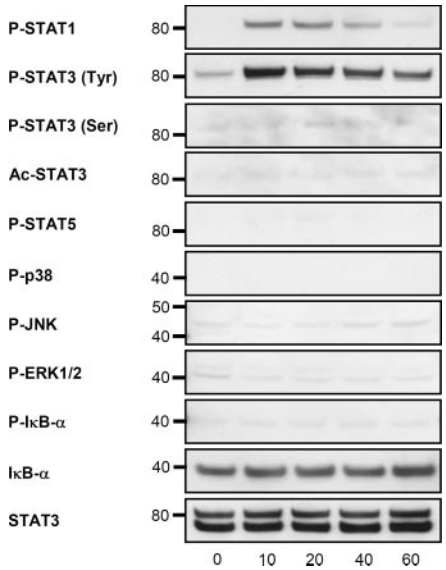

FIGURE 5. IL-22 activates STAT-dependent signal transduction pathways in HepG2. HepG2 cells were stimulated or not (0 min) with IL-22 for 10, 20, 40, and 60 min. Levels of phosphorylated (P-) STAT1 (Tyr), STAT3 (Tyr or Ser), STAT5 (Tyr), p38, SAPK/JNK, ERK1/2, IκB-α, acetylated (Ac-) STAT3, and total IκB and STAT3 were assessed by Western blot. One representative experiment of four is shown.

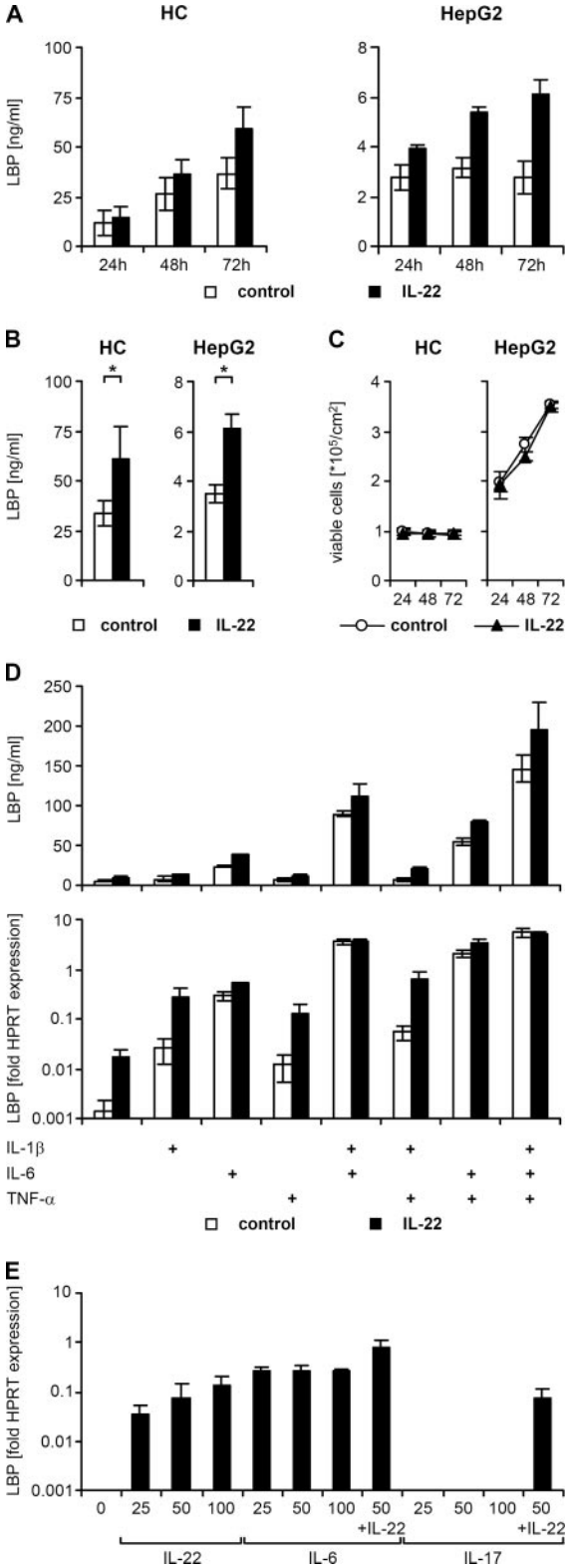

FIGURE 6. IL-22 up-regulates the production of LBP by hepatocytes. *A*, Primary human hepatocytes (HC) and HepG2 cells were each stimulated or not (control) with IL-22 for 24, 48, and 72 h. Cell culture supernatant was analyzed for LBP concentration using Immulite. Data from three independent experiments are given as the mean ± SEM. *B*, Primary human hepatocytes and HepG2 cells were each stimulated or not (control) with IL-22 for 48 h. Cell culture supernatant was analyzed for LBP concentration using Immulite. Data from five to six independent experiments are given as the mean ± SEM. Statistical significance of deviations was tested using the Wilcoxon matched pairs signed rank test. *, $p < 0.05$. *C*, To exclude the possibility that the IL-22 effect is an indirect product of hepatocyte vitality, primary human hepatocytes and HepG2 were stimulated or not (control) with IL-22 for 24, 48, and 72 h and analyzed afterward for their vitality based on their trypan blue exclusion capacity and culture vessel adherence. Data from three independent experiments are given as the mean ± SEM. *D*, HepG2 cells were stimulated or not (control) with either IL-22, IL-6, IL-1β, TNF-α, or combinations of these cytokines for 48 h. Cell culture supernatant was analyzed for LBP concentration using Immulite (*top*), and cellular expression of LBP mRNA was analyzed by real-time RT-PCR as relative to HPRT expression (*bottom*). Data from two independent experiments are given as the mean ± range. *E*, HepG2 cells were stimulated or not (control) with the indicated concentrations of IL-6, IL-22, IL-17A, or a combinations of IL-17A or IL-6 and 50 ng/ml IL-22 for 48 h. Cellular expression of LBP mRNA was analyzed by real-time RT-PCR as relative to HPRT expression. Data from three independent experiments are given as the mean ± SEM.

binding studies revealed a strong binding affinity of IL-22 to IL-22BP with an K_D of <3 nM, although the exact value could not be determined with this method (Fig. 3*A*). Additionally, kinetic binding experiments were performed either by running IL-22BP at different concentrations over a sensor chip surface coated with IL-22 or, vice versa, by running different concentrations of IL-22 over a sensor chip surface coated with IL-22BP, wheres association and dissociation data were recorded. The resulting K_D values were ~1 nM for both methods (Fig. 3*A*). The affinity between IL-22-IL-22BP therefore appears to be ~4–10 times higher than that of IL-22 to its membrane-associated receptor chain IL-22R1, as determined by Walter's group using similar experimental settings (35). This was mainly due to the much lower dissociation rate of IL-22-IL-22BP complexes ($K_d \leq 2 \times 10^{-4}$ s^{-1}; Fig. 3*A*) compared with the IL-22-IL-22R1 interaction, which was found by Walter's group to be $\sim 40 \times 10^{-4}$ s^{-1} (35). These binding data strongly suggest a mainly inhibitory role of IL-22BP on IL-22 in vivo.

Furthermore, we analyzed the expression of IL-22BP in the colon samples and lymph nodes from DSS and control mice used in Fig. 2, *C* and *D*. As demonstrated in Fig. 3*B*, IL-22BP showed clear constitutive expression in both colon and mesenteric lymph nodes and a decrease in the inflamed colon but not in the lymph nodes in the DSS colitis model. These data suggest a highly increased IL-22:IL-22BP ratio in the inflamed colon in this model that may be the cause of increased systemic IL-22 levels seen in inflammatory bowel disease.

We then questioned what the systemic role of IL-22 in inflammatory bowel disease could be. For this purpose, healthy mice were injected i.p. with either IL-22 or PBS as a negative control. Blood plasma samples were taken before and 1, 3, 6, 24, 48, and 72 h after injection and analyzed for possible differences in mediator levels between the treatment groups. As shown in Fig. 4*A*, we found elevated LBP levels in the IL-22-treated group at 48 h postinjection. LBP is a plasma protein that mediates the immune response to bacterial components at constitutive levels and neutralizes those components at levels observed during the acute phase response, thereby inhibiting their immune stimulatory effects (36). In a separate experiment with seven animals per group, we confirmed that IL-22 application at 48 h significantly elevated blood LBP levels ($p < 0.01$; data not shown). To identify the origin of the IL-22-induced LBP production, we characterized the mRNA expression of LBP in diverse IL-22R1-expressing, LBP-producing tissues after cytokine application. As shown in Fig. 4*B*, the IL-22-induced increase of LBP plasma levels was associated with an elevated hepatic, but not pulmonary or renal LBP mRNA expression.

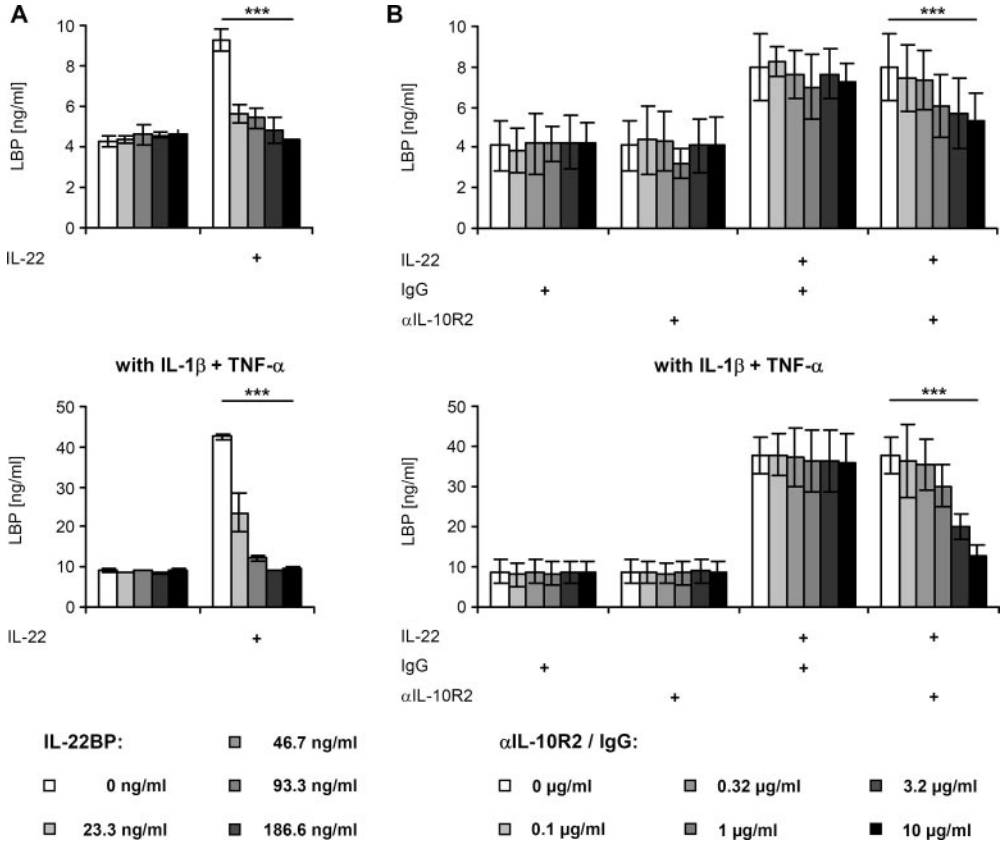

FIGURE 7. IL-22 up-regulated production of LBP by HepG2 cells is inhibited by IL-22BP or anti-IL-10R2 Abs. *A*, HepG2 cells were stimulated or not (control) with IL-22 in the absence (*top*) or presence (*bottom*) of IL-1β and TNF-α for 48 h. At this, the IL-22 used had been preincubated or not with the indicated concentrations of IL-22BP for 2 h. Cell culture supernatant was analyzed for LBP concentration using Immulite. Data from two independent experiments are given as the mean $\pm$ range. *B*, HepG2 cells were stimulated or not (control) with IL-22 in the presence (*bottom*) or absence (*top*) of IL-1β and TNF-α for 48 h. Additionally, neutralizing anti-IL-10R2 Abs or respective control Abs were present at the indicated concentrations during this stimulation. Cell culture supernatant was analyzed for LBP concentration as seen in *A*. Data from two (*top*) or three (*bottom*) independent experiments are given as the mean $\pm$ range or the mean $\pm$ SEM, respectively. Dose dependency of the IL-22BP and Ab inhibitory effects was investigated using Page's L test. ***, $p < 0.001$.

Hepatocytes have already been identified as IL-22 targets in earlier studies (14, 20, 21), which is in line with our previous data showing expression of both components of the IL-22 receptor complex (IL-22R1 and IL-10R2) in primary human hepatocytes and the human hepatocyte carcinoma cell line HepG2 (3, 20). We analyzed the IL-22-induced signal transduction in HepG2 cells in detail. Western blot analysis showed that 10 min of IL-22 stimulation led to clear tyrosine phosphorylation of STAT1 and, even stronger, of STAT3 in these cells, which decreased with time until 40 min of stimulation (Fig. 5). In contrast to other reports investigating signal transduction of murine IL-22 in H4IIE rat hepatoma cells, we did not observe serine phosphorylation or lysine acetylation of STAT3; tyrosine phosphorylation of STAT5; activation of the MAPKs JNK/SAPK, ERK1/2, or p38 kinase, or IκB-α, an inhibitor of the transcription factor NF-κB, by IL-22 in these cells (37).

The next step was to verify whether IL-22 induces LBP production in hepatocytes in vitro. Primary human hepatocytes as well as HepG2 cells were stimulated with IL-22 for 24, 48, and 72 h, and LBP concentrations in culture supernatants were analyzed by the Immulite system. We actually found that IL-22 induced the production of LBP in both cellular systems (Fig. 6*A*). Based on a higher number of performed experiments using 48 h culture, this induction could be proved to be significant (Fig. 6*B*). IL-22 does not appear to be a general inducer of acute phase proteins, given that no clear induction of C-reactive protein expression was observed by IL-22 (data not shown).

Recently, IL-22 was reported to exhibit antiapoptotic and mitogenic effects on serum-starved HepG2 cells (22). To exclude the possibility that the observed IL-22-dependent increase of LBP production was due to an increase in cell count, we determined the number of vital primary human hepatocytes and HepG2 cells after 24, 48, and 72 h of culture in the presence and absence (control) of IL-22. As shown in Fig. 6*C*, IL-22 did not affect the number of living cells under the culture conditions used here.

Using HepG2 cells, we then compared the effect of IL-22 on LBP production with the effects of the well-known inducers of hepatic proteins, IL-1β, IL-6, and TNF-α, and analyzed the synergistic potency of all these cytokines after 48 h of culture. In these experiments, IL-22 was somewhat more potent than IL-1β and TNF-α but was less potent than IL-6 in inducing LBP production (Fig. 6*D*). Additional experiments ($n = 6$) comparing the potency of the different LBP inducers applied at identical concentrations showed that IL-22 was slightly, although significantly, more potent than IL-1β ($p < 0.05$) and significantly less potent than IL-6 ($p < 0.05$), but there was no significant difference compared with the TNF-α-treated group (data not shown). Furthermore, the IL-22 action was synergistic with the actions of all other inducers tested (Fig. 6*D*). Thus, the effects of IL-1β, TNF-α, and IL-6, alone or in all possible combinations, were more pronounced in the presence of IL-22. The highest fold increase of IL-22-induced LBP ($\sim$3-fold) was observed when added to IL-1β-TNF-α-stimulated cells.

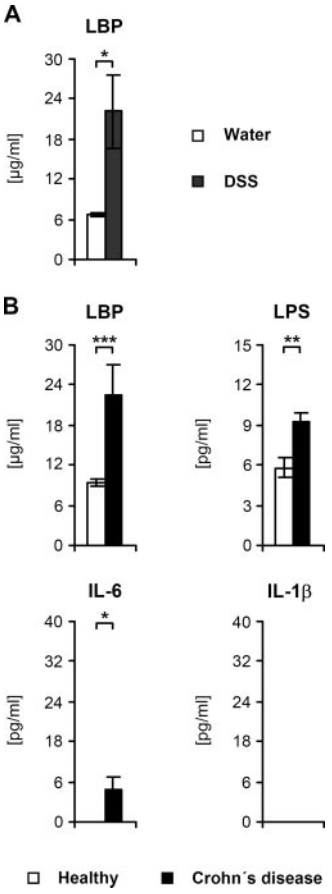

FIGURE 8. Systemic LBP levels are elevated in DSS colitis and patients with CD and may contribute to the prevention of LPS-induced systemic inflammation. *A*, Colitis was induced in BALB/c mice by providing them with DSS-containing drinking water for 5 days. Control mice received water only. Afterward, blood plasma was recovered and plasma LBP concentration was quantified by ELISA. Data from four (DSS) or three (water) mice per group are given as the mean ± SEM. *B*, Blood plasma from CD patients and healthy participants was analyzed for the concentrations of LBP, LPS, IL-6, and IL-1β by Immulite and ELISA. Data from 10 participants per group are given as the mean ± SEM. Statistical significance of deviations was tested using the Mann-Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The IL-22-mediated induction of LBP was additionally observed at the mRNA level (Fig. 6*D*).

In a very recent study, IL-17A and IL-17F were shown to have similar effects and act synergistically with IL-22 on keratinocytes and colonic subepithelial myofibroblasts with respect to the induction of antimicrobial protein (β-defensin 2 and S100 proteins) expression and cytokine (IL-6, IL-8, IL-11, and leukemia-inhibitory factor) secretion, respectively (6, 29). We therefore questioned whether IL-22 and IL-17 would also cooperatively increase hepatocyte LBP expression. As assessed by real-time RT-PCR, IL-17A, even at high concentrations, did not induce LBP mRNA expression and did not modify IL-22-induced LBP mRNA expression in these cells (Fig. 6*E*).

In parallel studies, we attempted to confirm that the observed IL-22-induced LBP production was specific, using two approaches. First, we investigated whether IL-22BP inhibits the IL-22-induced LBP production in HepG2 cells. Cells were stimulated or not (control) for 48 h with IL-22 or with IL-22 in the presence of the costimuli IL-1β and TNF-α. In the first approach, the IL-22 used had been preincubated for 2 h with IL-22BP at increasing molecular ratios (from 1:0.75 to 1:6) or control medium. As shown in Fig. 7*A*, IL-22BP dose-dependently inhibited the IL-22 response in all cases. At a molecular ratio of 1:1.5, the inhibition was already >50%. In the second approach, we performed IL-22 stimulation in the presence of increasing concentrations of neutralizing anti-IL-10R2 Abs or respective control Abs for 48 h. As shown in Fig. 7*B*, anti-IL-10R2 Abs, but not control Abs, also dose-dependently blocked the IL-22 affect on LBP production. These data clearly verified the specificity of the observed IL-22 effect.

Finally, we investigated whether there is an increase in LBP levels in inflammatory bowel disease. As assessed by ELISA, blood plasma LBP was clearly increased in both mouse DSS colitis and patients with CD (Fig. 8). The increased LBP levels in CD are likely to be induced by the cooperative action of other cytokines such as IL-6, which was also systemically present in these patients (Fig. 8*B*). CD patients are known to have an impaired intestine epithelial barrier function associated with increased permeability. Because elevated LBP concentrations neutralize bacterial LPS, we then determined free blood LPS levels in CD patients. As demonstrated in Fig. 8*B*, free LPS levels were actually only slightly higher in CD patients than in healthy donors. In addition, blood levels of IFN-γ, IL-17A (Fig. 1*A*), and IL-1β (Fig. 8*B*) were not increased in these patients. This means that, despite impaired epithelial barrier function, massive systemic inflammation is unlikely in CD. Our data may suggest that in CD, systemic LBP, which is at least partially induced by the hepatic action of IL-22, contributes to the prevention of massive systemic inflammation triggered by translocated LPS.

Discussion

The currently known facts regarding IL-22 let us assume that this cytokine is an example of a type of immune mediator that has been unknown until now. IL-22 is mainly produced by activated Th1 and Th17 cells, although it only seems to act on tissue cells (3, 20). This means that IL-22 is a distal regulator of the inflammatory cascade. Explaining the biology of IL-22 is therefore important not only in expanding our knowledge about the immune system but also in developing novel therapeutic strategies for chronic T cell-mediated autoimmune diseases with limited side effects.

After it was shown that IL-22 is strongly expressed in the inflamed intestine in CD (29, 30), several groups tried to illuminate the meaning of this cytokine in this disorder. The Fujiyama, Dambacher, and Mizoguchi groups investigated the local effects of IL-22 in the intestine (29, 30, 38). However, the exact role of this cytokine in the intestinal inflammation still remains ambiguous. In contrast to these studies, our work focused on possible systemic (endocrine) effects of IL-22.

We show here for the first time that IL-22 levels were also increased in the blood of CD patients. Systemic IL-22 should originate from the sites where activated T cells are present. In a mouse model of inflammatory bowel diseases, IL-22 mRNA expression was found not only in the inflamed intestine but also (to a lesser amount) in the mesenteric lymph nodes. In parallel, the expression of IL-22BP, a specific soluble receptor for IL-22 that was constitutively expressed in lymph nodes and the intestine of healthy animals, was decreased in the intestine, but not in the mesenteric lymph nodes in the inflammatory bowel disease model. To gain an insight into the potential in vivo role of IL-22BP, we also analyzed the affinity between IL-22BP and IL-22. These binding data strongly support an IL-22-inhibitory role of IL-22BP in vivo. We found that IL-22 has a 4- to 10-fold higher affinity to IL-22BP than to its membrane-associated receptor IL-22R1 (35). Although the association rate between IL-22 and IL-22BP was weaker, the dissociation rate was as much as 20 times lower than that between

IL-22 and IL-22R1. That means that IL-22 binds somewhat faster to IL-22R1 than to IL-22BP and that IL-22 may have local effects at the inflammation site even if all three binding partners are present. However, formed IL-22/IL-22BP complexes are stable. In the case of the CD mouse model where the intestinal expression of IL-22BP decreased 5-fold, IL-22 produced at this site should have local as well as systemic effects.

Surprisingly, no increased systemic levels were observed in the case of IFN-γ and IL-17A, although these cytokines are also produced in the inflamed intestine of CD patients (8, 9, 28, 39). Therefore, the systemic presence of IL-22 in CD patients seems to represent a unique trait. The systemic absence of IFN-γ and IL-17 may be due to lower intestinal expression levels of these cytokines compared with IL-22 as suggested by our data from the corresponding mouse model.

In an attempt to identify the role of systemic IL-22, we found that IL-22 increased LBP production by hepatocytes both in vivo and in vitro. This IL-22 effect was mostly transcriptionally regulated and time as well as dose dependent. Compared with previously known inducers of acute phase proteins, the IL-22 effect was clearer than that of IL-1β and was less potent than that of IL-6. The IL-22-induced LBP plasma levels in mice were only 2-fold lower than that induced by 100 μg of LPS i.p. (data not shown), although this amount of LPS is known to massively stimulate proinflammatory cytokine production including IL-6, IL-1β, and TNF-α (20). LBP is a soluble pattern recognition molecule constitutively present in blood plasma (40). During bacterial infection, LBP concentrations increase up to 10-fold. This rise is caused by transcriptional activation of the LBP gene (41). At constitutive concentrations, LBP is essential for the host to sense bacteria and stimulates cells such as monocytes, macrophages, dendritic cells, and endothelial cells to initiate an appropriate inflammatory response (36). In fact, LBP binds bacterial components such as LPS and peptidoglycans and catalyzes their transfer to cellular pattern recognition receptors consisting of membrane-bound or soluble CD14 and members of the Toll-like family. This finally triggers cell activation leading to production of reactive oxygen species and secretion of chemokines and inflammatory cytokines (such as IL-1β and TNF-α; Refs. 36, 42, and 43). In contrast, high concentrations of LBP neutralize bacterial components and therefore limit their immune stimulatory activity (44–46). Injection of high-dose LBP was shown to protect mice from LPS-induced septic shock by neutralizing LPS (44). LBP has also been demonstrated to opsonize bacteria and to increase their phagocytosis by monocytes and macrophages thereby promoting bacterial clearance, an effect that does not seem to be related to specific LBP concentrations (47, 48).

In our study, increased LBP blood levels were indeed present in both the blood of animals with experimentally induced colitis and in patients with CD. IL-22 is apparently not solely responsible for the increased LBP levels, but it more likely works together with other cytokines. In fact, we found that IL-6, a strong LBP inducer, was also elevated in the blood of such patients, although its increase was not as massive as in the case of IL-22. By neutralizing LPS, the increased LBP concentration in the blood of CD patients could contribute to the absence of a massive systemic inflammatory reaction to LPS, which is present in the blood of such patients.

Apparently, IL-22 induces LBP in conditions other than inflammatory bowel disease, e.g., in bacterial infection. Our speculations on the meaning of such mechanisms begin with the fact that IL-22 is mainly produced by activated T cells. The generation of these cells occurs in the later, i.e., adaptive phase of the immune response to a bacterial infection. In this phase, the initially induced inflammation seems to no longer be appropriate and is terminated. It is fitting that IL-22 did not elevate the production of C-reactive protein in hepatocytes (data not shown). However, IL-22 did elevate LBP secretion 48 h after application in vivo. High LBP levels induced by T cell-derived IL-22 in the later phase of the immune reaction might then neutralize LPS and other bacterial products in addition to promoting phagocytosis and therefore the clearance of pathogens. The fact that cells of the adaptive immune system produce a mediator that limits the reaction of the innate immune system presents an interesting aspect of immunity.

Through our in vitro experiments with IL-22 in human hepatocytes, we have additionally made two observations which add to the facts concerning IL-22 in the current literature: 1) regarding the IL-22-induced signal transduction in hepatocytes; and 2) regarding the lacking influence of IL-22 on the hepatocyte proliferation. Regarding the IL-22-induced signal transduction, in our study IL-22 activated the signaling molecules STAT1 and STAT3 (tyrosine phosphorylation), but not STAT5, MAPKs, or the NF-κB pathway. This contrasts with previously shown data on rat hepatoma cells, in which IL-22 activated STAT1, STAT3, STAT5, and all three major MAPK pathways (37), and indicates an IL-22-induced signal transduction dependent not only on the cell type but also on the species examined. LBP gene promoter analysis has formerly revealed active binding sites for STAT3, AP-1, C/EBP-β, glucocorticoid-responsive elements and NF-κB (49, 50). This suggests a possible activation of LBP transcription by IL-22 via STAT3 in liver cells and may explain the strong synergistic action on LBP transcript levels of IL-22 with IL-1β and TNF-α, which activate NF-κB and MAPK pathways, as well as the lower synergistic action with IL-6, which also activates STAT3. In comparison to the mRNA levels, the IL-6-induced up-regulation of LBP protein was strongly enhanced by the presence of IL-22, suggesting that IL-22 additionally promotes the translation of LBP mRNA. Regarding the second point, the influence of IL-22 on the hepatocyte viability and proliferation, we found that IL-22 had no effect when culturing primary hepatocytes and HepG2 cells under standard conditions. In contrast, IL-22 was reported to exhibit antiapoptotic and mitogenic effects on serum-starved HepG2 cells (22).

In conclusion, we show that CD patients show increased systemic levels of IL-22 able to induce hepatic LBP production. These results potentially provide a novel mechanism whereby the severe inflammation in CD is limited to the intestine and does not spill over into a generalized systemic inflammation.

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Disclosures

The authors have no financial conflict of interest.

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