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ORIGINAL CONTRIBUTION

Bovine lactoferrin induces interleukin-11 production in a hepatitis mouse model and human intestinal myofibroblasts

Tetsuya Kuhara · Koji Yamauchi · Keiji Iwatsuki

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Abstract
Purpose Orally administered bovine lactoferrin (bLF) exerts an anti-inflammatory effect on hepatitis and colitis animal models. To investigate the mechanism underlying the action of bLF, we explored the expression of inflammation-related factors in the intestine of a hepatitis mouse model after the oral administration of bLF and in several human intestinal cell lines treated with bLF.
Methods The effects of bLF on the expression of interleukin-11 (IL-11) and bone morphogenetic protein 2 (BMP2) in the intestinal mucosa of a hepatitis mouse model as well as in cell cultures of human intestinal epithelial cells, myofibroblasts, and monocytes were examined using the real-time reverse transcription polymerase chain reaction. Epithelial cells and myofibroblasts were also cocultured using transwells. bLF transport, and IL-11 and BMP2 induction, as well as the interactions between the two cell types, were then analyzed after bLF treatment.
Results In vivo, oral bLF administration increased the production of IL-11 and BMP2 in intestinal specimens. In vitro, bLF only stimulated the production of IL-11 in human intestinal myofibroblasts; i.e., it had no effect on BMP2 production in any cell type. In the transwell cocultures, bLF passed through the epithelium and directly stimulated IL-11 production in the myofibroblasts on the basolateral side. The IL-11 produced in the myofibroblasts subsequently acted protectively on the epithelial cells of the coculture.
Conclusions bLF upregulated the activity of anti-inflammatory factors, such as IL-11, in the intestine of a hepatitis mouse model and human intestinal myofibroblasts.

T. Kuhara (✉) · K. Yamauchi · K. Iwatsuki
Food Science & Technology Institute,
Morinaga Milk Industry Co., Ltd.,
5-1-83 Higashihara, Zama, Kanagawa 228-8583, Japan
e-mail: t_kuhara@morinagamilk.co.jp

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Introduction

Lactoferrin (LF) has a wide range of properties [1, 2] including anti-microbial/viral, anti-inflammatory, anti-tumor, and immunoregulatory activities and also plays a role in the regulation of iron homeostasis. Independent mechanisms have been described for each of these properties, for example, its ability to bind and sequester iron limits microbial growth, and the modulation of the cytokine imbalance suppresses inflammation. The beneficial effects of bovine LF (bLF), which can be orally ingested and is widely available, have been investigated in numerous clinical trials. These trials have demonstrated several promising outcomes, such as the inhibition of hepatitis C virus viremia in chronic hepatitis C patients [3–6], the improvement of dermatological symptoms in tinea pedis [7], the suppression of colorectal adenomas [8], and the enhancement of peripheral natural killer (NK) cell activity [8]. These results also indicated that ingested bLF exerts various biological responses without directly interacting with target organs, since barely detectable amounts of bLF and its large fragments are absorbed by the intestines [9]. In animal models, the oral administration of bLF has been reported to attenuate colitis [10], inhibit hepatic inflammation derived from oxidative liver damage [11], and protect against oocyte depletion induced by cyclophosphamide [12]. Previously, we demonstrated that orally

ingested bLF stimulated interleukin-18 (IL-18) production in the intestinal epithelium and type I interferon production in Peyer's patches, thereby enhancing peripheral NK cell activity in mice [13]. In addition, oral bLF inhibited metastasis by enhancing NK cell activity in a murine metastasis model [14]. In the present study, to explore the underlying mechanism of the anti-inflammatory effects of oral bLF, we investigated the activity of bLF using a murine hepatitis model. In a rat colitis model induced by trinitrobenzenesulfonic acid (TNBS), oral bLF administration reportedly reduced inflammation by correcting the cytokine imbalance [10], although it was hard to determine whether bLF induced the production of anti-inflammatory cytokines and improved tissue damage by direct or indirect mechanisms. In order to detect the direct anti-inflammatory effects of bLF on the intestinal tissue in an inflammation status, we used a murine hepatitis model. In addition, to avoid directly affecting the intestinal organs via the intraperitoneal injection of liver injury-inducing agents (e.g., D-galactosamine or carbon tetrachloride), we analyzed the activity of bLF using a mouse hepatitis model induced by zymosan plus lipopolysaccharide (LPS), in which human LF reportedly protects against the development of hepatitis [15]. In the present study, the oral administration of bLF suppressed hepatic inflammation and stimulated IL-11 and bone morphogenetic protein 2 (BMP2) production in the intestine.

IL-11, a pleiotropic cytokine, has a variety of hematopoietic and non-hematopoietic effects [16]. The anti-inflammatory activities of IL-11 are reportedly involved in the inhibition of inflammatory cytokine production through the inhibition of NF-κB [17] and the enhancement of cytoprotection via heat shock protein induction in intestinal epithelial cell lines [18]. In patients with hematological malignancies who are undergoing chemotherapy, IL-11 prevents gastrointestinal epithelial disintegrity and gut-associated infections [19]. IL-11 has also been reported to exert therapeutic anti-inflammatory effects in patients with Crohn's disease [20, 21]. Recently, it has been suggested that IL-11 can be used to treat patients with hepatic inflammation and advanced liver disease associated with hepatitis C virus infection [22].

BMP2 has also been implicated in a variety of functions [23]. In the intestines and the liver, BMP2 reportedly acts as a tumor suppressor that promotes apoptosis [24] and plays important roles in the wound-healing response [25].

Some previous studies have reported that LF directly inhibits the production of cytokines in specific cells [2], but a few reports have demonstrated that LF displays direct cytokine-inducing activity. In addition, there have been no reports about food ingredients that stimulate IL-11 and BMP2 production in the intestine. In order to understand the physiological role and beneficial effects of oral bLF, it is extremely important to clarify which cells in the intestine produce IL-11 and BMP2 in response to bLF. In the present study, we explored the effect of bLF treatment on the expression of IL-11 and BMP2 in several human intestinal cell lines because it is hard to obtain murine intestinal myofibroblasts and the human cell lines that we employed have been well characterized. Using human cell lines also allowed us to produce clinically useful results. Furthermore, the intercellular interactions that occurred between intestinal cells treated with bLF were examined using a coculture system.

Materials and methods

Hepatitis mouse model

All animal studies were approved by the Committee of Animal Care and Oversight of Morinaga Milk Industry in accordance with guidelines for the care and use of laboratory animals. A total of 60 male 7–8-week-old Balb/c mice (Charles River, Kanagawa, Japan) were housed under constant conditions (temperature: 21–25 °C; humidity: 40–60%; 12-h light/dark cycle), fed a non-purified diet (MR stock: Nihon Nosan Kogyo, Kanagawa, Japan), and allowed free access to tap water. We analyzed the effects of bLF using a hepatitis mouse model induced by the intravenous injection of zymosan plus LPS because the injection of inflammation-inducing agents into the intestine in a murine colitis model markedly affected the immune system and the cytokine balance [10], which would have made it difficult to analyze the action of bLF in the intestine. In addition, the present model allowed us to avoid directly affecting the internal organs by intraperitoneally injecting liver injury-inducing agents [15]. The mice were intravenously injected with zymosan (Sigma, St. Louis, MO) at a dose of 25 μg/g of body weight on day 0. bLF (Morinaga Milk Industry, Tokyo, Japan) was suspended in sterile water and orally administered from day 1 to day 7 via a gastric tube at a dose of 300 μg/g of body weight once daily. The mice in the other group were treated with bovine serum albumin (BSA; Sigma). At 3 h after the last oral administration, 15 ng/g of body weight of LPS (0111:B4; Sigma) was intravenously injected into the mice. Then, 18 h later, the mice were anesthetized with diethyl ether, their blood was harvested, and their serum alanine transaminase (ALT) activity level was measured using a colorimetric diagnostic kit (Wako Pure Chemical Industry, Osaka, Japan). Non-treated mice, which did not receive any intravenous injections or oral administrations, were also sampled during the experiments.

Evaluation of the effect of bLF on small intestinal tissue

On days 0, 1, 3, and 6, and at 18 h after the LPS injection, a mucosal tissue sample was excised from the middle section of the small intestine, immediately frozen, and kept at −80 °C until further analysis. Semi-quantitative real-time RT–PCR analysis was performed to detect the mRNA expression levels of the anti-inflammatory factors IL-4, IL-10, IL-11, transforming growth factor beta (TGF-β), and BMP2, as well as the inflammatory factors IL-1β, IL-6, and TNFα. Total RNA was extracted from the mucosal tissue using Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed using a cDNA synthesis kit (TaKaRa Bio, Shiga, Japan). Real-time PCR was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA) with SYBR premix ExTaq II (TaKaRa Bio) and a commercial primer set for real-time PCR (TaKaRa Bio). The relative mRNA expression levels of the abovementioned genes were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

Immunoblot analyses of IL-11 and BMP2 production in the small intestine were performed on the basis of the real-time RT–PCR analysis results. The mucosal tissue samples were homogenized in mammalian cell lysis buffer (Sigma) and then centrifuged, and the protein concentrations of the resulting supernatants were then measured using the BCA protein assay reagent (Thermo Fisher Scientific, Lafayette, CO). Equal amounts of the samples were subjected to reduced SDS–PAGE followed by immunoblot analyses using anti-mouse IL-11 (R&D Systems, Minneapolis, MN), BMP2, and β-actin (Abcam, Cambridge, MA).

Cell cultures

To quantify the production of IL-11 and BMP2 in intestinal mucosal tissue after treatment with bLF, we tested human epithelial cell lines (Caco2, T84, and HT29), myofibroblasts (CCD-18Co), and monocytes (THP-1). All the cell lines are included in the European Collection of Cell Cultures and were obtained from Dainippon-Sumitomo Pharma (Osaka, Japan). Dulbecco's modified Eagle's medium (Caco2, HT-29), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (T84), modified Eagle's medium with Earle's balanced salts (CCD-18Co), and RPMI-1640 medium (for THP-1) (all from Invitrogen) were supplemented with 5% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. A previous study reported that orally administered bLF was present at levels of 0.01–10 μM in the lower small intestines of adult rats [9]. Consequently, bLF was added to the culture at concentrations ranging from 1.56 to 400 μg/mL (nearly 0.02–5 μM). The effects of human LF, which was purified from the milk of healthy volunteers by Morinaga, and human transferrin (Sigma) were also examined at concentrations of 400 μg/mL. The cultured cells were harvested after 4 h of treatment with bLF and analyzed using real-time RT–PCR. G3PDH expression was not affected by any of the culture treatments. The concentrations of IL-11 and BMP2 in the 24-h culture media treated with bLF were measured using an ELISA (R&D Systems). The addition of bLF to the culture did not affect cell viability, which was determined by tryphan blue exclusion.

Determination of interactions between intestinal epithelial cells and myofibroblasts following exposure to bLF

Caco2 cells were grown on 12-well collagen-coated transwell inserts (BD Biosciences, Bedford, MA) in enterocyte differentiation medium (BD Biosciences). The tight junction permeability of the Caco2 cell monolayers was monitored by measuring their transepithelial electrical resistance (TEER) using a Millicell ERS instrument (Millipore, Bedford, MA). Moreover, a lucifer yellow permeability assay was performed according to the manufacturer's instructions. The TEER values of the cells had increased to more than 400 Ω/cm^2 at 48 h after the start of the culture and were sustained for more than 48 h. Lucifer yellow transport across the monolayers was not detected during these periods.

The CCD-18Co cells were cultured in a 12-well culture plate at a density of 5×10^5 cells/well. After being cultured for 1 day, they were combined with transwell inserts containing cultured Caco2 cells, which had started being cultured 48 h beforehand, and used for the coculture experiments. The culture media on the apical and basolateral sides, which were separated by the Caco2 cell monolayer, were harvested at 0, 1, 3, 6, 12, 24, and 36 h after the addition of bLF (400 μg/mL) on the apical side. To confirm the action of IL-11 itself, human IL-11 (R&D Systems) was added to the basolateral side at 1 ng/mL. The Caco2 and CCD-18Co cells were also harvested at these time points. bLF transcytosis was detected by measuring the bLF concentrations in the media on both sides of the Caco2 cell monolayer using an ELISA (Bethyl Laboratories, Montgomery, TX). The concentrations of IL-11 and BMP2 in the 24-h culture media treated with bLF and BSA were also measured using an ELISA (R&D Systems). These cocultures were examined in duplicate for each time point and repeated 3 times. A portion of the harvested cells was used for RNA extraction, and the remaining cells were solubilized in lysis buffer and used to detect phosphorylated protein

(Bio-Rad). The relative mRNA levels of IL-11, BMP2, suppressor of cytokine signaling 3 (SOCS3), and SMAD1 were analyzed using real-time RT–PCR. In order to detect the effect of IL-11 production on the epithelial cells, the levels of phosphorylated signal transducer and activator of transcription 3 (STAT3) and Hsp27, which are signal transmitters for IL-11, and SMAD1, which is a signal transmitter for BMP2, were measured. Samples containing equal amounts of protein were subjected to immunoprecipitation with antibodies against STAT3, SMAD1 (both Santa Cruz Biotechnology, Santa Cruz, CA), and Hsp27 (R&D Systems). Each immunoprecipitated sample was examined by immunoblotting with anti-STAT3 and anti-phospho-STAT3 (Millipore) antibodies and anti-SMAD1, anti-Hsp27, and anti-phospho-serine (Invitrogen) antibodies.

Statistical analysis

Numerical data are expressed as the mean ± SD. Post hoc testing of the differences between groups was performed using the Tukey–Kramer test with Stat View software (SAS Institute, Cary, NC). The Student's unpaired *t*-test was used to compare the mean values of two groups. Differences were considered significant at $p < 0.05$.

Results

bLF administration prevented the progression of hepatic failure in mice and enhanced IL-11 and BMP2 expression in the small intestine

The BSA group exhibited a significant elevation of serum ALT activity compared with the non-treated group at 18 h after the LPS injection; however, this elevation was suppressed in the bLF-treated group (bLF group, 533.3 ± 378.2 karmen units [KU]; BSA group, 1007.4 ± 383.1 KU; and non-treated group, 146.2 ± 98.7 KU).

Our examination of the expression of inflammatory and anti-inflammatory factors in the small intestine revealed that the mRNA expression level of the anti-inflammatory factor IL-11 gradually increased during bLF administration (Fig. 1a) and that the mRNA expression of another anti-inflammatory factor, BMP2, was significantly elevated by bLF after LPS injection, although it was maintained at the basal level prior to the LPS injection (Fig. 1b). On day 6, increases in IL-11 and BMP2 expression were detected using immunoblot analyses (Fig. 1c). The small intestinal expression levels of the other anti-inflammatory and inflammatory factors tested did not change significantly during the experimental period (data not shown).

bLF administration enhanced IL-11 production in intestinal myofibroblasts

Since the IL-11 and BMP2 levels in the small intestine increased without increases in the expression of inflammatory factors, it was suggested that bLF directly stimulated intestinal cells to produce IL-11 and BMP2. Next, we determined whether bLF directly stimulates IL-11 production in human epithelial cells, myofibroblasts, and a monocyte cell line, which together constitute intestinal mucosal tissue. Figure 2a shows that bLF administration did not induce IL-11 mRNA expression in the intestinal epithelial cell lines or the monocyte cell line; however, IL-11 mRNA expression was markedly increased in the myofibroblast (CCD-18Co) cell line after bLF stimulation. On the other hand, bLF did not influence the expression of BMP2 mRNA in any of the cell lines (Fig. 2b). In the CCD-18Co cells, the single administration of bLF resulted in the sustained upregulation of IL-11 mRNA expression for at least 6 h, although no change was observed in the other cell lines at any time. In the CCD-18Co cell cultures, bLF increased IL-11 mRNA expression (Fig. 2c) as well as the IL-11 protein concentration (Fig. 2d) in a dose-dependent manner. Human LF, but not human transferrin, exerted similar activity in CCD-18Co cells. bLF did not affect the expression of TNFα, IL-1β, or TGF-β in any of the cells (data not shown). bLF treatment did not enhance the concentration of BMP2 in the cell cultures examined.

Transepithelially transported bLF induced IL-11 production in basolateral myofibroblasts leading to anti-inflammatory reactions in the cocultured cells

Intestinal subepithelial myofibroblasts are located in the lamina propria beneath the epithelial cells. In the present study, the ability of transepithelially transported bLF to induce IL-11 production in myofibroblasts seeded beneath the epithelium and the resultant effects were examined. bLF administration had almost no effect on TEER. At 1 h after the addition of bLF to the apical side, immunoreactive bLF appeared on the basolateral side of the transwell chamber at a concentration of 189.3 ± 16.7 ng/mL. The bLF concentration on the basolateral side increased gradually, reaching 451.1 ± 27.9, 493.0 ± 26.9, 645.6 ± 77.4, 981.7 ± 59.6, and 939.6 ± 129.1 ng/mL at 3, 6, 12, 24, and 36 h, respectively. The bLF flux values were 169.7 ± 10.1, 15.5 ± 15.7, and 21.0 ± 14.4 ng/h/cm^2 at 0–3 h, 3–6 h, and 6–9 h after the start of the experiment, respectively. No bLF flux was observed after 24 h. IL-11 mRNA expression in the CCD-18Co cells located on the basolateral side was significantly enhanced at 3 h after the bLF administration, and this enhancement was maintained until 12 h after its administration (Fig. 3a). After 24 h of

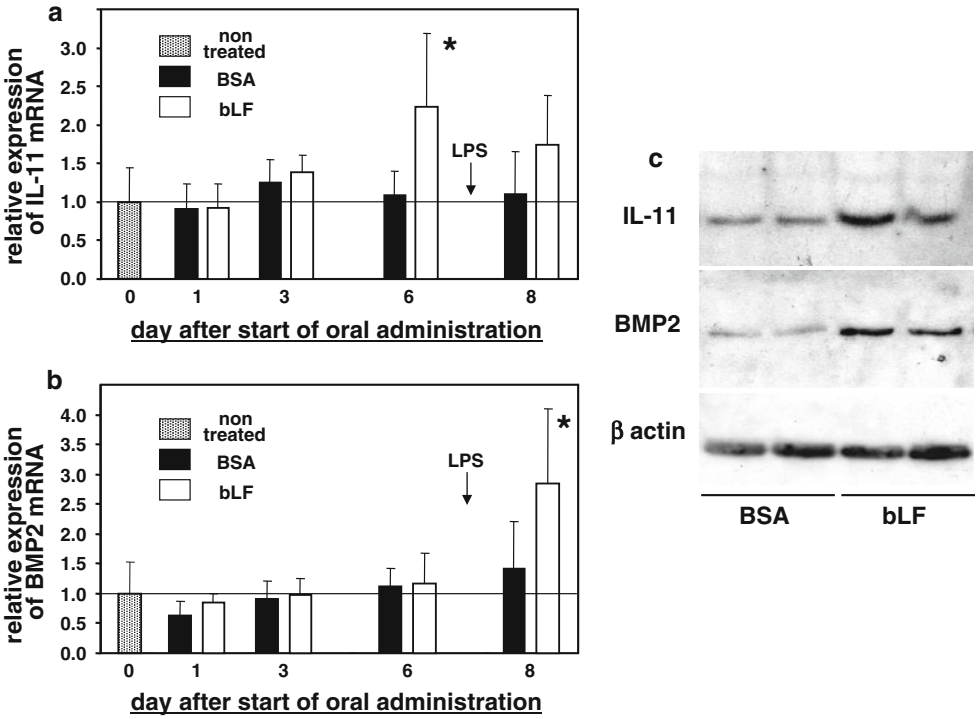

Fig. 1 Expression of murine IL-11 and BMP2 in intestinal mucosa after the oral ingestion of bLF. The mice were initially intravenously injected with zymosan and then injected with LPS 7 days later to establish a hepatitis mouse model. bLF or BSA was administered orally during the same period. Mucosal tissue samples were obtained from the small intestine on days 0, 1, 3, 6, and 8 and subjected to (**a**, **b**) real-time PCR analysis and (**c**) immunoblot analysis of IL-11 and BMP2 expression. Data are presented as the mean ± SD ($n = 6$). The image shown here is representative of three separate analyses. *$p < 0.05$ versus the value on day 0 using the Tukey–Kramer test

culture, the IL-11 concentration of the bLF-treated cells was significantly higher than that of the BSA-treated control cells (Fig. 3b). While bLF treatment did not enhance the expression of IL-11 in the Caco2 cells, the relative amount of phosphorylated STAT3, which is a critical signaling molecule of IL-11 that acts downstream of gp130, increased in the cells from 3 h after the bLF administration (Fig. 4a). Moreover, the mRNA expression of SOCS3, which is a suppressor of the cytokine signaling mediated through gp130, was significantly enhanced in the epithelial cells from 6 h after the bLF administration (Fig. 3c). The addition of human IL-11 to the basolateral side at 1 ng/mL also markedly increased the expression of SOCS3 in the Caco2 cells (data not shown). The administration of bLF did not affect the expression of BMP2 mRNA in the Caco2 or CCD-18Co cells (Fig. 3d). Furthermore, SMAD1, a transcriptional factor for BMP2, was not activated by bLF treatment (Fig. 4b). Finally, we determined the activated Hsp27 level in the epithelial cells. Figure 4c shows that the amount of phosphorylated Hsp27 gradually increased from 12 h after the bLF treatment.

Discussion

Numerous studies have examined the relationships among the structure, function, and physiological effects of LF. However, the mechanisms underlying the biological activities of LF have not been fully elucidated. Also, it is unclear whether orally ingested LF is absorbed into the human body and subsequently exerts biological activity within it. While a few reports have shown that intact, immunoreactive LF is absorbed from the intestine into the bloodstream and is subsequently transported to various tissues [26], xenogenous proteins are unlikely to function for long periods of time, preventing antibody formation. In the present study, we confirmed that bLF directly promotes IL-11 production in intestinal subepithelial myofibroblasts.

Subepithelial intestinal myofibroblasts play important roles in the organogenesis of the intestine, and the growth factors and cytokines secreted by these cells promote epithelial restitution and proliferation; i.e., wound repair [27]. Intestinal subepithelial myofibroblasts are located in the lamina propria beneath epithelial cells. Human LF was reportedly transported across intestinal epithelial cells (HT29) in an Ussing chamber, and the degree of movement was similar to that of other proteins with similar molecular weights [28]. In our coculture experiments, very small amounts of bLF (around 1/1,000 at 3 h after administration) were transported across intestinal epithelial cells in an immunoreactive form, resulting in enhanced IL-11 production in myofibroblasts on the basolateral side of the culture chamber (IL-11 mRNA expression was significantly elevated from 3 to 12 h after bLF administration). In our animal experiments, the murine IL-11 mRNA expression induced by oral bLF gradually increased from day 3

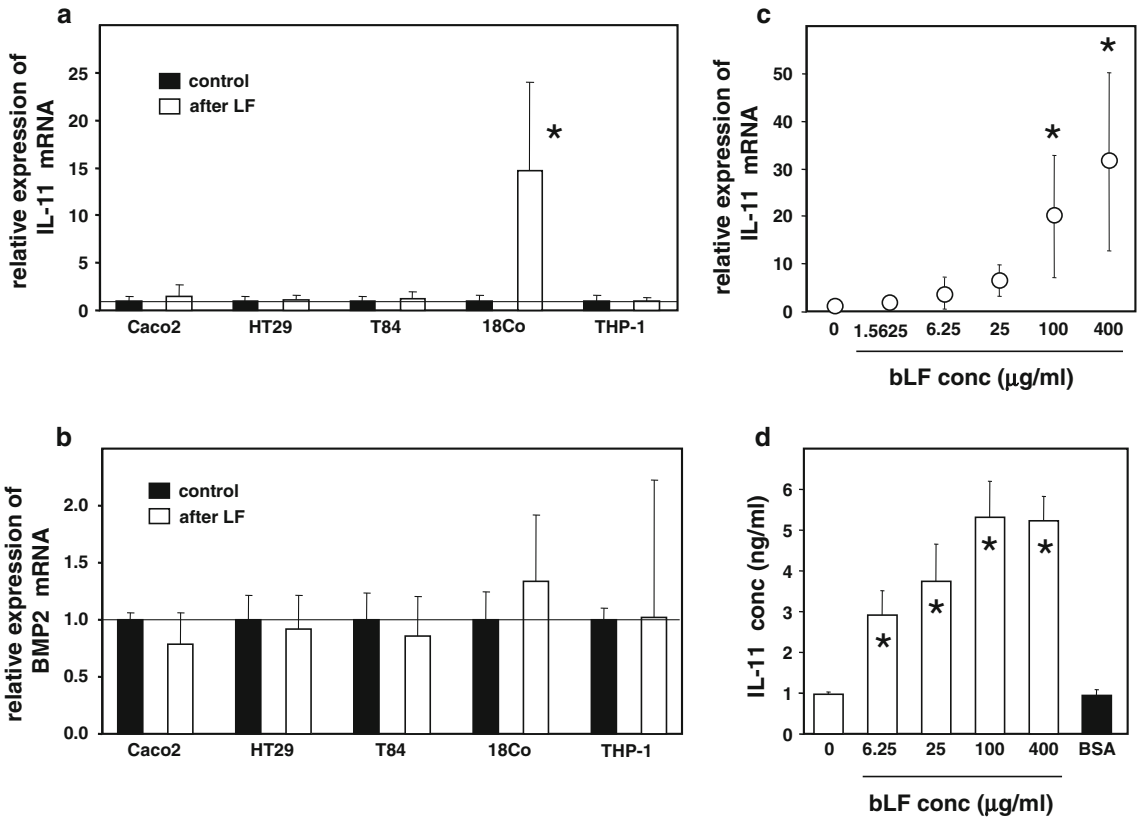

Fig. 2 Expression of human IL-11 and BMP2 induced by bLF in intestinal epithelial, myofibroblast, and monocyte cell lines. Each cell line was cultured with 400 μg/mL bLF and then prepared for the real-time PCR analysis of **a** IL-11 and **b** BMP2 mRNA expression. Moreover, the CCD-18Co cell line was analyzed for the dose dependence of the effects of bLF on **c** IL-11 mRNA expression and **d** the IL-11 protein concentration of the cell culture. Data are presented as the mean ± SD ($n = 6$). *$p < 0.05$ using the Student's unpaired *t*-test (**a**) or Tukey–Kramer test (**c**, **d**)

and had increased significantly by day 6. Therefore, it was supposed that the successive administration of bLF enhanced and sustained endogenous IL-11 production.

Some of the clinical effects of IL-11 treatment overlap with those of LF. A clinical study of hepatitis C virus patients who had not responded to anti-viral therapy suggested that recombinant human IL-11 has beneficial anti-inflammatory effects [21]. The oral administration of bLF was found to improve hepatic inflammation in non-responders to anti-viral therapy [4–6]. The oral administration of bLF also improved dermatological inflammatory symptoms in patients with tinea pedis [7]. In patients with psoriasis, the subcutaneous delivery of recombinant human IL-11 reduced cutaneous inflammation [29]. Furthermore, IL-11 therapy preserved the mucosal integrity of the gastrointestinal tract and reduced bacteriemia in patients with hematological malignancies undergoing chemotherapy [18]. bLF supplementation also prevented the late onset of sepsis in very low-birth-weight neonates [30]. Moreover, in animal models, IL-11 and LF exhibited similar levels of effectiveness as treatments for TNBS-induced colitis in rats [10, 31] and cytokine-mediated liver injury in mice [32]. bLF induced the activation of Hsp27 in intestinal epithelial cells in the present study, similar to the reported induction of Hsp25 by IL-11 in rat epithelial cells, which conferred intestinal epithelial-specific cytoprotection [17]. These results indicate that there are numerous similarities between the biological effects of IL-11 and LF and suggest that the activity of LF is mediated, at least in part, through the induction of IL-11. The present results support the idea that oral LF exerts anti-inflammatory and cytoprotective effects on the intestine via IL-11.

BMP2 has a wide potential in development of not only bone but also numerous organs such as the heart, skin, and the nervous system [22]. Recently, it has been reported that BMP2 plays important roles in the wound-healing response of the injured liver [25]. While the oral administration of bLF increased BMP2 expression in the murine intestine, in vitro experiments did not reveal a similar effect in the present study; namely, bLF elevated IL-11 expression, but did not induce BMP2 expression in intestinal epithelial cell lines, myofibroblasts, or monocytes. As shown in Fig. 1a and b, murine IL-11 mRNA expression was elevated prior to the LPS injection, but an increase in BMP2 mRNA

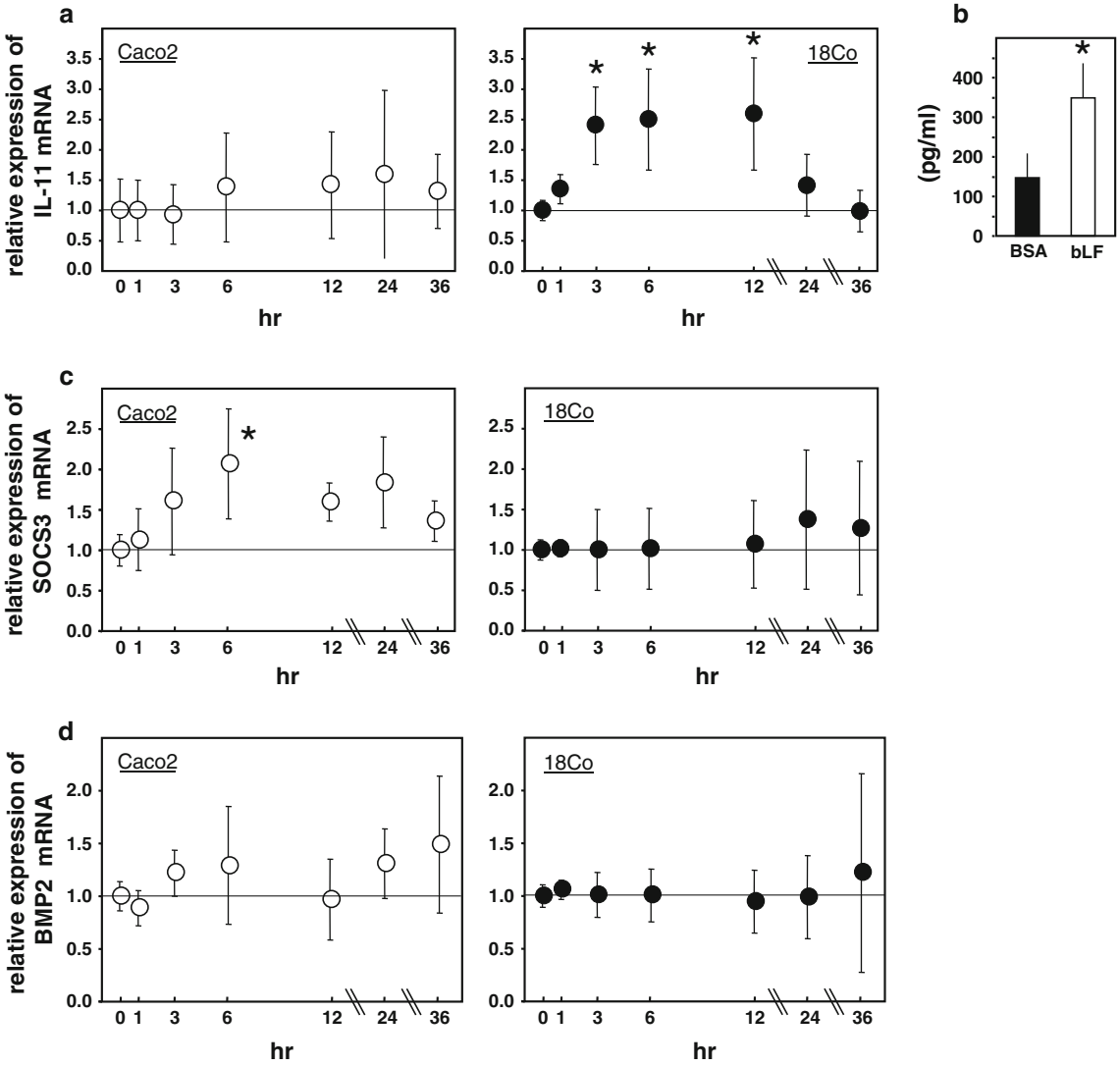

Fig. 3 bLF enhanced IL-11 expression in CCD-18Co cells and IL-11 induced SOCS3 expression in Caco2 cells under coculture conditions. Caco2 cells were seeded in a transwell and cocultured with CCD-18Co cells seeded in a lower well. bLF (400 μg/mL) was then added to the apical side. The relative mRNA expression levels of **a** IL-11, **c** SOCS3, and **d** BMP2 were analyzed in both cell types using real-time PCR. **b** The IL-11 concentrations on the basolateral side of the culture medium were determined over a 24-h period ($n = 6$). *$p < 0.05$ versus the value at 0 h using the Tukey–Kramer test or Student's unpaired t-test (**b**)

expression was noted after the LPS injection. Hence, it is supposed that BMP2 expression requires stronger inflammatory stimulation than IL-11 induction. bLF reportedly increases the expression of IL-4 and IL-10 in the colonic mucosa under inflammatory conditions but not under physiological conditions [10]. This result implies that other factors present under inflammatory conditions are necessary for the induction of BMP2 expression after bLF administration. BMP2 has been shown to act as a tumor suppressor, promoting apoptosis in colonic epithelial cells, and indeed, BMP2 expression was lost in the microadenomas of patients with familial adenomatous polyposis [23]. Recently, the ingestion of bLF has been reported to significantly retard the growth of adenomatous colorectal polyps in human patients [8]. This previous study also indicated that oral bLF significantly enhanced NK cell activity throughout the therapeutic period. The oral administration of bLF stimulates IL-18 production in the intestinal epithelium and type I interferon production in Peyer's patches, thereby increasing NK cell activity in mice [13]. Although there have been no reports indicating a direct action of LF on IL-18 production in intestinal epithelial cells, BMP2 has been reported to stimulate IL-18 production [33]. In our animal study, we could not prove that the upregulation of IL-11 and BMP2 expression in the intestine induced by oral bLF improved hepatic failure. Further studies are necessary to demonstrate that the production of these factors in the intestine affects other organs.

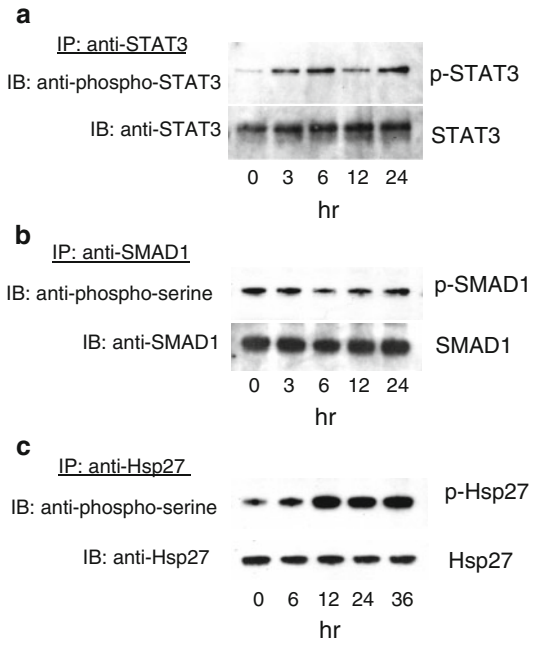

Fig. 4 bLF activated STAT3 and Hsp27, but not SMAD1, in Caco2 cells cocultured with CCD-18Co cells. The Caco2 cells in the upper transwell and CCD-18Co cells in the lower well were cocultured and treated as described in Fig. 3. After being stimulated with bLF for 0–36 h, the Caco2 cells were lysed. The cell lysates were then immunoprecipitated (IP) with **a** anti-STAT3, **b** SMAD1, and **c** Hsp27 antibodies and then immunoblotted (IB) with anti-phospho-STAT3 (**a** *top*) or anti-phosphoserine (**b**, **c** *top*). The amounts of STAT3, SMAD1, and Hsp27 in the immunoprecipitates were determined by immunoblotting with anti-STAT3 (**a** *bottom*), SMAD1 (**b** *bottom*), and Hsp27 (**c** *bottom*) antibodies, respectively. The figure is representative of three independent duplicated cultures

We are currently conducting research analyzing the relationship between the physiological functions of bLF and IL-11 using IL-11 receptor knockout mice. IL-11 has also been reported to increase the expression of endogenous IL-11 in the skin [29].

In conclusion, the present study suggests that bLF in the small intestine passes through the intestinal epithelium and stimulates the production of IL-11 in intestinal subepithelial myofibroblasts. The anti-inflammatory effect of oral bLF might arise as a result of its effect on IL-11 production.

Conflict of interest None of the authors has any potential conflicts of interest related to the present study to declare.

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