Effects of bovine lactoferrin on the immature porcine intestine

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Abstract
Bioactive milk proteins may be important in protecting preterm infants from developing inflammation and necrotising enterocolitis (NEC). A preterm pig model was used to investigate the protective effects of enteral bovine lactoferrin (bLF) against NEC development and inflammation. Caesarean-delivered preterm pigs were fed parenteral and minimal enteral nutrition for the first 2 d followed by 2 d of total enteral nutrition before euthanasia. Pigs were stratified into two groups and fed with either a control formula (CON, n = 15) or a 10 g/l of bLF-enriched formula (LF, n = 13). NEC incidence, gut functions and inflammatory cytokines were analysed. NEC incidence and nutrient absorption were similar between the two groups. In pigs that developed NEC, disease outcome was more severe in the colon accompanied by increased intestinal permeability in LF pigs. In contrary, the LF pigs had a lowered IL-1B level in the proximal small intestine. Dose-dependent effects of bLF on cell proliferation, intracellular signalling and cytokine secretion were tested in porcine intestinal epithelial cells (PsIc1) in vitro. Low doses (0.1–1 g/l) increased cell proliferation via extracellular signal-regulated kinase (ERK), limited IL-8 secretion and prevented NF-κB and hypoxia-inducible factor-1α (HIF-1α) activation, suggesting anti-inflammatory effects. In contrast, at a higher dose (10 g/l), bLF exerted adverse effects by reducing cell proliferation, stimulating IL-8 release, inhibiting ERK activation and up-regulating NF-κB and HIF-1α activation. Overall, at a dose of 10 g/l, bLF exacerbated disease severity in pigs that developed NEC, while the in vitro studies indicated the positive effects of bLF at low doses (0.1–1 g/l). Supplementation of infant formulas with bLF should therefore be optimised carefully.

Key words: Inflammatory cytokines: Lactoferrin: Necrotising enterocolitis: NF-κB

Necrotising enterocolitis (NEC) is one of the most common and severe intestinal diseases in human preterm infants with an incidence of 7% in very-low-birth-weight infants (<1500 g) and a mortality rate of 20–30% (1). The causes of NEC are still poorly understood, but some major predisposing factors are prematurity, aggressive enteral feeding and improper microbial colonisation in the intestine (1). Human milk is considered as the optimal diet for both term and preterm infants (2,3), but a large proportion (e.g. 20–40%) of preterm and term infants are not breast-fed (3). In such cases, breast milk is replaced by human donor milk, bovine milk-derived human milk fortifiers or infant formulas, but these may increase the risk of NEC development. Several clinical studies have indicated that exclusively feeding human milk to preterm infants decreases NEC incidence and lowers mortality significantly (4–6), compared with adding a bovine milk-based human milk fortifier (1.7–3.2 g/l) or feeding an infant formula (7,8). The reason for this may be that specific bioactive components in human milk can contribute to the protection against NEC. Studies focusing on the bioactivity of these individual components can provide knowledge for the development of a new generation of improved formulas containing proteins supplemented at doses similar to those in human milk.

Lactoferrin is an important 80 kDa Fe-binding protein present in mammalian milk and in several secreted fluids such as saliva and mucosal secretions (9–11). It is a major human milk protein having a concentration of up to 7 g/l in the humancolostrum and 2 g/l in mature milk (9). In contrast, it is present in bovine milk as a minor protein (1.5 and 0.2 g/l in the colostrum and mature milk, respectively) (9). The structure of lactoferrin comprises an N-terminal and a C-terminal domain linked by a hinge region, which causes protein flexibility (11,12). Human lactoferrin and bovine lactoferrin (bLF) are

Abbreviations: bLF, bovine lactoferrin; CON, control formula; HIF-1α, hypoxia-inducible factor-1α; IkB, inhibitor kB; LF, bovine lactoferrin-enriched formula; LPS, lipopolysaccharide; NEC, necrotising enterocolitis; PARP-1, poly(ADP-ribose) polymerase 1; PsIc1, porcine intestinal epithelia cells; TEN, total enteral nutrition.

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animal and clinical studies in the future.

appropriate range of bLF doses that can be selected for further

as well as the mediation of ERK, NF-κB on cell proliferation, pro-inflammatory cytokine release

pig model of NEC(26) to study the effects of bLF supplemen-

which may benefit the immature intestine in preterm infants.

Several studies have indicated that bLF attenuates lipopolysac-
charide (LPS)-induced inflammatory cytokines such as IL-8,
IL-6, IL-1β and TNF-α in monocyctic cells(19) and IL-8 in Caco-2

However, lactoferrin stimulates in vitro cell proliferation(17,18),

that of this protein is its high Fe-binding affinity (approximately

Importantly, bLF and human lactoferrin share 77 % amino

acid homology and have similar structures(24). As a conse-
quence, their biological activities are similar; for instance,

they have comparable in vitro anti-microbial activities(16).

Therefore, bLF could be a key component in protecting new-
born infants from diseases in early life. For instance, oral

administration of bLF at a dose of 240–400 mg/kg per d
defeated positive effects in vitro against intestinal diseases

such as reducing the incidence of dextran sulphate sodium-

induced colitis in rats and attenuating inflammatory cytokines
(IL-6, TNF-α and IL-1β)(25). In addition, administration of bLF

at a dose of 100 mg/d to preterm neonates for the first 30 d of

administration before placing vascular catheters (4F, Portex;,

maintained at 37°C, 80–100 % humidity and a suitable oxygen

administration and orogastric feeding tubes (6F, Portex;,

Smiths Medical). Pigs were then provided with maternal serum

of 4 ml/kg after catheterisation, and subsequently 5 ml/kg after

12 h and 7 ml/kg after 20 h to provide passive immunological

protection. Pigs were stratified into two groups according to

sex and weight with two types of enteral nutrition: control formu-

lma CON, n 15) and 10 g/l of bLF-enriched formula (LF, n

13). The two formulas were made by mixing various commercial

ingredients to meet the optimal macronutrient and energy

requirements for preterm pigs(26,27); Lacprodan DI-9224 (Arla

Foods Ingredients); Ross Polycose (Abbott Nutrition); SHS Sera-

vit, Liquigen Medium-chain TAG and Calogen Long-chain TAG

(Nutricia); with or without bLF (Morinaga Milk Industry). The

lactoferrin used was bLF with 15 % Fe saturation and a low

endotoxin content (1-6 endotoxin units/mg protein) detected

by the Limulus Amebocyte Lysate Chromogenic Endotoxin

Quantitation Kit (Fisher Scientific). The two formulas had similar

composition except for the presence (LF) or absence (CON) of

bLF, and they were adjusted to an equal intake of energy and

macronutrients (Table 1). During the first 2 d, pigs were adminis-

tered parenteral nutrition through their umbilical catheters at

rates of 4 and 6 ml/kg per h for days 1 and 2, respectively,

supplemented with either the CON or LF formula as minimal enteral

nutrition with doses of 3 ml/kg per 3 h at day 1 and 5 ml/kg per

3 h at day 2. Parenteral nutrition was based on Kabiven (Fre-

niius Kabi) and adjusted nutrient composition to meet the

requirements of pigs with an energy of 3210 kJ/l and a macronu-

trient composition of 36 % fat, 24 % protein and 40 % carbohydrates.

From day 3, parenteral nutrition was stopped, and the pigs were fed either the CON or LF formula as TEN administered at a dose of 15 ml/kg per 3 h for another 2 until euthanasia and tissue collection on day 5. The animal

protocol was approved by the Danish National Committee on

Materials and methods

Animal experiments

A total of twenty-eight preterm pigs from four sows were delivered by caesarean section (Large White × Danish Landrace × Duroc, Askelygaard Farm, Roskilde, Denmark) at 92 % of gestational age (105 d, term 116 (SEM 2) d). After delivery, pigs were immediately transferred to individual incubators maintained at 37°C, 80–100 % humidity and a suitable oxygen administration before placing vascular catheters (4F, Portex) in the umbilical artery and orogastric feeding tubes (6F, Portex;,

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Table 1. Macronutrient composition of the formulas used for enteral feeding in the control group (CON) and the lactoferrin-enriched formula group (LF)*

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ/l)</td>
<td>4508</td>
<td>4508</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>79</td>
<td>69</td>
</tr>
<tr>
<td>bLF (g/l)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Carbohydrates (g/l)</td>
<td>62.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Maltodextrin (g/l)</td>
<td>47.5</td>
<td>47.5</td>
</tr>
<tr>
<td>Lactose (g/l)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Fat (g/l)</td>
<td>57</td>
<td>57</td>
</tr>
</tbody>
</table>

bLF, bovine lactoferrin.

*These were calculated based on macronutrient analysis from suppliers.
Animal Experimentation, and it has been described in detail previously\(^{26,27}\).

**Clinical evaluation and sample collection**

Every 3 h from birth to euthanasia, pigs were evaluated for NEC symptoms including bloody diarrhoea, abdominal distension, lethargy and cyanosis. Pigs with severe symptoms were euthanised immediately for tissue collection. Following euthanasia, the stomach, three regions of the small intestine (proximal, middle and distal) and the colon were macroscopically evaluated for NEC severity according to our standard scoring system: 1 = no or minimal abnormality; 2 = mild focal gastroenterocolitis; 3 = moderate locally extensive gastroenterocolitis; 4 = severe focal gastroenterocolitis; 5 = severe locally extensive haemorrhage and NEC; 6 = severe extensive haemorrhage and NEC. Scores above 2 in any of the five gastrointestinal regions were considered as positive for NEC. After euthanasia, individual organs including the heart, lungs, liver, kidneys, spleen, stomach, small intestine and colon were weighed. The length of the small intestine was measured at a relaxed stage. From the middle of each intestinal region, five pieces of whole-wall intestinal tissue samples were taken and immediately snap-frozen in liquid N\(_2\) and stored at \(-80^\circ\text{C}\) for further analyses of enzymatic activities and cytokines. Another two samples of intestinal tissue from each region were stored in parafomaldehyde solution for histology. In addition, 10 cm segments of each intestinal region were sampled and slit longitudinally for the measurement of circumference and mucosal proportion. The mucosa was gently scraped to determine the proportion both wet weight and dry weight as described previously\(^{26,27}\).

**In vivo sugar absorption and intestinal permeability tests**

**In vivo** sugar absorption and intestinal permeability tests were conducted as described previously\(^{28}\) with modifications. Plasma levels of galactose in response to oral boluses of galactose and lactose solutions on days 3 and 4, respectively, were analysed to measure the digestive and absorptive function of the gut. Blood samples were collected before and 20 min after an oral bolus (15 ml/kg) of 5% galactose for the galactose test on day 3 before conversion to TEN, and before and 20 and 40 min after an oral bolus (15 ml/kg) of 10% lactose for the lactose test 30 h after conversion to TEN. For the assessment of intestinal permeability, 3 h before euthanasia, an oral bolus (15 ml/kg) of 5% lactulose and 5% mannitol solutions was given to pigs and post-mortem urine samples were collected at euthanasia. Permeability was quantified by the ratio of lactulose:mannitol concentrations in the urine as reported elsewhere\(^{28}\).

**Intestinal morphology, functions and tissue cytokine analyses**

Formaldehyde-fixed tissues from the proximal and distal small intestine and the colon were embedded in paraffin, transversely sectioned (5 \(\mu\)m) and stained with haematoxylin and eosin for measurements of villus height and crypt depth\(^{26}\). Gut morphology including villus height and crypt depth was quantified from scanned photos using ImageJ software (National Institute of Health). For each pig, three representative cross-sections in each region were sampled and ten representative measurements of villus height and crypt depth were made. Intestinal brush-border enzymes, including sucrase, lactase, maltase, aminopeptidase A, aminopeptidase N and dipeptidyl-peptidase IV, were analysed according to previous protocols\(^ {26}\) and are expressed as U/g wet tissue. The cytokine levels of IL-8 and IL-1\(\beta\) in the proximal and distal small intestine and the colon were quantified using ELISA following tissue homogenisation as described previously\(^ {29}\). The levels of cytokines are expressed as pg/mg wet tissue.

**Cell culture, cell proliferation and cytokine analysis**

Psc1 intestinal epithelial cells from 6-month-old weaning pigs (Bionutritech) were cultured in advanced Dulbecco’s modified Eagle’s medium with supplementation of 2% heat-inactivated fetal bovine serum, 40 U penicillin/ml, 40 \(\mu\)g streptomycin/ml and 2 mM-glutamine (Life Technologies) at 37 \(^\circ\)C and 5% CO\(_2\). Cells were used at passage 5–25. bLF was prepared by dissolving in serum-free DMEM medium and sterile filtered (0·2 \(\mu\)m) (Millipore).

For the cell proliferation assay, cells were seeded in ninety-six-well plates (5 \(\times\) 10\(^3\) cells/well) and allowed to adhere for 24 h before treatments with bLF (0–20 g/l) for another 48 h. For assessment of the effects of ERK, cells were pre-incubated with the mitogen-activated/extracellular signal-regulated kinase (MEK) 1/2 inhibitor PD98059 (70 \(\mu\)M; Sigma-Aldrich) for 1 h before the treatments with bLF (0–10 g/l) for 48 h. Cell proliferation was quantified by the Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s instructions. For cytokine analysis, cells were cultured until reaching 90% confluence in twenty-four-well plates and then serum-starved for 24 h. The cells were then incubated with bLF (0–10 g/l) and/or DPS (0·1 \(\mu\)g/ml) (from Escherichia coli O26:B6; Sigma-Aldrich) for 24 h. Thereafter, supernatants were collected for the analysis of IL-8 and IL-6 by ELISA, following the manufacturer’s instructions (R&D Systems). Cytokine concentrations were normalised to the total intracellular protein content (ng/mg protein). After the treatments and supernatant collection, cell lysates were taken and analysed using the BCA Protein Assay Kit (Fisher Scientific) for the total protein content.

**Intracellular signalling by Western blot**

Western immunoblotting was conducted as indicated previously\(^ {10}\) with minor modifications. Cells at 90% confluence were serum-starved for 24 h before stimulation with bLF at a dose of 0.1, 1 or 10 g/l for different time periods (5–90 min). Following the treatments, cells were lysed in radio-immuno-precipitation assay lysate buffer containing 1 \(\times\) protease and phosphatase inhibitors (Fisher Scientific). Proteins in lysates (10 \(\mu\)g) were separated by SD–PAGE using 12.5% Tris–Glycine gels in non-reducing conditions before electro-transfer onto polyvinylidene difluoride membranes (Life Technologies)\(^ {10}\). Membranes were then probed with...
anti-IkB-α, anti-phospho-ERK, anti-HIF-1α and anti-poly
(ADP-ribose) polymerase 1 (PARP-1) antibodies (Santa Cruz)
to detect the levels of intracellular inhibitor IκB-α (IκB-α), phos-
phorylated ERK 1/2, HIF-1α and PARP-1 cleavage following the
bLF treatment. In order to confirm the equal protein loading in
each lane, membranes were stripped and re-probed to detect the
total ERK or β-actin as the loading control. For the detection
of NF-κB/p65, cells were treated with bLF as described above.

The incidence of NEC was 60% (n 9/15 pigs) in the CON group
compared with 62% (n 8/13 pigs) in the LF group, with no
difference in either NEC incidence or NEC severity (Fig. 1(a)).
Within the subgroup of pigs that developed NEC, the degree
of NEC severity in the colon was greater in the LF pigs than
in the CON pigs (Fig. 1(b)). Average NEC severity scores for
both the whole gastrointestinal tract and the small intestine of
NEC pigs tended to be greater in the LF pigs than in the CON
pigs (P=0.07 and 0.08, respectively). The two groups did not
differ in body weight at birth (965.2 (SEM 8.9) g), body-weight
gain (−3.4 (SEM 2.0) g/d) and the relative weights (g/kg body
weight, pooled values) of lungs (24.3 (SEM 0.4)), liver (30.1
(SEM 1.1)), kidneys (10.6 (SEM 0.3)), stomach (9.2 (SEM 0.7))
and large intestine (16.2 (SEM 0.7)) (Table 2). Only the relative
heart weight was greater in the LF pigs.

Mucosal structure, digestive functions and
cytokine levels

The dry mucosal proportion (Table 2), villus height and crypt
depth (data not shown) were similar in the CON and LF pigs.
When considering healthy pigs, the crypt depth of the proximal
regions was greater in the LF pigs (88.6 (SEM 6.0) μm)
than in the CON pigs (80.9 (SEM 3.6) μm, P<0.05) (Fig. 2).
All the analysed activities of the brush-border membrane
enzymes across the three small-intestinal regions did not
differ between the two groups (Supplementary Fig. S1, avail-
able online). Sugar absorption measured by a galactose and
a lactose test did not differ either. In NEC pigs only, intestinal
permeability was greater in the LF pigs than in the CON pigs
(P<0.05; Fig. 3). In contrast, higher levels of IL-1β were
observed in the proximal small intestine of the CON pigs
than that of the LF pigs (P<0.05; Fig. 4(a)). In addition,
pooled IL-1β contents of the two groups were greater in the
colon than in the distal small intestine (P<0.05; Fig. 4(a)).
Conversely, there were no differences in the levels of IL-8
between the two groups in both the proximal and distal
small intestine as well as in the colon (Fig. 4(b)).

Cell proliferation

bLF exerted dose-dependent effects on cell proliferation in vitro
(Fig. 5). At low doses (0·01 and 0·1 g/l), bLF stimulated cell
proliferation, while at excessive doses (10–20 g/l), it
decreased cell proliferation, when compared with the control
(no treatment, P<0.01) (Fig. 5(a)). The addition of the MEK 1/2
inhibitor PD98059 before the treatments, which also inhibits
the phosphorylation of ERK 1/2, prevented the proliferative
effects induced by bLF at the 0·1 g/l dose (P<0.05; Fig. 5(b)).

Cytokine secretion modulated by bovine lactoferrin
and lipopolysaccharide

PsIcl cells did not respond to LPS for the secretion of IL-8
(Fig. 6(a)), whereas at doses of 1–10 g/l, bLF stimulation
induced IL-8 release. In addition, co-stimulation of bLF and
LPS did not differ from the bLF treatments alone. IL-6 release
was increased by LPS alone or by bLF at low concentrations

Statistical analysis

All data are reported as means with their standard errors.
Differences in NEC incidence between the two groups were
tested using a χ² test (JMP 9; SAS Institute). Continuous data
in the animal study were analysed by a linear mixed model
using diet and region (when applicable) as fixed factors and
sow and pig as random factors. All cell studies were per-
formed in triplicate or quadruplicate. Comparisons between
groups were performed using one-way ANOVA and Tukey’s test
in GraphPad Prism 5.0 (GraphPad Software, Inc.). Signifi-
cance was considered at P<0.05. P values in the range of
0.05–0.1 were considered as non-significant trends.

Results

Clinical assessments

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was increased by LPS alone or by bLF at low concentrations
No differences in IL-6 secretion between LPS and LPS + bLF (0.01–1 g/l)-treated cells were observed (Fig. 6(b)). In contrast, at a dose of 10 g/l, bLF attenuated LPS-induced IL-6 release ($P$, 0.05).

Intracellular signalling regulated by bovine lactoferrin

bLF treatments affected the activation of the ERK pathway in a dose-dependent manner. bLF treatments at doses of 0.1–1 g/l increased ERK activation observed by the phosphorylation of ERK 1/2 (15 min for bLF at 0.1 g/l and 30 min for bLF at 1 g/l compared with the control before gradually decreasing to the control levels after 60–90 min, $P$, 0.05; Fig. 7(a), (b) and (d)). However, no changes in phosphorylated ERK 1/2 occurred after the treatment with bLF at the dose of 10 g/l (Fig. 7(c) and (d)). At higher doses (10 g/l), bLF did not activate ERK and did not stimulate cell proliferation compared with the control. bLF stimulation at doses of 1 and 10 g/l, but not at 0.1 g/l, caused the cleavage of PARP-1 ($P$, 0.05) to form a 50 kDa fragment (a marker of necrosis$^{31,32}$). No fragment of 89 kDa (a marker of apoptosis$^{31,32}$) from PARP-1 was formed following bLF stimulation at all the doses tested.

We also studied the role of bLF in the regulation of NF-$\kappa$B signalling. I$\kappa$B-a is a protein bound to NF-$\kappa$B, which prevents the translocation of NF-$\kappa$B from the cytoplasm into the nucleus$^{33,34}$. Once I$\kappa$B-a is degraded, NF-$\kappa$B is released and subsequently translocated into the nucleus to act as a transcription factor to induce cytokine expression. Fig. 8 illustrates a dose-dependent effect of bLF on NF-$\kappa$B activation. I$\kappa$B-a was stable at the bLF dose of 0.1 g/l, slightly decreased after 60–90 min at the bLF dose of 1 g/l and was degraded within 30 min after the bLF treatment at the dose of 10 g/l (Fig. 8 (a)–(c) and (e)). These results suggest that only at bLF doses of 1 and 10 g/l, I$\kappa$B-a degradation will occur and may cause NF-$\kappa$B translocation, and that the bLF dose of 10 g/l may activate NF-$\kappa$B more rapidly than that of 1 g/l. Therefore, these doses were chosen to confirm the levels of NF-$\kappa$B/p65 (p65) in the cell nucleus following stimulation (Fig. 8(d) and (f)). Fig. 8(d) shows that p65 was translocated into the nucleus within 15–30 min and then decreased gradually. Nuclear p65 levels were up-regulated faster and

![Fig. 2. (a) Villus height, (b) crypt depth and (c) villus:crypt ratio in the proximal and distal small intestine of pigs. Only healthy proximal and distal sections (necrotising enterocolitis (NEC) score 1 or 2) were considered. □ Control pigs, ▪ lactoferrin-enriched formula pigs. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P$, 0.05).](https://www.cambridge.org/core/static/b916a0e00815712b7b00a720e5a063f0)
more intensely with the bLF dose at 10 g/l than at 1 g/l (P<0.05; Fig. 8(d) and (f)).

Regarding the HIF-1α signalling pathway, only the bLF dose at 10 g/l led to the up-regulation of HIF-1α levels, compared with the control (no treatment) and lower bLF doses (Fig. 8(g) and (h)).

Discussion
Various bioactive milk components, which are deficient in mature milk and infant formulas, may contribute to the protective effects of human and bovine colostrum against NEC(7,8,35). Supplementation of bioactive proteins at doses similar to those in human or bovine colostrum might exert prophylaxis against inflammation, and may be important for the development of more effective infant formulas for preterm infants. We hypothesised that a high lactoferrin content in human colostrum is one of several important factors necessary to provide protection against NEC development in preterm infants. Moreover, based on the high degree of amino acid homology between human lactoferrin and bLF, a bLF-enriched formula may exert effects similar to those of human lactoferrin. We investigated this in our preterm pig model by administering 10 g/l of bLF-enriched formula, close to the lactoferrin concentration in human colostrum and equivalent to 1·2 g/kg per d in the period of TEN. This dose was greater than those used in previous studies which showed negligible effects against NEC(24,25) and, consequently, was hypothesised to exert stronger protective effects against NEC. Enteral feeding of a 10 g/l of bLF-enriched formula did not, however, decrease the sensitivity of the pigs to develop NEC. At this dose, bLF appeared to exacerbate disease severity in those pigs that developed NEC compared with CON pigs, as greater NEC severity and intestinal permeability were induced in the LF pigs. In addition, the LF pigs showed a tendency of a less mature gut with a lower small-intestinal weight than the CON pigs. In contrast, an important effect of bLF was the lower levels of IL-1β observed in the proximal small intestine of LF pigs. This may be explained by the ability of bLF to bind LPS derived from intestinal bacteria and thereby restrict LPS in inducing IL-1β release from enterocytes and immune cells(39). bLF may act in a similar manner to limit IL-8 release; however, bLF itself can also activate the NF-κB pathway and stimulate the release of IL-8 from enterocytes, as shown in the present in vitro data.

Therefore, two contrary effects of bLF on IL-8 release may cause similar levels of IL-8 in the intestinal tissues of CON and LF pigs. Lower doses of bLF administration could be beneficial to the immature intestine exerting bLF–LPS binding and also limiting excessive amounts of unbound bLF from stimulating unnecessary immune responses leading to a high release of IL-8.

To our knowledge, the present animal study is the first study to investigate the effects of bLF against NEC in preterm pigs at a dose close to that present in human colostrum. In germ-free newborn pigs, a high dose of bLF (20 g/l of bLF in formulated pigs every 4 h) evoked protective effects against lethal shock induced by endotoxin(30). The opposing effects of bLF demonstrated in the present study may be explained by both

Fig. 3. Nutrient absorption and intestinal permeability in pigs: arterial plasma levels of galactose following (a) a given oral bolus of 5% galactose (15 ml/kg) for 20 min or (b) an oral bolus of 10% lactose (15 ml/kg). (c) Ratio of 5% lactulose:5% mannitol (15 ml/kg) in urine samples after 3–5 h of an oral bolus of lactulose and mannitol before euthanasia. [Control pigs; lactoferrin-enriched formula pigs. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of the control group (P<0.05). NEC, necrotising enterocolitis.]

Fig. 4. IL-1β and IL-8 levels in the proximal and distal small-intestinal tissues and the colon tissues. [Control pigs; lactoferrin-enriched formula pigs. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of the control group (P<0.05). †Pooled mean of the two pig groups was significantly different from that for the distal region (P<0.05).]
Effects of lactoferrin on the intestine

Dose-dependent effects of bovine lactoferrin (bLF) in vitro on cell proliferation regulated by extracellular signal-regulated kinase (ERK). (a) Cells were stimulated with bLF (0.01–20 g/l) in serum-free medium for 48 h. (b) Cells were treated with the ERK inhibitor PD98059 (75 μM) or vehicle (dimethylsulphoxide) for 1 h before stimulation with bLF (0–10 g/l) in either serum-free medium or serum medium for 48 h. Values are absorbance at (dimethylsulphoxide) for 1 h before stimulation with bLF (0–10 g/l) in either serum-free medium or serum medium plus vehicle. (b) Serum-free medium or serum medium for 48 h. Values are absorbance at (dimethylsulphoxide) for 1 h before stimulation with bLF (0–10 g/l) in either serum-free medium or serum medium plus vehicle. (b) Mean value was significantly different from that of the vehicle (Fig. 6).

Fig. 5. Dose-dependent effects of bovine lactoferrin (bLF) in vitro on cell proliferation regulated by extracellular signal-regulated kinase (ERK). (a) Cells were stimulated with bLF (0.01–20 g/l) in serum-free medium for 48 h. (b) Cells were treated with the ERK inhibitor PD98059 (75 μM) or vehicle (dimethylsulphoxide) for 1 h before stimulation with bLF (0–10 g/l) in either serum-free medium or serum medium for 48 h. Values are absorbance at (dimethylsulphoxide) for 1 h before stimulation with bLF (0–10 g/l) in either serum-free medium or serum medium plus vehicle. (b) Mean value was significantly different from that of the vehicle (Fig. 6).

Fig. 6. Dose-dependent effects of bovine lactoferrin (bLF) (0–10 g/l) on lipopolysaccharide (LPS) (0.1 μg/ml)-induced pro-inflammatory cytokine secretion: (a) IL-8 and (b) IL-6. Values are means (n 3), with their standard errors represented by vertical bars. (a) Mean value was significantly different from that of the control (no treatment) (P<0.05).

A direct comparison

administered at lower doses of bLF (0·24–0·4 g bLF/kg per d(25)) and 0·1 g bLF/d(24) together with our observations of the detrimental effects of bLF at a higher dose (1·2 g/kg per d) against NEC, but a positive effect on the IL-1β reduction led to the hypothesis that the protective effects of bLF against NEC and inflammation may be dose-dependent. For this purpose, different doses of bLF were tested in vitro. The present results illustrate that bLF interacts with intestinal epithelial cells in a dose-dependent manner between bLF concentrations of 0·1, 1 and 10 g/l. These amounts of bLF are similar to those found in bovine term milk, bovine colostrum/human term milk and human colostrum, respectively(9–11). Lactoferrin has been reported to be stable during gastrointestinal digestion in infants(10,58) and therefore its major proportion from enteral feeding will be present in the gut lumen to interact with enterocytes. Levels of bLF administered during enteral feeding will, however, not be equivalent to the concentrations finally interacting with the epithelium due to dilution of the administered dose by gastric and pancreatic secretions. However, in some locations of the small intestine, a large amount of water and solutes are absorbed by passive or active transport(59), which will result in higher concentrations of bLF. Therefore, the range of 0·1–10 g/l of bLF doses tested in vitro was reasonable for mechanistic investigation. In the present study, low doses

gut prematurity and colonisation. The morphology of the immature gut and the immune system of preterm pigs may lead to different immune responses and inflammatory cascades under the presence of bLF at high doses (10–20 g/l), compared with that in term pigs. Therefore, the amounts of bioactive protein supplemented to infant formulas may be very crucial depending on whether it is administered to term or preterm infants. An oral administration of bLF decreased dextran sulphate sodium-induced colitis, inflammatory lesions, degree of colon shortening, as well as attenuated pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α in 7-week-old rats when administered at lower doses of bLF (0·24–0·4 g bLF/kg per d)(25) than that used in the present study. A direct comparison of the results is, however, difficult due to the differences in species and the degree of prematurity. Although the dextran sulphate sodium model of colitis correlates in several parameters with human inflammatory bowel disease, it does not reflect the intestinal inflammatory state that we observe in preterm pigs that developed NEC spontaneously in response to enteral feeding(16,37). In another study, bLF (0·1 g bLF/d) given to low-birth-weight infants prevented late-onset sepsis, but not NEC(24). However, when a bLF dose of 100 mg/d was administered together with Lactobacillus rhamnosus GG, an attenuation of NEC incidence was observed(24). This suggests that bLF may influence the gastrointestinal tract in synergistic ways with other bioactive components, such as probiotics.

The earlier studies showing the prophylaxis of bLF against dextran sulphate sodium-induced colitis and sepsis but not NEC (0·24–0·4 g bLF/kg per d)(25) and 0·1 g bLF/d(24) together with our observations of the detrimental effects of bLF at a higher dose (1·2 g/kg per d) against NEC, but a positive effect on the IL-1β reduction led to the hypothesis that the protective effects of bLF against NEC and inflammation may be dose-dependent. For this purpose, different doses of bLF were tested in vitro. The present results illustrate that bLF interacts with intestinal epithelial cells in a dose-dependent manner between bLF concentrations of 0·1, 1 and 10 g/l. These amounts of bLF are similar to those found in bovine term milk, bovine colostrum/human term milk and human colostrum, respectively(9–11). Lactoferrin has been reported to be stable during gastrointestinal digestion in infants(10,58) and therefore its major proportion from enteral feeding will be present in the gut lumen to interact with enterocytes. Levels of bLF administered during enteral feeding will, however, not be equivalent to the concentrations finally interacting with the epithelium due to dilution of the administered dose by gastric and pancreatic secretions. However, in some locations of the small intestine, a large amount of water and solutes are absorbed by passive or active transport(59), which will result in higher concentrations of bLF. Therefore, the range of 0·1–10 g/l of bLF doses tested in vitro was reasonable for mechanistic investigation. In the present study, low doses

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of bLF (0–1 g/l) stimulated cell proliferation through the activation of the ERK pathway, preventing IL-8 secretion and inhibited the activation of both NF-κB and HIF-1α. In contrast, at higher concentrations (1–10 g/l), bLF exerted a suppression of cell growth and inhibited ERK activation, leading to IL-8 secretion and NF-κB and HIF-1α activation.

Similar results from low doses of bLF (0.4 g/l) on intestinal cell proliferation have been observed previously, which were also mediated through ERK activation. A dose-dependent effect has also been reported for concentrations of bLF at the lower range of 0.1–1000 μg/ml. Additionally, a greater stimulatory effect has been observed for apo-bLF on proliferation. Moreover, the increase in cell proliferation following stimulation with bLF at doses of 0.1–1 g/l and the suppressive effect of the inhibitor PD98059 on cell proliferation at the bLF dose of 0.1 g/l suggest that ERK 1/2 activation is involved in cell proliferation induced at low doses of bLF. This effect implies that at appropriate levels, bLF is likely to stimulate the maturation of the small intestine in newborn infants through ERK 1/2. The activation of ERK 1/2, a member of mitogen-activated protein kinase pathway family, has been extensively correlated with cell proliferation in various studies, and these results also support this involvement. In contrast, at a higher dose (10 g/l), bLF did not activate ERK 1/2, and a lower rate of cell proliferation than in control cells was observed, indicating that cell death may occur. The generation of the 50 kDa fragment from PARP-1 observed in the present study suggests that bLF concentrations between 1 and 10 g/l stimulate cell necrosis, but not apoptosis, and these results agree with the observation of poor cell proliferation at the bLF dose of 10 g/l.

Data from both the in vivo pig study and also from the in vitro cell proliferation studies raised questions whether high bLF doses led to a negative impact and excessive inflammation in preterm neonates. Several studies have indicated that inflammation and NEC are related to high levels of inflammatory cytokines such as IL-8 and IL-6 in the gut. Therefore, we tested the release of IL-8 and IL-6 following stimulation with both LPS and bLF in vitro. LPS stimulation of PsIC1 cells induced IL-6 but not IL-8 secretion. bLF treatments caused changes in the levels of IL-6, but at the dose of 10 g/l, it attenuated LPS-induced IL-6 release. bLF may in this way bind to LPS and thereby limit the inductive effect of LPS on IL-6. This may also explain the greater levels of IL-1β observed in the proximal small intestine of the group CON group compared with the LF group in the pig study. The lack of changes in the levels of IL-8 between the LPS and LPS + bLF treatments illustrates that bLF itself may be the main factor for IL-8 release in the range of 1–10 g bLF/l. On the other hand, some bioactive proteins decreased LPS-induced IL-8 release in the more LPS-sensitive cell line.
Dose-dependent effects of bovine lactoferrin (bLF) on the activation of NF-κB and hypoxia-inducible factor-1α (HIF-1α). Cells were stimulated with bLF at a dose of (a) 0.1, (b) 1 or (c) 10 g/l for the time periods indicated, and cell lysates were blotted using the inhibitor of NF-κB (IκB-α) antibody (d) Cells were treated with bLF at a dose of 1 or 10 g/l for 15–90 min, and then nuclear extracts were blotted with the NF-κB/p65 antibody. Quantitative analysis by the densitometry of representative (e) IκB-α, (f) NF-κB/p65 and (h) HIF-1α bands normalised relative to the control (no treatment), which is assigned an arbitrary unit of 1.

Effects of lactoferrin on the intestine

HIF-1α and NF-κB activation by bLF itself. NF-κB in the intestine of the LF pigs in an excessive manner, thereby causing more severe inflammation rather than protecting them against NEC. Our findings regarding the activation of NF-κB following stimulation with bLF are in accordance with several studies performed in neutrophils. In contrast, some studies have shown that bLF may exert anti-inflammatory effects by attenuating LPS-induced IL-8 or TNF-α, or inhibit NF-κB, TNF receptor-associated factor 6 (TRAF-6) and p38 pathways induced by LPS. These studies were conducted at bLF concentrations below 1 g/l, which may be the reasons for no observed negative effects such as NF-κB activation by bLF itself.

The up-regulation of HIF-1α is normally observed under hypoxic conditions and during Fe deficiency, which limit the
automatic degradation of HIF-1α. Up-regulated HIF-1α stimulates the transcription of a multitude of genes involved in cell survival under stress conditions\(^{(53)}\). The apo-form of bLF has been found to bind intracellular free Fe in rats, thereby subsequently stabilising HIF-1α\(^{(22,23,51)}\). Stabilisation or up-regulation of HIF-1α was indicated as a possible therapy for intestinal bowel disease due to the promotion of barrier functions\(^{(53)}\). However, this protein has been correlated with hypoxic inflammation, and it is up-regulated as a consequence of NF-κB and cyclo-oxygenase-2 activation induced by IL-1β.\(^{(22)}\) HIF-1α is also activated by cell stimulation with TNF-α or NO, and it participates in several inflammatory responses\(^{(22)}\). Therefore, an up-regulation of HIF-1α and NF-κB after the bLF treatment at a dose of 10 g/l, as shown in the present study, can be detrimental to the cells, for instance causing cell necrosis, inhibiting cell proliferation and releasing high levels of IL-8. To our knowledge, this is the first time bLF has been shown to exert a dose-dependent effect on HIF-1α up-regulation in intestinal cells. In the future, the crosstalk and synergistic effects of HIF-1α and NF-κB could be interesting to pursue to clarify the roles of these pathways mediated by bLF.

Overall, this preterm pig study indicates that a bLF dose of 10 g/l lowers IL-1β levels in the proximal small intestine, but does not protect preterm pigs against NEC. bLF at this dose tends to exacerbate NEC severity and decrease intestinal functions by lowering small-intestinal weight and increasing intestinal permeability in pigs that develop NEC. In combination with the in vitro experiments exploring a range of bLF doses, the present study suggests that only lower doses of lactoferrin may potentially be beneficial for preterm neonates to dampen the inflammatory mediators. Consequently, the supplementation of infant formulas with appropriate levels of bLF should be more carefully studied before its usage for preterm infants. Further pig studies are necessary to investigate the effects of various bLF doses before concluding any protective effects of this protein in vivo.

Supplementary material
To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513002456

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