Immunomodulatory properties of human recombinant lactoferrin in mice: Implications for therapeutic use in humans

Jolanta Artym[1,C,E,F], Maja Kocięba[1,B,E], Ewa Zaczyńska[1,B,C,F], Barbara Adamik[2,B,C,E,F], Andrzej Kübler[2,B,E], Michał Zimecki[1,A–F], Marian Kruzel[3,A,F]

[1] Laboratory of Immunobiology, Institute of Immunology and Experimental Therapy, Wrocław, Poland
[2] Department of Anesthesiology and Intensive Therapy, Wroclaw Medical University, Poland
[3] University of Texas, Houston Medical School, USA

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Address for correspondence
Michał Zimecki
E-mail: zimecki@iitd.pan.wroc.pl

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Abstract

Background. Trauma and major surgery cause extensive immune hyporeactivity in patients. Thus, the preventive, preoperative application of immunoregulatory therapeutics may normalize this immune reactivity and decrease morbidity and mortality in these subjects.

Objectives. The aim of this study was to investigate the immunomodulatory actions of recombinant human lactoferrin (rhLF) in mice, and to relate these effects to in vitro actions of rhLF on tumor necrosis factor alpha (TNF-α) production in lipopolysaccharide-stimulated whole blood cell cultures (LPS-stimulated WBCC) from patients admitted to intensive care units.

Material and methods. BALB/c and CBA mice were used. rhLF was tested for allergic response to ovalbumin (OVA), delayed-type hypersensitivity (DTH) to OVA, and carrageenan-induced inflammation in an air pouch. Blood samples from 30 patients diagnosed with severe sepsis/septic shock (Apache II 21 ±1, mortality rate 40%) were collected on days 1, 3 and 5 of observation. The effects of rhLF on LPS-induced TNF-α production were measured in WBCCs.

Results. Recombinant human lactoferrin reduced the parameters of OVA-induced inflammation and inhibited the elicitation phase of DTH and carrageenan-induced inflammation in mice. The majority of patients from whom whole blood cell cultures (WBCC) were established showed a strong hyporeactivity to LPS upon admission. rhLF exerted differential effects on the production of LPS-induced TNF-α in those cultures on days 1, 3 and 5 of observation. Cytokine production was upregulated only in patients with sustained anergy to LPS, and inhibited or unchanged in moderately reactive patients.

Conclusions. Evidence for the potential preventive or therapeutic utility of rhLF in patients with impaired immune reactivity has been demonstrated.

Key words: human recombinant lactoferrin, mice, pleurisy, septic patients, tumor necrosis factor alpha

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Introduction

Trauma and major surgical interventions cause extensive immune hyporeactivity in patients at all ages. Immune dysfunction may further provoke organ failure and lead to the development of septic conditions in some patients. This is often heralded by depressed ability to produce lipopolysaccharide (LPS)-inducible cytokines in blood cultures.[1] The response of individual patients to clinical insult may vary considerably, being either excessive or insufficient, and both types of reactions may be harmful and may lead to postoperative complications.[2] Unfortunately, preventive measures regarding postoperative complications have not yet been implemented into clinical practice. Therefore, the complex immunological monitoring of patients elected for surgery is strongly recommended, as part of a personalized therapy to prevent sepsis.[3] Alternatively, the preventive, preoperative application of immunoregulatory therapies, which can normalize the immunological temperament of patients, would be an attractive approach to decrease morbidity and mortality in patients undergoing surgery.

Lactoferrin (LF), a protein involved in iron metabolism, represents a key element of the innate immunity in mammals. LF is contained in 2 reservoirs: the excretory fluids and the secondary granules of neutrophils.[4] It exhibits a wide array of immunological activities in vitro and in vivo.[5] The concentration of LF in circulation may increase several-fold during sepsis, trauma, hemodialysis, or extracorporeal circulation, which classifies LF as an acute-phase protein.[6–9] The strategies for preventive and/or therapeutic systemic or local applications of LF have already been proven effective in clinical trials on several categories of patients, including septic ones.[10–13]

In our earlier studies, we showed that bovine milk-derived LF (bLF) exhibited immunoregulatory in vitro effects on the immune reactivity of blood lymphocytes derived from septic and trauma patients.[14,15] In addition, orally applied bLF appeared to be immunoregulatory with respect to cytokine production in healthy human individuals and ameliorated postsurgical hyporeactivity in patients undergoing thyroid resection.[16,17] In turn, bLF significantly tempered the surgery-induced blood levels of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in mice.[18] Bovine milk-derived LF used in some clinical trials and the rhLFs expressed in yeast or transgenic plants are given orally because of the potential immunogenicity of parenteral administration.[12,13,19–21] Amino acid sequence homology and sugar moiety have to be compatible with the human immune system. In fact, sugar moieties in LF play a crucial role in the interaction with the host's cell receptors.[22,23] Therefore, there is an urgent need for LF that is fully compatible with humans in order to avoid species-specific incompatibilities and to ensure adequate therapeutic effect.

The aim of this report was: 1) to validate the immunomodulatory activity of Chinese hamster ovary (CHO)-expressed recombinant human LF (rhLF) in several mouse in vivo models, including ovalbumin (OVA)-induced pleurisy, carrageenan inflammation in an air pouch and delayed-type hypersensitivity (DTH) to OVA, and 2) to translate these results from mice into human in vitro studies of the immune reactivity of whole blood cell cultures (WBCC) in septic patients admitted to intensive care units (ICU). The effects of rhLF on LPS-induced TNF-α levels in WBC cultures, as measured on days 1, 3 and 5 following a diagnosis of severe sepsis/septic shock in ICU patients was our main criterion in the evaluation of LF as a potential protective measure against sepsis.

Material and methods

Mice

BALB/c and CBA female mice, 8–10 weeks old, were delivered by the Institute of Laboratory Medicine in Łódź, Poland. The mice were fed with commercial, granulated food and water ad libitum. The studies obtained the permission of the Local Ethics Committee from the Institute of Immunology and Experimental Therapy, Wrocław, Poland (No. 2/2011 and No. 57/2012).

Reagents

CHO-derived rhLF was supplied by PharmaReview Corporation (Houston, USA); dexamethasone (Dexaven®) was from Jelfa (Jelenia Góra, Poland); Maalox (*aluminii hydroxydum* 3.5 g and *magnesi hydroxydum* 4.0 g in 100 mL) were from Rhone-Poulenc Rorer Theraplix (Montrouge Cedex, France); AErrane (Isofluranum) was from Baxter (Warszawa, Poland); Freund's complete adjuvant (cFa), Freund's incomplete adjuvant (iFa), and fetal calf serum (FCS) came from BD Biosciences (San Jose, USA); RPMI-1640 medium and Hanks' medium were from CytoGen GmbH (Wetzlar, Germany). Ovalbumin, LPS from *E. coli* (serotype O111:B4), carrageenan, trypan blue, Giemsa, May-Grünwald stains, and all other reagents were acquired from Sigma-Aldrich (St. Louis, USA). Human TNF-α ELISA Ready-Set-Go was provided by eBioscience (San Diego, USA).

Mice in vivo models

Delayed-type hypersensitivity to ovalbumin

CBA mice (6 per group) were sensitized subcutaneously with 5 µg OVA emulsified in cFa at the base of the tail (Fig. 1A). After 4 days, the mice were challenged with 50 µg OVA in iFa (50 µL total) in each of their hind footpads. After 24 h, the footpad thickness was measured using a spring caliper (Swiss Precision Instruments; Garden Grove, USA). rhLF was administered intraperitoneally (i.p.) to mice in doses of 50 µg–5 mg, 30 min after the administration of the eliciting dose of the antigen. The reference

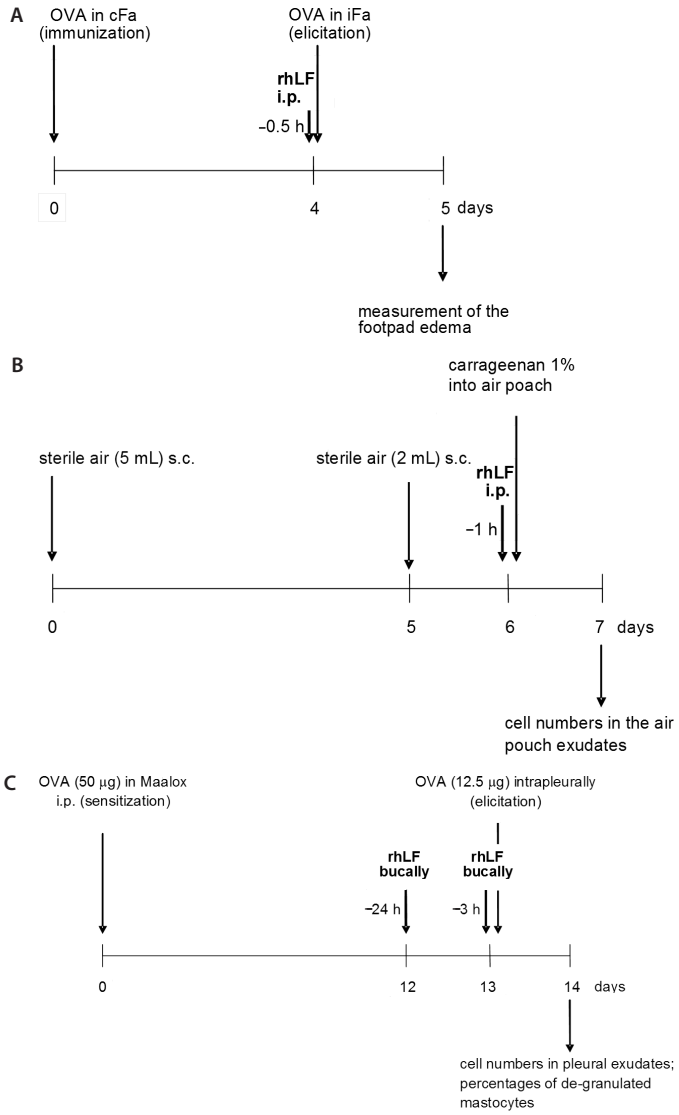

Fig. 1. Experimental design of the tests

A – delayed-type hypersensitivity to OVA; B – carrageenan-induced inflammation in the air-pouch model; C – allergic pleurisy to OVA. See Material and methods section for a description of the experimental protocols.

drug – dexamethasone (Dex) – was given at a dose of 50 µg. Background mice (BG) were not sensitized, but received the challenging dose of OVA in iFa; the value from this non-specific inflammatory response was subtracted from the responses measured in sensitized mice. All results were presented as a mean value of antigen-specific increase of footpad thickness measured in 6 mice per group (12 feet per determination) and expressed in DTH units (1 DTH unit = 0.01 cm) ±standard error (SE).

Carrageenan-induced inflammation in the air-pouch model

An air pouch was formed by subcutaneous injection (s.c.) into the dorsal region of BALB/c mice (7 per group), under halothane anesthesia, of 5 mL of sterile air (23G × $1^1/_4$ needle, 5 mL syringe). On day 5, the air pouch was given an additional 2 mL of sterile air, and an inflammatory process was elicited by the injection of 1% carrageenan in 0.9% NaCl into the air pouches. Recombinant human lactoferrin was administered i.p. at 100 µg, 500 µg, and 2.5 mg doses, 1 h before the injection of carrageenan (Fig. 1B). The mice from the BG group were administered 0.9% NaCl into the air pouches. Control mice were given 0.2 mL of 0.9% NaCl i.p., followed by carrageenan, into the air pouches. Dex was administered i.p. at a dose of 50 µg, 1 h before the injection of carrageenan. On the next day, 1 mL of 0.9% NaCl was injected into the pouches, and the exudates were aspirated with a syringe and transferred into tubes and centrifuges at 800 × g for 5 min. Viable cells in the pellets were enumerated in a light microscope using trypan blue dye.

Generation of the allergic, humoral immune response to ovalbumin

BALB/c mice (7 per group) were immunized i.p. with 50 µg of OVA in 0.2 mL of Maalox (as an adjuvant) (Fig. 1C). After 14 days, the mice were given the eliciting dose of OVA intrapleurally (12.5 µg in 50 µL of 0.9% NaCl), using a syringe with a 2 mm needle. This group of mice is hereafter referred to as the sensitized control mice. Recombinant human lactoferrin (100 µg/dose) was given buccally, using a pipette, in a volume of 25 µL of 0.9% NaCl, 24 h and 3 h before the administration of the eliciting dose of OVA (a total dose of rhLF 200 µg/mouse). These mice are described as the rhLF groups. Control mice were given Dex in a single dose of 20 µg i.p., 3 h before the elicitation of the allergic response (Dex group). Mice treated i.p. with Maalox only (without a sensitizing dose of OVA), but receiving the eliciting dose of OVA intrapleurally, are named the BG group. Twenty-four hours after the elicitation of an allergic pulmonary inflammation, the cell numbers in the pleural cavity were determined. The mice were killed by cervical dislocation. The skin from the abdomen was removed, the chest opened with scissors, and the pleural cavities were washed with 0.2 mL of 0.9% NaCl containing ethylenediaminetetraacetic acid (EDTA) (10 mM) for each cavity. Then, 50 µL of the pleural lavage was taken in order to determine the number of cells. Cell numbers were enumerated in a Bürker hemocytometer. The degree of mastocyte degranulation in the pleural fluid was determined by a histologist at 1000× magnification, following the staining of the cell pellet smears with Giemsa and May-Grünwald reagents.

Human studies (ex vivo)

Patients

Thirty adult patients diagnosed with severe sepsis/septic shock according to the definitions for sepsis and organ failure were consecutively added to the study group.[24]

Table 1. Baseline characteristics of patients under study

Parameter	Patients (n = 30)
Age [years]	64 ±3
Gender M/F	19/11
APACHE II score	21 ±1
SOFA score day 1 day 3 day 5	 9 ±1 8 ±1 7 ±1
PCT [ng/mL] day 1 day 3 day 5	 23.4 ±4.2 19.4 ±5.7 7.1 ±3.2
Reason for ICU admission, n (%) surgical medical trauma	 18 (60) 9 (30) 3 (10)
Diagnosis on admission, n (%) intra-abdominal infection pneumonia other	 14 (47) 13 (43) 3 (10)
Septic shock, n (%)	22 (73)
Length of ICU stay	24 ±4
ICU mortality, n (%)	12 (40)

The results are presented as mean values ±SE. M/F – male/female; APACHE II – Acute Physiology and Chronic Health Evaluation II; SOFA – Sequential Organ Failure Assessment; PCT – procalcitonin; ICU – intensive care unit.

Patients were hospitalized in the mixed ICU at the University Teaching Hospital in Wrocław, Poland. The Ethics Committee of Wroclaw Medical University approved the study protocol (KB-424/214), and the need for informed consent from unconscious patients was waived due to the observational nature of the study. Blood samples for analysis were collected from a blood catheter to tubes with sodium citrate as an anticoagulant at baseline (the day of severe sepsis/septic shock diagnosis), and on days 3 and 5 of treatment. The mean age of the patients was 64 years, with a predominance of males (63%). The main diagnosis on admission was intra-abdominal infection (47%) and pneumonia (43%); 1 patient was diagnosed with a urinary tract infection; 1 with skin and soft tissue infection; and 1 with meningitis. The mean length of ICU stay was 24 days. Septic shock was diagnosed in 73% of patients, and the ICU mortality rate was 40%. Gram-negative pathogens were identified in 16 patients (53%) and Gram-positive ones were found in 14 patients (47%). The clinical status of the patients was assessed with the Acute Physiology and Chronic Health Evaluation II score (APACHE II) upon admission to the ICU, and with the Sequential Organ Failure Assessment score (SOFA) upon inclusion into the study, and on day 3 and 5 of observation. The baseline characteristics of patients are shown in Table 1.

The control group consisted of 7 healthy individuals (22–64 years old) with only a single blood draw for the evaluation of the effect of LF on LPS-induced cytokine production in whole blood cultures. Informed consent from these healthy donors was obtained.

Induction of cytokines in human WBCC

The venous blood samples were diluted 5 times with RPMI-1640 medium within 1 h, and distributed in 1 mL aliquots into 24-well culture plates. Four cultures were established and supplemented as follows: 1) 0.1 mL of the culture medium (control); 2) 100 ng/mL of LPS; 3) 50 μg/mL of LF; and 4) 50 μg/mL of LF followed 1 h later by 100 ng/mL of LPS. After an overnight incubation at 37°C, the cultures were terminated and supernatants frozen at –80°C until immunoassays could be done to determine cytokine determination. The concentrations of TNF-α were measured by an ELISA kit according to the manufacturer's instructions.

Statistics

The results are presented as mean values ±SE. Brown-Forsythe's test was used to determine the homogeneity of variance between the groups. When the variance was homogenous, the analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with Tukey's test to estimate the significance of the differences between the groups. Nonparametric data were evaluated with the Kruskal-Wallis analysis of variance, as indicated in the text. Significance was determined at $p < 0.05$. Statistical analysis was performed using Statistica v. 7 for Windows.

Results

Suppressive effect of rhLF on the effector phase of delayed-type hypersensitivity to OVA in mice

As shown in Fig. 2, rhLF given to mice together with the eliciting dose of the antigen (OVA) suppressed the manifestation of DTH, in a dose-dependent manner, as measured by footpad edema. The most effective dose of rhLF was 5 mg (reduction of footpad edema by 50%), which was similar to Dex (47%).

Inhibitory effect of rhLF on exudate cell numbers in the air-pouch model in mice

The results presented in Fig. 3 revealed a dose-dependent inhibitory effect of rhLF administration on the number of cells infiltrating the air pouches. The 500 μg dose appeared to be the most effective, whereas a dose 5 times higher (2.5 mg) showed only negligible activity. Such a dose-dependent anti-inflammatory action of rhLF resembles the similar kinetics of bLF action in the model of OVA-induced pleurisy and indicates that the anti-inflammatory

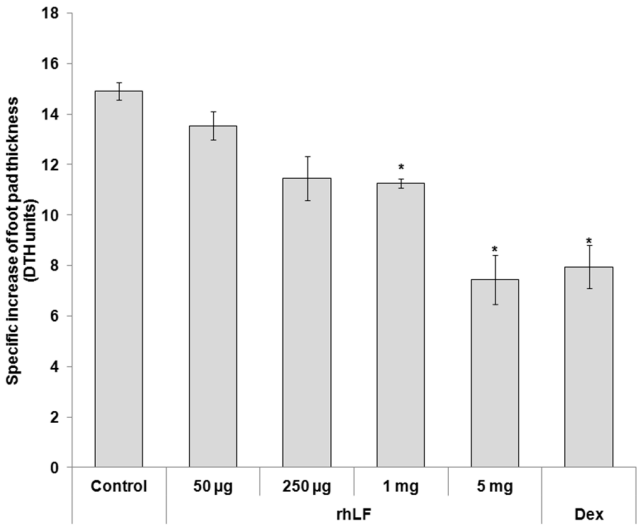

Fig. 2. Suppression of the manifestation of OVA-induced delayed-type hypersensitivity by rhLF

Mice were sensitized with OVA in cFa at the base of the tail and 4 days later, the reaction was elicited by injection of OVA in iFa in each of the hind foot pads, as described in Material and methods section. Recombinant human lactoferrin was administered i.p. to the mice in the indicated doses, 30 min after the administration of the eliciting dose of antigen. Dex was given at a 50 µg dose. All results are presented as the mean value of antigen-specific increase in footpad thickness measured in 6 mice per group (12 feet/determination) and expressed in DTH units (1 DTH unit = 0.01 cm) ±SE; * $p < 0.05$ when compared with the control group.

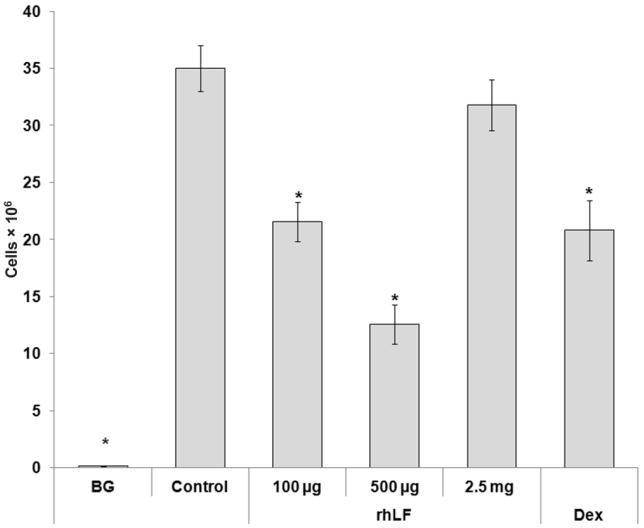

Fig. 3. Inhibition of exudate cell numbers in carrageenan-induced inflammation in air pouches by rhLF

The air pouches were formed by subdermal injection of 5 mL and then 2 mL of sterile air into the dorsal region of the mice. An inflammatory process was elicited by an injection of 1% carrageenan solution into the formed air pouches. Recombinant human lactoferrin was administered i.p. in the indicated doses, 1 h before the administration of carrageenan. Dex was given i.p. at a 50 µg dose. The cell numbers in the pouch exudates were determined 24 h later. The results are presented as the mean values from 7 mice per group ±SE; * $p < 0.05$ when compared with the control group.

action of bLF has, in fact, an immunoregulatory character.[25] The 100 µg rhLF dose was similarly effective as Dex used at a dose of 50 µg.

Amelioration of ovalbumin-induced pleurisy by rhLF in mice

As shown in Fig. 4, sensitized control mice contained almost 7-fold higher cell numbers in their pleural exudates then BG (non-sensitized) mice (14.2×10^6 vs 3.2×10^6). The application of rhLF reduced the cell number to 3.9×10^6 (by 72.5%). Dex lowered the cell numbers to background levels. Recombinant human lactoferrin was also effective in reducing the numbers of degranulated mastocytes in the pleural cavities from 80.4% in sensitized control mice to 64.9%. Dex was also effective (a reduction of 66.3%) (data not shown).

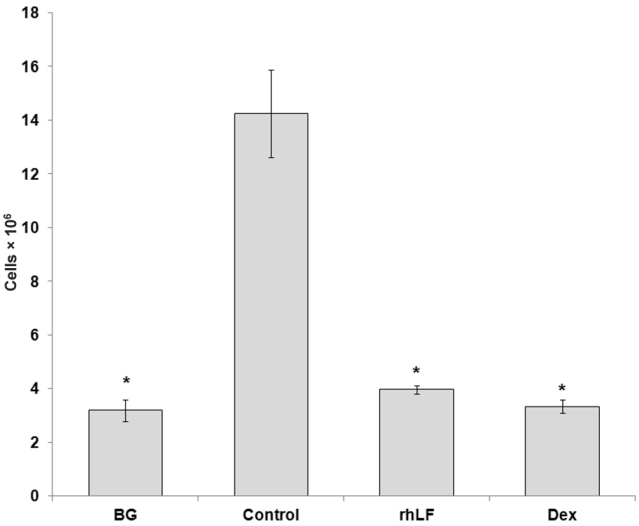

Fig. 4. The effect of rhLF on the cell numbers in the pleural exudates

The mice were sensitized with OVA and an allergic reaction to OVA was elicited after 14 days as described in Material and methods section. Recombinant human lactoferrin was administered bucally (100 µg/dose) at 24 and 3 h before the eliciting dose of OVA. Dex was used at a single dose of 20 µg i.p., 3 h before the elicitation of the allergic response. The cell numbers in the pleural exudates were determined 24 h later. The results are presented as the mean values from 7 mice per group ±SE; * $p < 0.05$ when compared with the control group.

Effects of rhLF on LPS-induced TNF-α production in WBCC

Venous blood from patients admitted to the ICU was taken on day 1, and again on days 3 and 5. WBCC cultures were established and the effects of rhLF on cytokine production were investigated in LPS-stimulated cultures as described in Material and methods section.

Among the 30 patients, 6 subjects did not survive a 5-day hospitalization period and 1 was discharged before day 5 (the incomplete data from these patients are inconclusive, and thus are not shown). The patients underwent routine evaluation of cellular and biochemical laboratory parameters in their blood and urine. Here, we focused on the effects of rhLF in WBC cultures on LPS-induced TNF-α production, a major pro-inflammatory mediator which serum levels predict survival rates in ICU patients.[26]

Seven healthy individuals constituted the control group. The majority of patients exhibited a strong hyporeactivity to LPS stimulus 1 day after the admission to the ICU, as assessed by TNF-α production, with a tendency for recovery on subsequent days. The analysis of the effects of rhLF on LPS-induced TNF-α production revealed several major patterns. Therefore, for the visualization of the regulatory effects of rhLF, the patients were classified into 3 categories. Such an approach was used in the past to enable the proper evaluation of the regulatory effects of LF in patients and normal subjects.[14–17] The tables also include additional information, such as survival, presence or absence of septic shock, reason for admittance to the ICU, and type of bacterial infection. Although the subgroups of patients were very small, it may be concluded that mortality of patients was associated with septic shock, strong hyporeactivity, Gram-negative infection, and surgical intervention as the reason for ICU admittance; and that survival was correlated with increased immune reactivity of blood cells to LPS, Gram-positive infection, and other than surgical reasons for ICU admittance.

One of the responsiveness patterns – hyporeactive patients with spontaneous recovery for TNF-α production (patients No. 1, 2, 4, 5, 13, 17–19, and 23–25) – showed

Table 2. The effects of rhLF on LPS-inducible TNF-α production in hyporeactive patients with spontaneous immune recovery for cytokine production

Patient No.	Day	Control	LPS	rhLF	rhLF/LPS	Septic shock	Reason for admission to ICU	Infection type (Gram+/–)
1	1	18	52	12	67	yes	medical	Gram–
	3	8	26	9	162			
	5	3	179	6	100			
2	1	ND	114	ND	126	yes	surgical	Gram–
	3	ND	102	ND	172			
	5	ND	174	ND	308			
4	1	ND	31	ND	56	no	trauma	Gram+
	3	ND	15	ND	64			
	5	ND	174	ND	178			
5	1	ND	ND	ND	13	yes	surgical	Gram–
	3	ND	305	ND	263			
	5	ND	805	ND	843			
13	1	ND	89	ND	106	yes	medical	Gram+
	3	ND	151	ND	283			
	5	12	356	ND	501			
17	1	23	65	17	103	yes	surgical	Gram–
	3	19	144	16	138			
	5	13	84	15	101			
18	1	14	74	16	90	yes	surgical	Gram–
	3	17	97	16	158			
	5	24	166	19	284			
19	1	13	20	12	38	yes	medical	Gram+
	3	26	123	28	146			
	5	22	189	43	235			
23	1	ND	49	ND	40	no	other	Gram–
	3	ND	66	ND	114			
	5	ND	128	ND	214			
24	1	ND	10	ND	14	yes	medical	Gram–
	3	ND	9	ND	12			
	5	ND	124	ND	140			
25	1	ND	8	ND	9	yes	medical	Gram–
	3	ND	30	ND	29			
	5	ND	393	ND	319			

ND – not detected; the WBC cultures were established and supplemented as follows: 1) 0.1 mL of the culture medium (control); 2) 100 ng/mL of LPS; 3) 50 μg/mL of LF; and 4) 50 μg/mL of LF followed 1 h later by 100 ng/mL of LPS; after an overnight incubation at 37°C, the cultures were terminated and supernatants frozen at –80°C until cytokine determination. This description also relates to Tables 3–5.

Table 3. The effects of rhLF on LPS-inducible TNF-α production in hyporeactive patients showing no spontaneous recovery for TNF-α production

Patient No.	Day	Control	LPS	rhLF	rhLF/LPS	Septic shock	Reason for admission to ICU	Type of infection (Gram+/–)
6	1	ND	ND	ND	ND	no	medical	Gram+
	3	ND	23	ND	105			
	5	ND	35	ND	153			
7	1	ND	69	ND	102	yes	surgical	Gram–
	3	ND	33	13	53			
	5	11	43	ND	116			
11	1	ND	38	ND	79	yes	medical	Gram–
	3	ND	113	ND	115			
	5	ND	25	ND	31			

Table 4. The effects of rhLF on LPS-inducible TNF-α production in patients with moderate and high cytokine production

Patient No.	Day	Control	LPS	rhLF	rhLF/LPS	Septic shock	Reason for admission to ICU	Type of infection (Gram+/–)
3	1	ND	464	ND	725	no	trauma	Gram+
	3	ND	269	ND	599			
	5	ND	180	ND	292			
14	1	23	599	18	482	yes	medical	Gram+
	3	38	1026	26	1100			
	5	27	789	24	781			
16	1	32	1942	27	2060	no	trauma	Gram+
	3	25	2296	26	2334			
	5	23	1430	28	1941			
22	1	ND	714	ND	574	no	medical	Gram+
	3	ND	764	ND	434			
	5	13	333	1	213			
26	1	ND	928	ND	540	no	surgical	Gram+
	3	ND	124	ND	159			
	5	ND	198	ND	136			
28	1	ND	141	ND	305	yes	surgical	Gram–
	3	ND	190	ND	359			
	5	9	529	11	829			

slight stimulatory effects of rhLF (Table 2). However, in the absence of spontaneous recovery in cytokine production (patients No. 6, 7 and 11), rhLF demonstrated a more marked, beneficial stimulatory effect (Table 3). In the cases of moderate and high cytokine production (patients No. 3, 14, 16, 22, 26, and 28), no significant changes or small inhibitory effects of rhLF were observed (Table 4). Interestingly, in patient No. 3, who exhibited a gradually decreasing ability to produce TNF-α, rhLF reversed that undesirable tendency.

Patient No. 8 was discharged from the hospital, and patients No. 9, 10, 12, 15, 21, and 30 died during the study, so the data is incomplete and inconclusive, and therefore is not shown.

In contrast to the septic patients, the healthy control subjects were characterized by moderate-to-high cytokine production (Table 5). TNF-α production (604, 584, 841, 786, 629 pg/mL) was weakly enhanced (15–30%) or unchanged (1,516 pg/mL) except for 1 subject (1,455 pg/mL), where

Table 5. The effects of rhLF on LPS-inducible TNF-α production in control subjects

Donor No.	Control	LPS	rhLF	rhLF/LPS
1	ND	604	ND	790
2	ND	1455	ND	558
3	ND	584	ND	729
4	ND	841	ND	1109
5	ND	786	ND	689
6	ND	1516	ND	1556
7	ND	629	ND	878

60% inhibition was observed. This effect of rhLF differed from that of the highly responsive patient No. 16, in whom TNF-α production was not inhibited, confirming our previous findings that high production of this cytokine in blood cell cultures was beneficial in postoperative patients.[14,15]

Discussion

This report represents the first comparison of rhLF immunomodulatory effects in vivo in a mouse model with human in vitro effects in whole blood cell cultures of patients with sepsis. Firstly, we demonstrated that rhLF exhibited similar anti-inflammatory properties to other types of LFs in mouse models.[18,25,27] Secondly, we analyzed in vitro immunoregulatory properties of rhLF in WBCC of septic patients in terms of LPS-inducible TNF-α production, and we revealed that the protein acted as an adequate sensor and regulator of the patients' cell response to the bacterial antigen.

Several mechanisms have been proposed to explain the anti-inflammatory actions of LFs in mouse models. In the pleurisy model, toll-like receptors (TLRs), in particular TLR4, could be a good candidate for the mediation of the protective action of LF in this experimental model since LF downregulates the function of these receptors.[28] Apart from normalization of the immune response, the parameters associated with this model and the composition of cell types in the pleural infiltrate, we showed that the concentration of IL-5 in the pleural exudates, a major mediator of allergic response, was strongly reduced.[25] Recombinant human lactoferrin was also able to suppress another type of antigen-specific immune response – the cellular response to OVA, similar to that previously demonstrated for bovine lactoferrin.[29] We showed that inhibition of the cellular response to OVA by endogenously LF-induced steroids may contribute to the suppressive action of LF in the cellular immune response.[29] In this study, we demonstrated for the first time that LF can also inhibit nonspecific inflammation in a classical pharmacological model, such as carrageenan-induced inflammation in air pouch. The suppressive effect on the number of air pouch-infiltrating cells was dose-dependent, with an optimal rhLF dose of 500 μg, which surpassed the effect of dexamethasone. Inhibition of IL-6 and TNF-α production by LF could play a role in this model, as demonstrated in the case of carrageenan-induced inflammation of footpad edema in rats.[30]

Having established that rhLF has the ability to suppress in mice the manifestations of the inflammatory processes of basic immunological types (antigen-specific and nonspecific), we wished to evaluate its potential ability to modulate the deeply altered immune reactivity of the peripheral blood cells of septic patients. As expected, we revealed the true immunoregulatory nature of rhLF in the WBCC of septic patients and confirmed our earlier observations on the immunoregulatory actions of bLF in several human models. Orally administered bLF appeared to be immunoregulatory with respect to cytokine production in healthy individuals and ameliorated hyporeactivity in surgical patients.[16,17] In addition, in vitro studies showed that bLF exhibited immunoregulatory properties with regard to LPS-inducible TNF-α and IL-6 production in the WBCC of septic and trauma patients.[14,15] Of particular importance in this study was the use of novel CHO-expressed rhLF, which should be more appropriate for clinical use than recombinant LFs manufactured in yeast or transgenic plants, due to the mammalian-type glycosylation profile.[12,21,22] In addition, by analyzing the responsiveness of patients' WBCC over 3 time points (days 1, 3 and 5 following the admission to the ICU), we were able to demonstrate the differential kinetics of the LPS-induced TNF-α production among the patients. In particular, we verified a maintained or even a decreasing hyporeactivity, spontaneous recovery of cytokine production, or a moderate cytokine production throughout that period. The effects of rhLF on LPS-induced TNF-α production in blood cell cultures were, in fact, anticipated, taking into account the immunoregulatory nature of the protein. The spontaneously recovering hyporeactivity was not significantly affected, but the sustaining anergy or decreasing reactivity to LPS were upregulated. These effects of rhLF on healthy individuals demonstrating normal responsiveness to LPS stimulus were regulatory, as previously described.[16]

Several mechanisms may account for the immunoregulatory effects of lactoferrin in the presented studies. The protein, by downregulating TLR-dependent signaling pathways, may inhibit NF-κB activation, and therefore TNF-α production.[28] In the mouse model of endotoxemia, LF given prior to LPS inhibited all the studied cytokines (TNF-α, IL-6, and IL-10) in serum, as well as inducible nitric oxide.[27] On the other hand, when administered to mice after endotoxic shock, LF strongly inhibited TNF-α and nitric oxide, but the levels of IL-6 and IL-10 did not significantly change.[27] Such results indicate that pretreatment of mice with LF induces a strong hyporeactivity to LPS, or to surgery alone.[18] On the other hand, the sustained levels of IL-6 and IL-10, when LF is given in the course of endotoxemia, ensure adequate protection by counteracting the activity of the proinflammatory mediators.[27] LF also has the unique ability to regulate the expression of cyclooxygenases (COX). It strongly downregulates the expression of COX-2, but also has the ability to weakly stimulate COX-1 expression in human WBCCs.[22] Since prostaglandin E2 (PGE2) mediates the inflammatory response after trauma, the ability of LF to inhibit PGE2 production is highly relevant in explaining its anti-inflammatory action in septic shock and trauma.[31] Such an assumption may be supported by the finding that both inducible nitric oxide and PGE2 were inhibited by LF in endotoxemic mice.[32] On the other hand, the upregulation of intrinsic COX-1 expression by LF may be significant in disrupting the hyporeactivity of LPS-tolerant cells.

In view of the data presented above, we postulate that apart from determining a patient's immunoreactivity prior to elective surgery, the application of LF before or even after surgery should be beneficial to the patient. Thanks to the immunoregulatory nature of LF, the protein will sense the immune status of the patient and modify it accordingly. Overall, these studies provide a strong argument for continuing trials aimed at the application of LF as an agent reducing the risk of septic conditions in patients scheduled for surgery.

Conclusions

In conclusion, this study presents evidence for the potential preventive and therapeutic use of rhLF in patients with impaired immune reactivity.

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