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Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice

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SUMMARY

Mice injected with endotoxin develop endotoxaemia and endotoxin-induced death, accompanied by the oxidative burst and overproduction of inflammatory mediators. Lactoferrin, an iron binding protein, provides a natural feedback mechanism to control the development of such metabolic imbalance and protects against deleterious effects of endotoxin. We investigated the effects of intraperitoneal administration of human lactoferrin on lipopolysaccharide (LPS)-induced release of tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10) and nitric oxide (NO) *in vivo*. Lactoferrin was administered as a prophylactic, concurrent or therapeutic event relative to endotoxic shock by intravenous injection of LPS. Inflammatory mediators were measured in serum at 2, 6 and 18 h post-shock induction. Administration of lactoferrin 1 h before LPS resulted in a rather uniform inhibition of all mediators; TNF by 82%, IL-6 by 43%, IL-10 by 47% at 2 h following LPS injection,and reduction in NO (80%) at 6 h post-shock. Prophylactic administration of lactoferrin at 18 h prior to LPS injection resulted in similar decreases in TNF-α (95%) and in NO (62%), but no statistical reduction in IL-6 or IL-10. Similarly, when lactoferrin was administered as a therapeutic post-induction of endotoxic shock, significant reductions were apparent in TNF-α and NO in serum, but no significant effect was seen on IL-6 and IL-10. These results suggest that the mechanism of action for lactoferrin contains a component for differential regulation of cellular immune responses during *in vivo* models of sepsis.

Keywords endotoxaemia LPS sepsis septic shock systemic inflammatory response syndrome

INTRODUCTION

Lactoferrin is a natural iron binding glycoprotein with multifunctional immunoregulatory properties that have been associated with both, systemic host defence and the mucosal surfaces host defence through its antibacterial properties [1–4]. Early host defence response during septicaemia and endotoxaemia includes a rapid rise in serum lactoferrin concentration [5]. The significance of this response is well established, but not clearly understood. Lactoferrin demonstrates protective effects when administered prophylactic or concurrent to *Escherichia coli*-induced endotoxic shock [6,7]. Similarly, lactoferrin can ameliorate symptoms and gut pathology resulting from experimental endotoxaemia induced using bacterial lipopolysaccharide (LPS) [8,9]. Antibacterial action of lactoferrin has been attributed in part to its iron-binding properties and in part to direct interactions with *E. coli* membranes [10], and competition of LPS binding to cell surface molecules [11]. Also, lactoferrin biological effects are mediated by two types of cell receptors, suggesting various roles of lactoferrin in host defence, including those that transduce intracellularly through different signalling pathways [12–14]. However, the full contribution of protective lactoferrin treatment on immunoregulatory responses contributing to endotoxaemia remains undefined.

Endotoxaemia manifests as a severe clinical syndrome characterized by cytokine release, increased expression of adhesion molecules and release of reactive oxygen species and expression of acute phase proteins [15–17]. Vascular inflammation occurs within minutes of Gram-negative bacterial infection and coincides with a burst of proinflammatory cytokines derived from activated monocytes–macrophages. There is increasing evidence that bacteraemia and endotoxaemia stimulate the immune system into a self-perpetuating, generalized state of hyperactivity. In particular, the systemic inflammatory response to bacterial LPS induces the gut-associated lymphoid tissue to produce and liberate pro-inflammatory cytokines which affects

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the gut mucosal permeability and may contribute to enteric bacterial translocation to distant sites [18–21]. The LPS induction of iNOS activity appears to be a necessary component for LPS-induced bacterial translocation events [22–24]. Similarly, the effects and balance of TNF-α and IL-10 production mediate lethal bacterial endotoxaemia [25–27].

The potential use of lactoferrin for amelioration of clinical sepsis is of great interest [28]. We chose to further characterize the molecular basis of lactoferrin's protective action during sepsis by examination of prophylactic, concurrent and therapeutic administration of lactoferrin during the well established LPS-induced model of endotoxic shock [29,30]. Indeed, injection of LPS into animals virtually reproduces the pathophysiological changes caused by live bacteria, and it is considered a standard model for sepsis. Previous investigations revealed that prophylactic administration of lactoferrin protects gut mucosal integrity during endotoxaemia induced by LPS in mice [8]. We extend these studies further to examine lactoferrin mediation of inflammatory mediators and nitric oxide following LPS-induced endotoxaemia.

MATERIALS AND METHODS

Animals

Male CF-1 mice (Harlan, Houston, TX, USA), 25–30 g initial body weight, were used throughout this investigation. Mice were housed in groups of three per cage and were given a stock diet (F6 Rodent Diet 8664, Teklad, Madison, WI, USA) and water *ad libitum*. All experiments were conducted under approved guidelines of the animal ethics committee at the University of Texas, Health Science Center at Houston.

Lactoferrin, LPS and other reagents

Low endotoxin human milk lactoferrin (< 10 endotoxin units (EU)/mg, < 20% iron saturated) and bacterial endotoxin (*E. coli*, serotype 0111:B4, 3×10^6 EU/mg) were purchased from Sigma Chemical Company, MO, USA. Standard TNF-α, IL-6 and IL-10 were purchased from R&D Systems, Minneapolis, MN, USA. All chemicals were of analytical grade.

Experimental design

Effects of human lactoferrin on the development of endotoxaemia induced by LPS were determined in three different sets of experiments. The first two sets were designed to treat animals with lactoferrin (18 h or 1 h) prior to LPS challenge, whereas in the third experiment mice were treated with lactoferrin 1 h after LPS injection. In each experiment LPS was given i.v. ($3{\cdot}75 \times 10^5$ EU per 25-g mouse; ~5 mg/kg body) and lactoferrin i.p. (5 mg per mouse). The dose of LPS was established in a separate experiment using CF-1 mice [8]. Blood samples were collected into heparinized containers at 2 h, 6 h or 18 h after last treatment. Plasma samples were stored frozen (– 20°C) until assessed.

Determination of IL-6 activity

The assay was performed according to Van Snick *et al.* [31]. Briefly, 7 TD1 indicator cells were washed three times with Hanks's medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics to a density of 2×10^4 cells/ml. Cells were distributed in 100 μl triplicate aliquots in 96-well flat-bottom plates containing 100 μl serially diluted plasma. After 72 h of culture the proliferation of 7TD1 cells was determined using the MTT colourimetric method [14]. The results of IL-6 activity are presented in pg per ml related to IL-6 standard, such that concentration of IL-6 corresponds to activity expressed in U/ml [31]. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

Determination of TNF-α activity

Determination of plasma TNF-α activity utilized the indicator clone, WEHI 164·13 [12]. Cells were washed three times with Hanks's solution and resuspended in RPMI-1640, supplemented with 10% FCS, glutamine, 1 μg/ml of actinomycin D and antibiotics at a concentration of 2×10^5/ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4/well). Triplicate samples of serially diluted plasma samples were added. After an overnight incubation the survival of cells was determined using the MTT colourimetric assay [14]. The results of TNF-α activity are presented in pg/ml where 10 pg TNF-α corresponds to 1 U of activity tested relative to recombinant human TNF-α. One unit of TNF-α was calculated as an inverse dilution of plasma sample resulting in 50% survival of WEHI 164·13 cells.

Determination of IL-10 activity

Determination of IL-10 activity utilized the indicator cell line MC/9. Cells were washed three times with RPMI medium and resuspended in RPMI, supplemented with 10% FCS and recombinant human IL-4 (1 ng/ml) at a concentration of 1×10^5/ml. The cell suspensions were transferred in triplicate 100 μl aliquots into 96-well plates containing 100 μl serially diluted supernatants. After 72 h of incubation, proliferation of the MC/9 cells was measured using colourimetric MTT assay. The optical densities (O.D.) at 550 nm for tested samples were corrected for background by subtracting the mean absorbance of well containing MC/9 cells cultured in the present of rhIL-4. The level of IL-10 was determined directly from a standard curve (concentration range from 50 to 0·39 ng/ml of recombinant human IL-10) and presented in ng/ml.

Nitrate/nitrite (NO_x) analysis

Plasma NO_x was measured as one pathway of NO metabolism. Plasma NO_x was measured by reducing NO_3 to NO_2 with anaerobically grown *E.coli* and then measuring NO_2 with the Griess reaction [32]. Nitrate standards were measured by using the full procedure. The samples were read in the 96-well plate reader (Molecular Devices) at 540 nm.

Statistics

Data are expressed as mean + standard error (s.e.m.), using six mice per group. Differences between groups were analysed by the Student unpaired *t*-test when two groups were analysed and analysis of variance (ANOVA) when more than two groups were analysed. *P*-value of 0·05 or less was considered significant.

RESULTS

Lactoferrin treatment reduces TNF-α and IL-6 in serum during LPS-induced endotoxaemia

The effects of lactoferrin on production of inflammatory mediators during initiation of endotoxaemia was examined. Human lactoferrin (5 mg), or saline control, was intraperitoneally delivered to naive mice 1 h prior to administration of LPS ($3{\cdot}75 \times 10^5$ EU per 25 g mouse). Mice were bled at 2, 6 and 18 h following

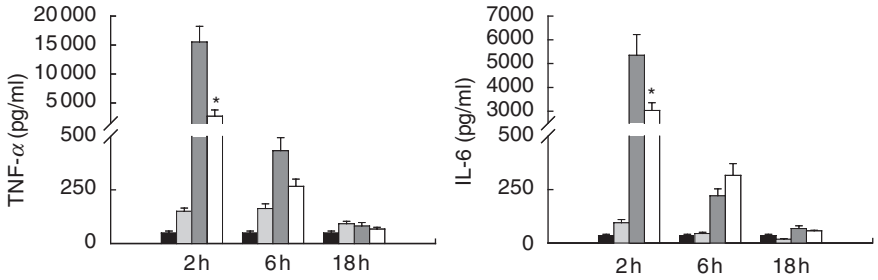

Fig. 1. Lactoferrin treatment reduces pro-inflammatory mediators TNF-α and IL-6 in serum during LPS-induced endotoxaemia. Mice ($n = 6$) were injected i.p. with 5 mg lactoferrin (LF/LPS) or with saline (LPS), 1 h prior to endotoxic shock with LPS ($3{\cdot}75 \times 10^5$ EU/mouse). TNF-α (left) and IL-6 (right) was measured (pg/ml) from serum at 2, 6 and 18 h post-induction of endotoxaemia. Lactoferrin treatment resulted in significant reduction in TNF-α and IL-6 ($^*P < 0{\cdot}05$), compared to LPS only. Mice ($n = 6$) given either saline (control) or 5 mg lactoferrin (LF) without LPS showed no significant elevation in TNF-α or IL-6 within serum (for all groups, $n = 6$). ■, Control; □, LF; ▩, LPS; □, LF/LPS.

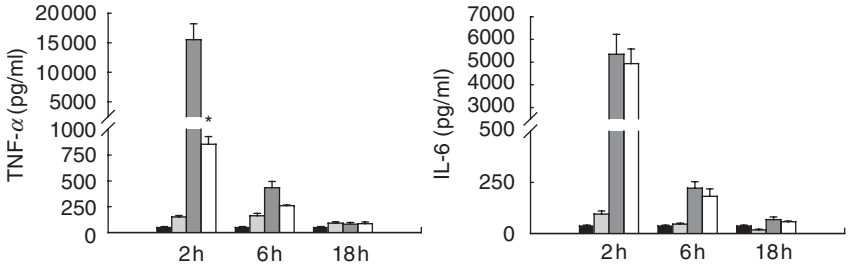

Fig. 2. Evaluation of serum TNF-α and IL-6: prophylactic effects of lactoferrin given prior to LPS-induced endotoxaemia. To rule out the possibility of lactoferrin interaction with LPS, mice ($n = 6$) were injected i.p. with 5 mg lactoferrin (LF/LPS) or with saline (LPS), 18 h prior to endotoxic shock with LPS ($3{\cdot}75 \times 10^5$ EU/mouse). TNF-α (left) and IL-6 (right) was measured (pg/ml) from serum at 2, 6 and 18 h post-induction of endotoxaemia. Lactoferrin treatment resulted in significant reduction in TNF-α ($^*P < 0{\cdot}05$), but not IL-6, compared to the LPS-only group. Mice ($n = 6$) given either saline (control) or 5 mg lactoferrin (LF) without LPS showed no significant elevation in TNF-α and IL-6 within serum (for all groups, $n = 6$). ■, Control; □, LF; ▩, LPS; □, LF/LPS.

LPS administration, and levels of TNF-α and IL-6 were quantified (Fig. 1). Significantly elevated levels of TNF-α ($15\,544{\cdot}2 \pm 2675{\cdot}2$ pg/ml) and IL-6 ($5348{\cdot}3 \pm 869{\cdot}0$ pg/ml) were present in blood 2 h post-LPS administration compared to levles in saline injected control mice. Levels of serum TNF-α were reduced significantly in the lactoferrin treated mice ($2741{\cdot}3 \pm 1068{\cdot}7$ pg/ml, $P < 0{\cdot}05$). Likewise, a significant reduction in IL-6 was demonstrated in the lactoferrin-treated group ($3028{\cdot}8 \pm 323{\cdot}2$ pg/ml, $P < 0{\cdot}05$). The effects of LPS endotoxaemia on TNF-α and IL-6 production were short-lived; the levels of TNF-α and IL-6 were reduced markedly by 6 h post-LPS administration. In all groups, TNF-α and IL-6 returned to background levels by 18 h post-endotoxic signal. Lactoferrin administered alone in the absence of LPS caused no significant change in TNF-α or IL-6 levels compared to saline injected controls at any time post-administration.

Prophylactic treatment with lactoferrin 18 h prior to LPS-induced endotoxaemia affects TNF-α but not IL-6 production

To rule out possible lactoferrin interaction with LPS (and thus reduced bioavailability of LPS), lactoferrin was administered i.p. 18 h prior to administration of LPS. Lactoferrin given prophylactively in this manner (18 h prior to administration of LPS) significantly lowered TNF-α levels at 2 h post-LPS treatment ($854{\cdot}0 \pm 75{\cdot}4$ pg/ml, $P < 0{\cdot}05$) (Fig. 2); TNF-α levels in the lactoferrin treated group were nearly 95% reduced. As demonstrated in the previous experiment, levels of TNF-α induced by LPS in the absence of lactoferrin were significantly reduced by 6 h post-LPS administration and these levels returned to background by 18 h post-endotoxic signal. Accordingly, prophylactic administration of lactoferrin did not alter TNF-α levels at these times post-LPS administration. IL-6 production was also evaluated. Again, LPS endotoxaemia caused significant increases in IL-6 production at 2 and 6 h post-administration. However, prophylactic treatment with lactoferrin 18 h prior to stimulation did not cause significant reduction in levels of IL-6 at any time point examined ($4938{\cdot}0 \pm 633{\cdot}3$ pg/ml at 2 h; $182{\cdot}0 \pm 35{\cdot}7$ pg/ml at 6 h) compared to the LPS-only group.

Therapeutic administration of lactoferrin post-LPS-induced endotoxaemia mediates pro-inflammatory responses

The therapeutic effects of lactoferrin on production of inflammatory mediators TNF-α and IL-6 when administered after initiation of endotoxaemia was further examined. Human lactoferrin (5 mg), or saline control, was intraperitoneal (i.p.) delivered to mice 1 h post-administration of LPS ($3{\cdot}75 \times 10^5$ E.U. per 25 g mouse). Mice were bled at 2, 6 and 18 h following LPS administration, and levels of TNF-α and IL-6 were quantified (Fig. 3). Results demonstrate that TNF-α, but not IL-6, was reduced significantly in the lactoferrin treated mice ($P < 0{\cdot}05$) at 2 h

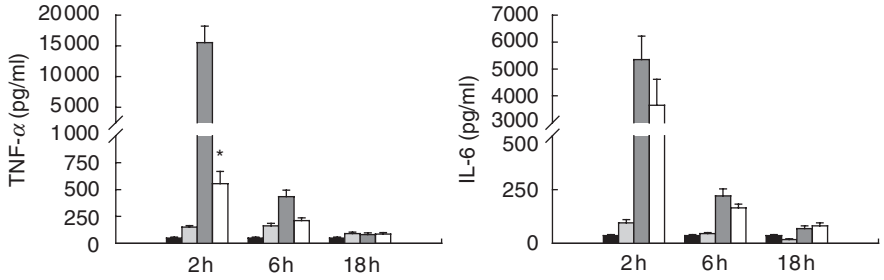

Fig. 3. Lactoferrin treatment reduces pro-inflammatory mediators present in serum when given post-induction of SIRS. Mice ($n = 6$) were injected i.p. with 5 mg lactoferrin (LF/LPS), 1 h post-endotoxic shock with LPS demonstrated significant reduction (*$P < 0{\cdot}05$) in TNF-α (left), but not IL-6 (right). Legend as Fig. 1. ■, Control; ▒, LF; ▓, LPS; □, LF/LPS.

post-administration of endotoxic signal. Specifically, therapeutic treatment with lactoferrin led to greater than 95% reduction in serum TNF-α levels. IL-6 was also decreased (31%); however, the reduction did not achieve statistical significance relative to LPS treatment only.

Lactoferrin does not significantly modulate IL-10 production during LPS-induced endotoxaemia

The effects of lactoferrin on production of IL-10 when administered after initiation of endotoxaemia were also examined. LPS treatment only led to a transient and significant production of IL-10 at 2 h post-administration (230·2 ± 91·6 ng/ml). Although lactoferrin given 1 h before LPS diminished IL-10 concentrations (47% and 32% reduction at 2 and 6 h post-LPS, respectively), the decrease observed was not statistically significant (Table 1). When lactoferrin was given either 18 h prior to LPS or 1 h after LPS administration there was no significant effect on levels or kinetics of IL-10 production. Similarly, there was no significant change in serum IL-10 levels when lactoferrin was given in the absence of LPS.

Inducible nitric oxide synthase activity reduced with lactoferrin treatment

Levels of NO_x were quantified in the sera of mice treated with lactoferrin and LPS. The kinetics of NO response following induction of endotoxaemia differed from those characterized for TNF-α, IL-6 and IL-10. No change in NO levels were apparent at 2 h post-administration of LPS only. Rather, significantly elevated NO concentrations were attained in circulation at 6 h following injection of LPS only (253·6 ± 48·8 μM), with elevated levels maintained 12 h later (191·7 ± 93·1 μM). Treatment with lactoferrin 1 h prior to LPS administration led to a profound and significant ($P < 0{\cdot}05$) decrease in NO levels at both 6 h (51·1 ± 19·1 μM) and 18 h (50·1 ± 24·5 μM) post-LPS-induced endotoxaemia (Fig. 4). Administration of lactoferrin only did not change background concentration of NO. Prophylactic treatment with lactoferrin 18 h prior to LPS treatment also resulted in significant reduction in NO activity at 6 h (95·1 ± 17·7 μM) and 18 h (68·9 ± 7·5 μM) post-endotoxic shock (Table 2), as did therapeutic administraton of lactoferrin at 1 h after LPS administration. (104·3 ± 16·9 μM) and 18 h (26·5 ± 3·6 μM).

Table 1. Lactoferrin does not modulate IL-10 during LPS-induced endotoxaemia

	Serum IL-10 (ng/ml) following treatment		
Treatment	2 h mean ± s.e.m.	6 h mean ± s.e.m.	18 h mean ± s.e.m.
No treatment	16·9 ±2·9	n.d.	n.d.
LF only	18·5 ±3·1	19·8 ±3·8	24·7 ±2·8
LPS only	230·2 ±91·6	55·8 ±29·2	18·2 ±4·8
LF 1 h before LPS	122·7 ±45·6	38·0 ±6·8	8·8 ±1·8
LF 18 h before LPS	271·0 ±41·5	97·3 ±51·3	30·5 ±3·2
LF 1 h post-LF	249·7 ±41·1	90·5 ±39·7	13·7 ±2·8

Table 2. Prophylactic and therapeutic treatment with lactoferrin reduces NO activity due to LPS endotoxaemia

	Serum NO_x (μM/ml) following treatment		
Treatment	2 h mean ±s.e.m.	6 h mean ±s.e.m.	18 h mean ±s.e.m.
No treatment	34·2 ± 4·1	n.d.	n.d.
LF only	27·7 ± 4·5	11·9 ± 2·5	26·1 ± 4·6
LPS only	46·1 ± 6·4	253·6 ± 48·8*	191·7 ± 93·1*
LF 18 h before LPS	43·2 ± 7·7	95·1 ± 17·7†	68·9 ± 7·5†
LF 1 h post-LF	53·4 ± 11·3	104·3 ± 16·9†	26·5 ± 3·5†

*$P < 0{\cdot}05$ *versus* no treatment; †$P < 0{\cdot}05$ *versus* LPS only.

DISCUSSION

These studies indicate that human lactoferrin modulates inflammatory mediators and nitric oxide release into circulation following LPS-induced endotoxaemia in mice. In previous experiments, it has been difficult to delineate how changes in mediator activity induced by lactoferrin relate to the protective effects in animals given lethal doses of bacteria or LPS. Moreover, protective actions of lactoferrin were strictly dependent on the time of administration in relation to challenging dose of endotoxic signal [33]. The damaging effects of trauma and the protective effects of lactoferrin may depend upon mediation of relative amounts of both pro-inflammatory (e.g. TNF-α and inducible NO) and

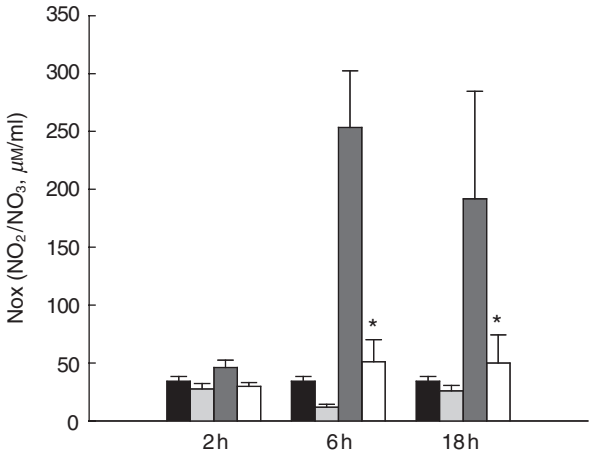

Fig. 4. Lactoferrin reduction in nitric oxide activity during LPS-induced endotoxaemia. Mice ($n = 6$) were injected i.p. with 5 mg lactoferrin (LF/LPS), or with saline (LPS), 1 h prior to endotoxic shock with LPS ($3{\cdot}75 \times 10^5$ EU/mouse). Nitric oxide levels (μM/ml) were then measured from serum at 2, 6 and 18 h post-induction of endotoxaemia. Lactoferrin treatment 1 h prior to toxic signal resulted in significant reduction in nitric oxide at 6 and 18 h post-endotoxemic insult ($^*P < 0{\cdot}05$). Mice ($n = 6$) given either saline (control) or 5 mg lactoferrin (LF) without LPS showed no significant elevation in nitric oxide. ■, Control; □, LF; ▩, LPS; □, LF/LPS.

anti-inflammatory (IL-10 and IL-6) molecules. Therefore, we analysed the activities of these mediators in mice given a sublethal dose of LPS when treated with lactoferrin at various time intervals in relation to the LPS injection.

The mechanism of action for lactoferrin appears to contain multiple components for differential regulation of cellular immune responses during *in vivo* models of sepsis. The protective effects of lactoferrin administered concurrent with LPS may result from generalized deactivation of the monocytes and macrophages as manifested by the significant suppression of both pro- and anti-inflammatory mediators of inflammation. Use of a concurrent administration protocol demonstrated significant reductions in TNF-α (82%), IL-6 (43%) and IL-10 (47%) at 2 h following LPS injection, and reduction in NO (80%) at 6 h post-shock. It has been shown *in vitro* that lactoferrin can down-regulate TNF-α production from immortalized human macrophages during LPS stimulation [34], perhaps via nuclear localization and complex transcriptional regulation of gene expression [31,35,36]. Alternatively, the decrease in endotoxic shock may simply result from direct interaction of lactoferrin with LPS [37], or via physical blocking of macrophage LPS-binding receptors [11]. The bioavailability of free lactoferrin within blood is approximately 1 h [38]. As such, the influence of lactoferrin on immunomodulation during administration concurrent with LPS may be only one component of reduced inflammatory responses. Moreover, in our recent studies (to be published) we demonstrate that LPS–lactoferrin complexes may be still active immunologically and lipopolysaccharide binding protein (LBP) is not essential for mediating LPS-induced cytokine production.

Lactoferrin has the ability to modulate cytokine production and immune responses [34], and can activate macrophages directly [39–41]. Paradoxically, lactoferrin can also inhibit effector phases of cellular immune responses [42]. The reason for these seemingly contradictory observations are not clear and require better understanding of the physiological circumstances surrounding the administration of lactoferrin. Indeed, it has been proposed that lactoferrin regulation of proliferative response of human PBMCs is dependent in part upon the activation status of cells being stimulated [43]. Collectively, these results indicate that lactoferrin has the ability to maintain physiological homeostasis through the modulation of pro-inflammatory responses.

It is difficult to conclude that the reduced IL-6 seen when lactoferrin was administered concurrent with LPS is anything less than mere physical interaction of the two molecules, as no reduction in IL-6 was shown when lactoferrin was given in prophylactic or therapeutic protocols. The changes in IL-6 may also be an indirect function of relative levels of TNF-α remaining following treatment, as IL-6 is reduced only in the presence of higher TNF-α. The finding that IL-6 remained elevated despite reductions in TNF-α may be related to changes in TNF-α receptor (not measured). Similarly, serum concentration of IL-10 was also reduced significantly only upon administration of lactoferrin concurrently with LPS, although suggestive reductions in IL-10 levels were apparent when lactoferrin was administered as a prophylactic 18 h prior to LPS. These findings for IL-6 and IL-10 are consistent with those reported earlier [9]. Indeed, it may be that the relative amounts of IL-10 to TNF-α may be more essential in mediation of immune responses, as in cases reported during modulation of mycobacterial response by the presence of Th2 cytokines [44]. Again, in this paper we demonstrate two effects of cytokine regulation by lactoferrin in relation to LPS administration: first, inhibition of pro-inflammatory mediators (TNF-α and NO), accompanied by a moderate reduction of the anti-inflammatory cytokines, and secondly a very strong reduction of TNF-α with no significant effect on anti-inflammatory cytokines. In fact, similar observations were reported for bovine lactoferrin delivered to endotoxaemic mice per os or i.v. [9]. We postulate that differential effects of lactoferrin given 18 h or 1 h prior to LPS are through delivering to relevant cells (being the direct target for LPS and/or other regulatory cells) stimulatory signals, resulting in their altered activation state or an ability to produce mediators. We [9] and others [45] have shown that lactoferrin is capable of inducing low to moderate levels of both pro- and anti-inflammatory cytokines such as TNF-α, IL-8, IL-6, IL-10 and NO. The cytokines appear in circulation at 2 h following i.v. LF injection [9], so the relatively long time lag (18 h) before LPS challenge would be sufficient for the released cytokines to exert various immunomodulatory functions relevant in changing the cytokine profile upon LPS stimulation. Such changes may include, for example, a diminished expression of LPS receptors or ahesion molecules [14]. Induction of IL-6 could also result in production of regulatory and protective acute phase proteins [46]. We have shown recently that blocking TNF-α resulted in a loss of lactoferrin activity in regulation of LFA-1 expression [14].

This study has demonstrated that the protective effects of lactoferrin in various models of bacteraemia and endotoxaemia may result from selective deactivation of the immune system, as manifested by the significant suppression of pro-inflammatory mediators. However, the ideal immunoregulator of inflammation should control both pro- and anti-inflammatory mediators. Selective inhibition of pro-inflammatory mediators or stimulation of anti-inflammatory cytokines may not always be beneficial [44]. For example, Haupt *et al*. [47] demonstrated that plasma from septic patients can decrease the production of pro-inflammatory TNF-α and anti-inflammatory IL-10 in healthy donor PBMCs.

Specifically, TNF-α is implicated as a major detrimental factor in systemic inflammatory response syndrome (SIRS) and septic shock [48]. The inverse correlation between plasma levels of TNF-α and survival in patients with meningococcal bacteraemia is a strong indicator that TNF-α has a primary role in the pathogenesis of sepsis [49]. Zimecki *et al.* demonstrated that oral administration of lactoferrin can regulate post-surgical responses [28]. Unfortunately, attempts to inactive TNF-α in septic patients has not been successful [50].

NO mediates gut integrity during intestinal injury [21,51]. Up-regulation of nitric oxide synthase (NOS) occurs during LPS-induced endotoxaemia [23], concurrent with increased bacterial translocation events, suggesting that LPS induction of iNOS activity is necessary for LPS-induced bacterial translocation [22,24]. The data presented confirm the finding of inhibition by lactoferrin of inducible NO synthesized in response to experimental *E. coli* infection [52]**.** Also, Tunctan *et al*. [53] demonstrated significant reduction in LPS-induced nitrate level and mortality with aminoguanidine, which is a selective inhibitor of iNOS. In the endotoxic model presented here we did not test any inhibitors on NOS; instead, we demonstrate that lactoferrin has the ability to reduce production of nitrites in both prohylactic and therapeutic protocols. At this time, we cannot define the mechanism of such inhibition. However, the ability of lactoferrin to interact with anionic molecules during normal and pathological responses may be relevant to some of its biological properties. Recently, lactoferrin was associated with an onset of Alzheimer's disease (AD) by demonstrating an active role in clearance of amyloid beta through the low density lipoprotein receptor-related protein (LRP) [54]. It is possible that such ability of lactoferrin to interact with anionic molecules can modulate specific transduction signalling pathways, which are responsible for selective deactivation of the immune system. For example, by virtue of iron sequestration, and further reduction of lipid peroxidation products such as 4HNE, lactoferrin can be linked with c-Jun NH2-terminal kinases (JNK) activation and c-Jun phosphorylation. We are currently in the process of investigating the mechanisms involved in LPS-mediated increase of AP-l phosphorylation of its DNA binding activity, which is a later event in the JNK signalling pathway.

The goal of this study was to investigate the molecular mechanisms of protective action of human lactoferrin in a defined model of SIRS. Our results indicate differential regulation of pro-inflammatory and anti-inflammatory mediators by lactoferrin during LPS-induced endotoxaemia. These findings may be of importance when considering clinical utilization of lactoferrin as a post-surgical therapeutic, with imparted protection through concerted actions such as reduced TNF-α production in endotoxic shock, ability to bind LPS [55], direct bacteriostatic effects [56] and protective effects on gut structure [8]. Further studies will be necessary to determine the mechanism(s) by which lactoferrin maintains biological homeostasis.

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