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ARTICLE

Effect of bovine lactoferrin on *Chlamydia trachomatis* infection and inflammation[1]

Rosa Sessa, Marisa Di Pietro, Simone Filardo, Alessia Bressan, Luigi Rosa, Antimo Cutone, Alessandra Frioni, Francesca Berlutti, Rosalba Paesano, and Piera Valenti

Abstract: *Chlamydia trachomatis* is an obligate, intracellular pathogen responsible for the most common sexually transmitted bacterial disease worldwide, causing acute and chronic infections. The acute infection is susceptible to antibiotics, whereas the chronic one needs prolonged therapies, thus increasing the risk of developing antibiotic resistance. Novel alternative therapies are needed. The intracellular development of *C. trachomatis* requires essential nutrients, including iron. Iron-chelating drugs inhibit *C. trachomatis* developmental cycle. Lactoferrin (Lf), a pleiotropic iron binding glycoprotein, could be a promising candidate against *C. trachomatis* infection. Similarly to the efficacy against other intracellular pathogens, bovine Lf (bLf) could both interfere with *C. trachomatis* entry into epithelial cells and exert an anti-inflammatory activity. In vitro and in vivo effects of bLf against *C. trachomatis* infectious and inflammatory process has been investigated. BLf inhibits *C. trachomatis* entry into host cells when incubated with cell monolayers before or at the moment of the infection and down-regulates IL-6/IL-8 synthesized by infected cells. Six out of 7 pregnant women asymptomatically infected by *C. trachomatis*, after 30 days of bLf intravaginal administration, were negative for *C. trachomatis* and showed a decrease of cervical IL-6 levels. This is the first time that the bLf protective effect against *C. trachomatis* infection has been demonstrated.

Key words: Chlamydia trachomatis, bovine lactoferrin, infection, inflammation, IL-6.

Résumé : *Chlamydia trachomatis* est un pathogène obligatoire, intracellulaire, responsable de la maladie transmise sexuellement bactérienne la plus courante à travers le monde, causant des infections aigües et chroniques. L'infection aigüe est sensible aux antibiotiques, alors que l'infection chronique nécessite des thérapies prolongées, augmentant ainsi le risque de développer une résistance aux antibiotiques. Des thérapies novatrices alternatives sont requises. Le développement intracellulaire de *C. trachomatis* requiert des nutriments essentiels dont le fer. Les médicaments chélateurs du fer inhibent le cycle développemental de *C. trachomatis*. La lactoferrine (Lf), une glycoprotéine pléiotropique qui lie le fer pourrait constituer un candidat prometteur pour contrer l'infection par *C. trachomatis*. Similairement son efficacité envers d'autres pathogènes intracellulaires, la Lf bovine (bLf) pourrait interférer avec l'entrée de *C. trachomatis* à l'intérieur des cellules épithéliales et exercer une activité anti-inflammatoire. Les effets *in vitro* et *in vivo* de la bLf envers le processus infectieux et inflammatoire de *C. trachomatis* ont été examinés. La bLF inhibe l'entrée de *C. trachomatis* dans les cellules hôtes lorsqu'elle est incubée avec des cellules en monocouches avant ou au moment de l'infection, et elle régule à la baisse l'IL-6 et l'IL-8 synthétisées par les cellules infectées. Six femmes enceintes asymptomatiques sur 7, infectées par *C. trachomatis*, se révélaient négatives quant à la présence de *C. trachomatis* après 30 jours d'administration intravaginale de bLf, et elles présentaient une diminution des niveaux cervicaux d'IL-6. C'est la première fois que l'effet protecteur de la bLf contre l'infection par *C. trachomatis* a été démontré. [Traduit par la Rédaction]

Mots-clés : Chlamydia trachomatis, lactoferrine bovine, infection, inflammation, IL-6.

Introduction

Chlamydia trachomatis, an obligate intracellular pathogen, is the leading cause of sexually transmitted bacterial infections in the world, with an estimated >131 million new cases per year (Newman et al. 2015). *Chlamydia trachomatis* genital infection manifests in women as cervicitis, salpingitis, and endometritis, and can progress leading to severe sequelae, such as pelvic inflammatory disease, ectopic pregnancy, and obstructive infertility (Shaw et al. 2011). Importantly, a major concern with chlamydial genital infections is that approximately 80% of women are asymptomatic, thus resulting in a reservoir for onwards transmission in the population (Shaw et al. 2011).

Chlamydia trachomatis is characterized by a unique biphasic developmental cycle alternating between the extracellular infectious bodies (elementary bodies, EBs), metabolically inactive, and the intracellular non-infectious bodies (reticulate bodies, RBs), metabolically active. The EBs adhesion and entry into mucosal epithelial cells initiate a signal transduction cascade of the host cell, leading to the recruitment and reorganization of the actin cytoskeleton at the site of attachment. Following the fusion of EB-containing endosomes, EBs develop into larger, metabolically active but noninfectious RBs. Using ATP and nutrients from the host cell, RBs grow and divide by binary fission within a membrane-bound vacuole, termed inclusion. Subsequently, the RBs asynchro-

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R. Sessa, M. Di Pietro, S. Filardo, A. Bressan, L. Rosa, A. Cutone, A. Frioni, F. Berlutti, and P. Valenti. Department of Public Health and Infectious Diseases, University of Rome, La Sapienza, Rome, Italy.
R. Paesano. Department of Gynecological-Obstetric and Urological Sciences, University of Rome, La Sapienza, Rome, Italy.
Corresponding author: Piera Valenti (email: piera.valenti@uniroma1.it).

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nously transform into EBs, which are released, after approximately 48 h, from the host cell by lysis (Wyrick 2010; Bastidas et al. 2013).

In recent years, it has been demonstrated that *C. trachomatis* can generate a persistent form during its developmental cycle as a consequence of several stress-inducing factors (Wyrick 2010; Di Pietro et al. 2013). As a result, normal RBs transform into enlarged and morphologically aberrant RBs, thus stopping the production of infectious EBs (Hogan et al. 2004; Wyrick 2010). In particular, *C. trachomatis* enters into the persistence state in the presence of iron-chelating drugs, which inhibit the developmental cycle and, hence, show its dependence on iron for the achievement of infectious cycle (Raulston 1997; Thompson and Carabeo 2011). In this regard, iron limitation in host cells has been shown to be of the utmost importance for the growth and survival of *Chlamydia* spp. (Raulston 1997; Al-Younes et al. 2001).

Following *C. trachomatis* infection, cervical epithelial cells produce several pro-inflammatory cytokines including TNF-α, IL-1α, IL-6, and IL-8 that augment the cell inflammatory response, thus inducing direct damage to genital tissues. Furthermore, IL-8, in turn, recruits innate immune cells, which are abundant in the genital mucosa and are able to further worsen chronic inflammation and tissue-damage of the reproductive system (Redgrove and McLaughlin 2014).

Interestingly, during the infection/inflammation, IL-8 recruits neutrophils that synthesize and secrete granules containing lactoferrin (Lf) (Masson et al. 1969).

Recently, a great interest in Lf, considered as a prominent component of the first-line defense of the host against infections and inflammation, has been raised.

Lf, an 80 kDa iron-binding glycoprotein, is found in most body fluids including vaginal fluid (Valore et al. 2002). Lf possesses several biological functions, dependent and independent from its iron-binding ability (Valenti and Antonini 2005). Among the biological properties related to its iron-withholding ability, Lf inhibits bacterial infections, whereas independently from iron chelation, its high positive charge favors the binding to microorganisms and (or) host cells, thus hindering the adhesion and entry into epithelial cells (Valenti and Antonini 2005). In addition to these activities, Lf demonstrates potent anti-inflammatory activity, protecting infected host cells from damages associated with pathological inflammation. In particular, independently from its iron-binding ability, Lf decreases the synthesis of proinflammatory cytokines in infected epithelial cells (Berlutti et al. 2006; Puddu et al. 2011; Valenti et al. 2011; Frioni et al. 2014).

Given the impact of asymptomatic chlamydial infection on disease outcomes and the multifunctional features of Lf, the aim of our study was to evaluate the effects of bovine milk derived Lf (bLf) on *C. trachomatis* infection and on the associated inflammatory state, in vitro and in vivo.

Materials and methods

Chlamydia trachomatis strain and cell culture

Chlamydia trachomatis L2 strain 434/Bu (ATCC VR-902B) was obtained from the American Type Culture Collection.

The human epithelial HeLa-229 cell line from cervix adenocarcinoma (ATCC CCL-2.1) was cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; Euroclone, Milan, Italy), supplemented with 10% fetal calf serum (FCS; Euroclone), in a humidified atmosphere with 5% CO_2.

Propagation and titration of *C. trachomatis*

Elementary body (EB) aliquots of *C. trachomatis* L2 were stored at –80 °C and propagated in HeLa-229 cells, grown in DMEM supplemented with 10% FCS, as previously described by Mastromarino et al. (2014). The infectious titer was assessed by immunofluorescence assay (IFA). Briefly, HeLa-229 cells grown on glass coverslips in 24-well plates were infected with 10-fold serial dilutions of bacterial stock, incubated for 48 h at 37 °C, fixed with methanol and stained with fluorescein isothiocyanate-conjugated monoclonal (FITC) antibody anti-*C. trachomatis* (MicroTrak, Trinity Biotech). The total number of *C. trachomatis* inclusion forming units (IFUs) was obtained by counting all fields using a fluorescence microscope (100× magnification).

Lactoferrin

Highly purified bovine milk derived lactoferrin (bLf) was kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan). The absence of bLf degradation fragments was checked by SDS–PAGE stained with silver nitrate. Lactoferrin concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The purity of bLf corresponded to about 98%, as also detected by high-performance liquid chromatography (HPLC) analysis. The bLf iron saturation was about 20%, as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation). LPS contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit; PBI International, Milan, Italy), was 0.7 ± 0.06 ng/mg of bLf. Before biological assays, bLf solution was sterilized by filtration using 0.2 μm Millex HV at low protein retention (Millipore Corp., Bedford, Massachusetts, USA). In all experiments bLf was used at non-cytotoxic concentrations corresponding to 100 μg/mL.

Effects of bovine lactoferrin on infection of HeLa-229 cells with *C. trachomatis* elementary bodies

Pre-incubation of bLf with C. trachomatis *EBs*

To detect the efficacy of bLf against *C. trachomatis*, 25 000 EBs/mL, corresponding to a multiplicity of infection (MOI) of 0.05, were pre-incubated in DMEM with FCS 2% (fresh medium), in the absence or presence of bLf (100 μg/mL), for 1 h or 3 h at 37 °C in humidified atmosphere with 5% CO_2. Subsequently, the *C. trachomatis* EBs suspension was centrifuged at 30 000*g* for 15 min, and the supernatant was removed. The pellet containing *C. trachomatis* EBs was suspended in fresh medium and used to infect a total of about 10^5 HeLa-229 cells. Briefly, after 1 h, the cells were washed with phosphate buffer solution without Ca^{2+} and Mg^{2+} (PBS) to remove the non-internalized *C. trachomatis* EBs and newly incubated in fresh medium. After 48 h post infection (h.p.i.) at 37 °C in 5% CO_2, the total number of *C. trachomatis* IFU was determined by IFA.

Pre-incubation of bLf with HeLa-229 cells before the infection with C. trachomatis *EBs*

HeLa-229 cells were pre-incubated in fresh medium in the absence or presence of bLf (100 μg/mL). After 1 h or 3 h of incubation at 37 °C in 5% CO_2, bLf was removed by washing the cell monolayers 3 times with PBS. Subsequently, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05 as above described. After 48 h.p.i. at 37 °C in 5% CO_2, the total number of *C. trachomatis* IFU was determined by IFA.

bLf addition to HeLa-229 cells at the moment of infection with C. trachomatis *EBs*

In this set of experiments, bLf was added to HeLa-229 cells at the moment of infection. Briefly, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05 in the absence or presence of bLf (100 μg/mL). After 1 h at 37 °C in 5% CO_2, the cells were washed with PBS to remove the non-internalized *C. trachomatis* EBs, and fresh medium was added. After 48 h.p.i at 37 °C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by IFA.

bLf addition to HeLa-229 cells 3 h after C. trachomatis *infection*

HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05. After 1 h of incubation at 37 °C in 5% CO_2, the cells were washed with PBS to remove the non-internalized *C. trachomatis* EBs and fresh medium was added. After further 3 h of incubation at 37 °C in 5% CO_2, fresh medium, with or without bLf (100 μg/mL), was

added to the infected cells. After 48 h.p.i. at 37 °C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by IFA.

Detection of cytokines

Preliminary experiments, carried out with *C. trachomatis* EBs at a MOI of 0.05, showed a very low cytokine expression by infected HeLa-229 cells. Therefore, HeLa-229 cells were infected with *C. trachomatis* EBs at a MOI of 5 to reach a higher expression of IL-6 and IL-8 than that observed at the MOI of 0.05. After 1 h of incubation, the cells were washed with PBS to remove the non-internalized *C. trachomatis* EBs and supplemented with fresh medium. After further 3 h of incubation at 37 °C in 5% CO_2, fresh medium, with or without bLf (100 μg/mL), was added to the infected cells. The cytokine production was determined in cell monolayer supernatants by ELISA using Human ELISA Max Deluxe Set (BioLegend, San Diego, California, USA) after 48 h of incubation at 37 °C in 5% CO_2.

Study design

We conducted an open-label cohort study in accordance with the ethical principles of the *Declaration of Helsinki*. Approval was granted by the Ethics Committee of Clinica Fabia Mater, Via Olevano Romano, 25 Rome, Italy (FM MOD 26022010). All of the pregnant women gave written informed consent.

One hundred ninety-eight pregnant women from 20 to 40 years without ascertained pathologies, with normal uterine cavity, and intact membranes were enrolled regardless of trimester. Women were excluded if they had a pathological pregnancy or if during this study they were affected by bacterial vaginal infections unrelated to *C. trachomatis.*

The exclusion of pregnant women during the clinical trial was also considered on the basis of voluntary declaration, lack of treatment effectiveness, side effects, protocol infringement, and missed programmed visits. The enrolled pregnant women were on a monthly scheduled visit.

Laboratory tests

At each scheduled visit, in addition to standard assays (haematocrit, glycemia, uricemia, bilirubin, glutamicoxaloacetic transaminase, glutamic pyruvic transaminase, cholesterol, triglyceride acid, and electrolytes), cervical specimens were collected with polyethylene terephthalate (Dacron) swabs to detect the presence of *C. trachomatis*. In addition, cervical fluids were analyzed to detect IL-6 concentrations.

Chlamydia trachomatis detection

Cervical specimens were analyzed by direct immunofluorescence assay (DFA) using Syva Microtrack kit (Syva Microtrack; Trinity Biotech) according to the manufacturer's instructions. Briefly, the smears were fixed with methanol and stained with fluorescein isothiocyanate conjugated (FITC) monoclonal antibody against *C. trachomatis* major outer membrane protein (MOMP) for 30 min at 37 °C in a humid chamber. The slides were examined for the presence of IFUs using fluorescence microscope (100× magnification).

Treatment against *C. trachomatis* infection in pregnant women

Among 198 pregnant women, 7 women, asymptomatically affected by *C. trachomatis*, were immediately treated with bLf intravaginal administration. The intravaginal tablet, containing 100 mg of lyophilized bLf 20% iron-saturated, was administered every 8 h for 30 days. The tablets were administered through a vaginal applicator to obtain a fast and adequate dissolution. If the treatment with bLf intravaginal administration for 30 days was ineffective, the pregnant women were submitted to antibiotic therapy (Workowski and Bolan 2015).

Maternal side effects

The side effects of bLf intravaginal administration were assessed by monitoring vaginal irritation, itching, and burning.

Fetal and newborn side effects

Fetal vital sign assessments were monitored by ultrasonographic measurements of intrauterine growth and through the amount of amniotic fluid, expressed as the amniotic fluid index (AFI). Newborn weight and Apgar score were registered. Apgar score is a practical method of evaluating the physical condition of a newborn shortly after delivery (Apgar 1953). An Apgar score of 0–3 at 5–10 min of age is predictive of high morbidity and mortality, while an Apgar score of 9–10 means the infant is in the best possible conditions.

Statistical analysis

All values are the mean ± standard deviation (SD) of 3 replicates from 3 independent in vitro experiments. The concentrations of IL-6 in cervical fluid of pregnant women were expressed as mean values ± SD. Comparison of means was performed by using a 2-tailed *t*-test for independent samples. A value for $P < 0.05$ was considered statistically significant.

Results

Effects of bovine lactoferrin on *C. trachomatis* infection

We evaluated the effects of bLf, at non-cytotoxic concentration corresponding to 100 μg/mL on *C. trachomatis* infections.

As shown in Fig. 1A, no significant reduction in the number of chlamydial IFUs was observed when chlamydial EBs were pre-incubated with bLf for 1 h or 3 h, indicating no direct effect of bLf on *C. trachomatis*.

In contrast, bLf inhibited *C. trachomatis* entry into host cells, as evidenced by a significant reduction of chlamydial IFU observed when HeLa-229 monolayers were pre-incubated with bLf for 1 or 3 h (1 h pre-incubation: $P = 0.0008$; 3 h pre-incubation: $P = 0.00007$) (Figs. 1B and 1C). The inhibitory effect of bLf on *C. trachomatis* entry was more pronounced when HeLa-229 cells were preincubated with bLf for 3 h compared with 1 h ($P = 0.0124$) (Fig. 1C).

To further confirm the inhibitory effect of bLf on *C. trachomatis* entry into host cells, bLf was added at the moment of HeLa-229 monolayer infection with *C. trachomatis*. The presence of bLf during the infection phase significantly inhibited *C. trachomatis* entry into HeLa-229 cells at the same extent evidenced when bLf was pre-incubated with cell monolayers for 1 or 3 h (Figs. 1B, 1C, and 1D). To determine whether bLf inhibited chlamydial replication, bLf was added after 3 h of *C. trachomatis* infection. The addition of bLf under these experimental conditions resulted in no significant reduction of the number of intracellular chlamydial IFUs ($P = 0.28$) (Fig. 1E).

Effect of lactoferrin on IL-6 and IL-8 cytokine production by HeLa-229 cells infected with *C. trachomatis*

To investigate the effect of bLf on the inflammatory response, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 5, and after 3 h of infection, bLf was added to the medium. Of note, the addition of bLf at 3 h post-infection did not influence the intracellular number of *C. trachomatis* infecting the cell monolayers at the MOI of 0.05 (Fig. 1E) or at the MOI of 5 (data not shown), thus allowing the detection of the actual synthesis of IL-6 and IL-8 by the same number of intracellular *C. trachomatis*. The production of IL-6 and IL-8 was evaluated in the supernatants (Figs. 2 and 3, respectively). The treatment with bLf did not raise the cytokine levels in noninfected cells compared with the cell monolayers alone. On the contrary, the infection with *C. trachomatis* induced a significant increase of both IL-6 and IL-8 levels. The addition of bLf to infected cells 3 h post-infection caused a significant decrease of both IL-6 and IL-8 levels compared with infected cells not treated with bLF ($P < 0.05$). In particular, bLf significantly decreased IL-6

Fig. 1. Inclusion forming units (IFU) of *Chlamydia trachomatis* observed by immunofluorescence assay in HeLa-229 cells infected at a MOI of 0.05 in the absence or presence of bLf added at different time. (A) One hour preincubation of *C. trachomatis* EBs in the absence or presence of bLf; (B) 1 h preincubation of HeLa-229 cells in the absence or presence of bLf; (C) 3 h preincubation of HeLa-229 cells in the absence or presence of bLf; (D) HeLa-229 cells infected with *C. trachomatis* and treated with bLf; (E) Addition of bLf to HeLa-229 cells at 3 h.p.i. with *C. trachomatis*.

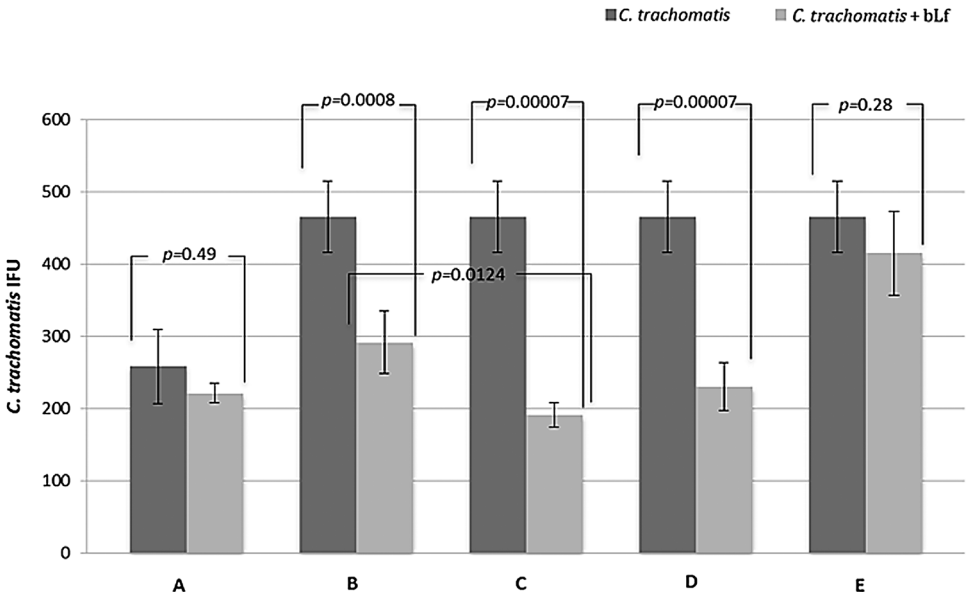
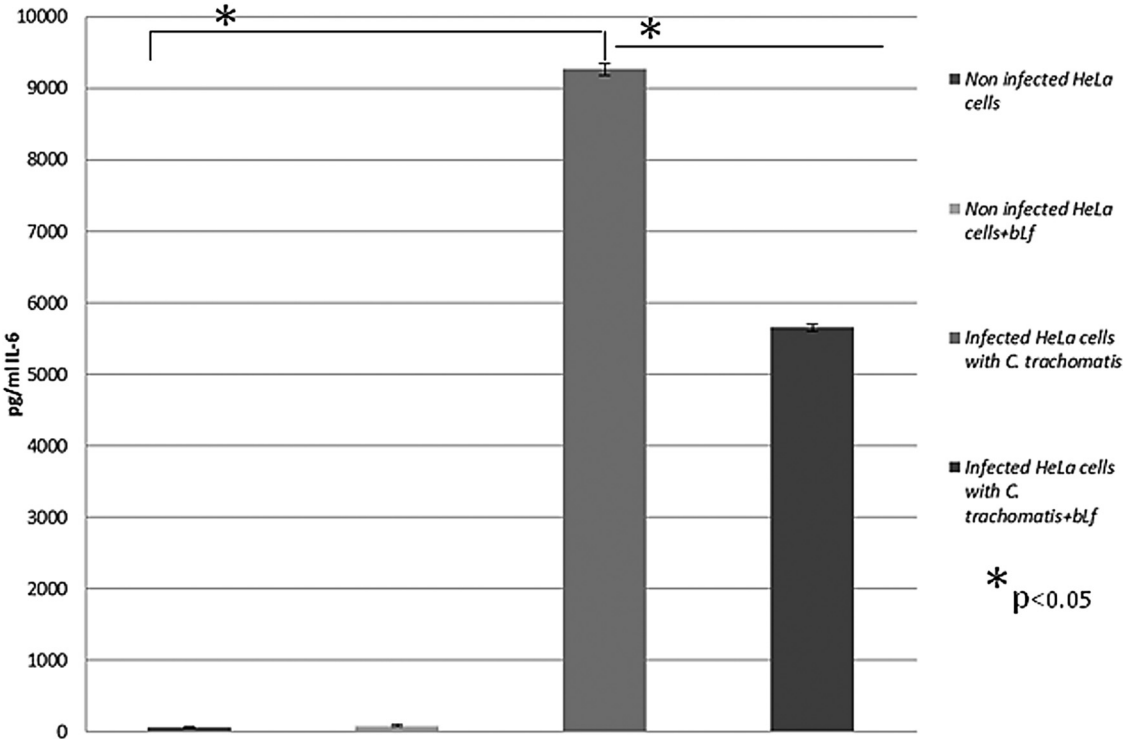


Fig. 2. IL-6 levels in the supernatants of HeLa-229 cell cultures after 48 h of incubation. The infection was performed at a MOI of 5, and bLf was added 3 h post-infection. The IL-6 concentrations are mean values ± SD. A value for $P < 0.05$ was considered statistically significant.



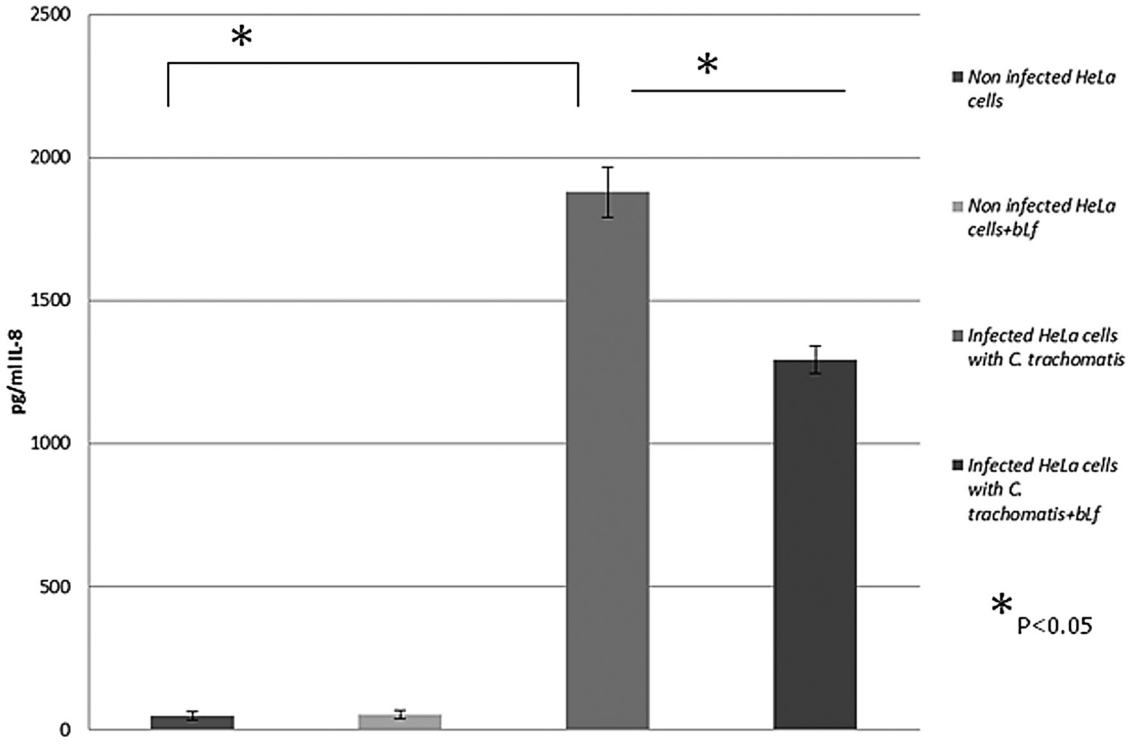
and IL-8 concentrations, even if cytokine levels remained higher than those synthesized by noninfected cell monolayers.

Clinical trials

Among 198 pregnant women, we excluded 16 women affected by bacterial vaginosis unrelated to *C. trachomatis*, and 6 women for protocol violation. One hundred seventy-six pregnant women completed the study and, among them, 7 asymptomatic pregnant women, positive to *C. trachomatis* DFA and showing high concentration of IL-6 in cervical fluids, were treated with the intravaginal administration of bLf (100 mg) every 8 h for 30 days.

After 1 month, 6 out of 7 cervical specimens were negative to *C. trachomatis* DFA, and the cervical fluids showed a decrease in IL-6 concentration (from mean values of 250 ± 19 to 50 ± 11 pg/mL). One out of 7 pregnant women was positive to *C. trachomatis* DFA and cervical IL-6 levels did not decrease, ranging between about

Fig. 3. IL-8 levels in the supernatants of HeLa-229 cell cultures after 48 h of infection. The infection was performed at a MOI of 5, and bLf was added 3 h post-infection. The IL-8 concentrations are mean values ± SD. A value of $P < 0.05$ was considered statistically significant.



270 and 300 pg/mL (Table 1). This patient was treated with antibiotics.

No maternal and neonatal side effects by bLf intravaginal administration were observed.

Discussion

Some mucosal pathogenic bacteria are not only capable of adhering but also of entering into nonprofessional phagocytes such as epithelial cells. Inside host cells, bacteria are in a protective niche in which they can replicate and persist, thus avoiding host defences. In addition, antibiotic therapies are not always effective in the eradication of intracellular pathogens (Armstead and Li 2011).

Chlamydia trachomatis is an obligate, intracellular pathogen responsible for the most common sexually transmitted bacterial disease worldwide, causing acute and chronic infections. Unlike acute infections, which can be cured with oral or topical administration of antibiotics, chronic infections are difficult to eradicate and need prolonged therapies, thus increasing the risk of developing antibiotic resistance (Kohlhoff and Hammerschlag 2015).

Therefore, novel alternative therapies are needed (Sessa et al. 2015). The difficulty in finding new agents against *C. trachomatis* infection resides in the complex life-cycle of this peculiar pathogen. In fact, *C. trachomatis* has a unique biphasic developmental cycle, alternating between the extracellular infectious EBs, metabolically inactive, and the intracellular non-infectious RBs, which are metabolically active. Of note, intracellular bacterial pathogens require intracellular nutrients, including iron, for replication in mammalian cells, and chlamydiae are no exception (Raulston 1997).

Concerning the first phase of *C. trachomatis* infection, classical antibacterial drugs are ineffective because EBs are metabolically inactive.

Conversely, antibacterial drugs could be active against intracellular replicative RBs, since they are metabolically active. However, antibacterial drugs cannot usually enter host cells.

Table 1. General characteristics of pregnant women positive for *Chlamydia trachomatis* before and after intravaginal administration of lactoferrin (100 mg every 8 h/day).

	Before treatment	After treatment
***n* = 6**		
C. trachomatis DFA	Positive	Negative
Mean values for cervical IL-6 (pg/mL)	250±19	50±11
Spontaneous delivery (week)	39–40	—
Mean values of birth weight (g)	3727±175	—
Apgar scores	9/10	—
***n* = 1**		
C. trachomatis DFA	Positive	Positive
Cervical IL-6 (pg/mL)	263	285
Caesarean section (week)	39	—
Birth weight (g)	3378	—
Apgar scores	9	—

Note: *n*, number of pregnant women; DFA, direct immunofluorescence assay.

A further key issue is represented by the intracellular redifferentiation of RBs (after intracellular replication) into EBs, which are released following the lysis of host cells, ready to infect neighboring epithelial cells and, hence, perpetuate the infectious process (Belland et al. 2003).

Therefore, an ideal drug against *C. trachomatis* infection should:

- inhibit adhesion and entry of *C. trachomatis* EBs into host cells;
- inhibit intracellular replication of *C. trachomatis* RBs;
- inhibit the reinfection of host cells by EBs, extracellularly released after the redifferentiation of RBs into EBs.

Lf is thought to play a pivotal role in the prevention of infections. Its ability to sequester iron from potential pathogens is considered an important feature to combat infections. Moreover, its cationic charge is responsible for the binding to bacterial and

cell surface components (Valenti and Antonini 2005). This Lf property has been shown to inhibit adhesion and entry into epithelial cells of several facultative intracellular bacteria (Longhi et al. 1993; Ajello et al. 2002; Di Biase et al. 2004; Willer et al. 2004; Berlutti et al. 2008); however, Lf activity against obligate intracellular bacteria as *C. trachomatis* has never been observed.

In this study, we utilized a preparation of bLf, iron -saturated at 20%, to consent further iron chelation, an essential nutrient for *C. trachomatis* developmental cycle (Raulston 1997). In fact, in the absence of free, available iron, *C. trachomatis* enters into a persistent state, as evidenced by the addition of iron-chelating agents, such as deferoxamine mesylate (DFO) or 2,2′-bipyridyl (Bpdl) to *C. trachomatis* infected cell monolayers, leading to small-sized inclusions containing enlarged, aberrant and nondividing RBs (Thompson and Carabeo 2011), unable to generate infectious progeny (Wyrick 2010).

Different from data reported by Thompson and Carabeo (2011), we found that the addition of bLf to HeLa cell monolayers 3 h post-infection resulted in no significant reduction of the number of intracellular chlamydial IFU ($P = 0.28$) (Fig. 1E) and of infectious progeny. These conflicting data could be due to the higher effective concentrations of iron-chelating agents (from 100 to 200 μmol/L) (Thompson and Carabeo 2011) compared with 1.25 μmol/L bLf, corresponding to 2.5 μmol/L iron binding sites, used in this study.

We believe it is very interesting that bLf does not affect either the replication of RBs or the induction of aberrant RBs, thus avoiding the "silent" reservoir that leads to chronic infection and inflammation.

In fact, aberrant RBs can contribute to chronic inflammation (although this aspect is still under debate). Of note, recurrent chlamydial disease may also result from the persistence of the microorganism after unresolved infections (Wyrick 2010).

To the best of our knowledge, we have demonstrated for the first time that the incubation of cell monolayers with bLf before the infection or at the moment of the infection significantly inhibited the adhesion and entry of *C. trachomatis* into epithelial cells. Therefore, the inhibition of *C. trachomatis* infectivity by bLf was dependent on its interaction with the cell surface. Moreover, bLf could bind to cell surface glycosaminoglycans as well as to heparan sulfate proteoglycans (Wu et al. 1995; Lang et al. 2011), which are potential receptors for *C. trachomatis* adhesion (Stallmann and Hegemann 2015).

Conversely, the preincubation of bLf with *C. trachomatis* did not influence its infectivity, supporting the idea that the specific interaction between bLf and epithelial host cells seems to be the sole pivotal mechanism responsible for the inhibition of *C. trachomatis* invasion.

Similar to the results obtained in epithelial cell monolayers infected with other facultative intracellular pathogens (Berlutti et al. 2006; Valenti et al. 2011; Frioni et al. 2014), the addition of bLf significantly decreased the IL-8 and IL-6 levels synthetized by *C. trachomatis* infected cells. To avoid the possibility that the IL-8 and IL-6 decrease was related to the different number of *C. trachomatis* IFUs, these experiments were carried out by adding bLf 3 h post-infection. These results demonstrated once again the ability of bLf to down-regulate pro-inflammatory cytokine synthesis. Although it has been known for years that exogenous Lf localized to cell nucleus (Ashida et al. 2004; Suzuki et al. 2008; Valenti et al. 2011), the mechanisms by which bLf could perform its anti-inflammatory activity are still under debate.

These in-vitro results, showing for the first time the protective effects of bLf against *C. trachomatis* infection, led us to investigate its efficacy also in asymptomatic pregnant women positive to *C. trachomatis* and with high levels of IL-6 in cervical fluids.

Seven out of 176 pregnant women enrolled in this pilot study, showing cervical specimens positive to *C. trachomatis*, were treated with the intravaginal administration of bLf (100 mg) every 8 h for 30 days.

After 1 month, 6 pregnant women tested negative for *C. trachomatis* and showed decreased IL-6 levels in their cervical fluids (from mean values of 250 ± 19 to 50 ± 11 pg/mL).

Similar to what we observed in the in-vitro model, intravaginal administration of bLf seems to act by protecting host cells against the adhesion and entry of chlamydial EBs, which are released extracellularly after redifferentiation of RBs to EBs. The simultaneous decrease of IL-6 levels could be a marker for the lack of reinfection by *C. trachomatis* EBs in the presence of bLf.

Even if other clinical trials are required, the protective effect of bLf against *C. trachomatis*, demonstrated for the first time in this study, suggests a further therapeutic approach based on its intravaginal administration in addition to that already reported in preventing and curing the preterm delivery (Paesano et al. 2012).

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