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Lack of effect of bovine lactoferrin in respiratory syncytial virus replication and clinical disease severity in the mouse model ☆

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ABSTRACT

Background: Lactoferrin (LF) is a glycoprotein present in human milk with known antimicrobial effects. *In vitro*, LF has demonstrated antiviral activity against respiratory syncytial virus (RSV). We sought to assess the effect of bovine (b)LF in RSV replication, lung inflammation and function, cytokine profiles and clinical disease in an *in vivo* murine model.

Methods: Female BALB/c mice were inoculated with 10^7 PFU RSV A2 or 10% EMEM. bLF or placebo (DPBS) were administered once or twice daily by oral gavage or intraperitoneal (IP) injection at doses ranging from 2 to 10 mg/animal/day, from 48 h before until 96 h post-RSV inoculation. Bronchoalveolar lavage (BAL), whole lung and serum samples were harvested on day 5 post-inoculation to asses RSV loads, lung inflammation and cytokine concentrations. Weight loss, airway obstruction and disease severity were assessed daily in all groups.

Results: On day 5 post-inoculation BAL RSV loads, lung inflammation and serum innate, Th1, Th2 and Th17 cytokine concentrations showed no differences between RSV infected mice treated with bLF and RSV infected but untreated mice independent of bLF dosing and administration route ($p > 0.05$). In addition, all bLF groups showed similar weight loss, degree of airway obstruction, and disease severity scores on days 1–5 post-inoculation which was comparable to infected untreated mice ($p > 0.05$) but higher than uninfected controls.

Conclusions: Administration of oral or IP bLF at different doses did not demonstrate antiviral activity or significant effects on disease severity in the RSV mouse model. Whether these observations could be extrapolated to infants at risk for RSV infection needs to be further explored.

1. Introduction

Respiratory Syncytial Virus (RSV) is the main cause of lower respiratory tract infection (LRTI) leading to hospitalization in infants and young children worldwide (Hall et al., 2009; Nair et al., 2010; Shay et al., 1999). There are well-characterized risk factors for severe disease (Bloemers et al., 2007; Garcia et al., 2010; Wang et al., 1996), however the vast majority of infants hospitalized with RSV are previously healthy with no known risk factors. Despite the burden of the disease there is no vaccine available and treatment remains mostly supportive and non-specific.

Several studies have shown the protective effect of breastfeeding against respiratory and gastrointestinal infections in infants (Beaudry et al., 1995; Dixon et al., 2010; Klein et al., 2008; Lopez-Alarcon et al., 1997). Lactoferrin (LF) is a multifunctional iron-binding glycoprotein, member of the transferrin family, which is produced in epithelial cells at the mucosa of many mammalians and it is expressed at high concentrations in breast milk (Bennett and Kokocinski, 1978; Metz-Boutigue et al., 1984). LF has demonstrated antibacterial, antifungal activity (Levy, 1996; Manzoni et al., 2009) and has also shown to have antiviral activity against a wide range of viruses such as hepatitis C virus (HCV), rotavirus, human immunodeficiency virus (HIV), herpes simplex virus (HSV), cytomegalovirus (CMV), and *in vitro* against RSV possibly by preventing viral entry into the target cell or inhibiting virus replication (Andersen et al., 2001; Fujihara and Hayashi, 1995; Grover et al., 1997; Hasegawa et al., 1994; Ikeda et al., 1998, 2000; Puddu et al., 1998; Superti et al., 1997). In addition, LF seems to exert an immunomodulatory effect by modulation of both the innate and adaptive host immune response (Legrand, 2012). Whether LF can

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modulate RSV disease *in vivo* and/or modify pro-inflammatory cytokine responses have not been characterized. The objectives of this study were to evaluate the therapeutic effect of bovine (b)LF in reducing RSV loads and modulating clinical disease severity, including lung function, lung inflammation and systemic cytokine concentrations in the RSV murine model.

2. Materials and methods

2.1. Animals and inoculation

Seven-week old female Balb/C mice were intranasally inoculated with 10^8 PFU/mL of RSV A2 or sham inoculated with 100 μL of sterile 10% EMEM. Animals were allowed to aspirate the inoculum for 30 s while held upright until fully recovered from the anesthesia. The Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children's Hospital approved this study and animals were maintained throughout the protocol under specific pathogen-free conditions.

2.2. Virus, growth conditions and RSV quantification

Human RSV A2 (courtesy of Dr. Pedro Piedra from Baylor College of Medicine, Houston, TX) was grown in Hep-2 cells and stored as previously described (Jafri et al., 2004; Mejias et al., 2004). Viral titers were determined by plaque assay, which had a lower limit of detection of $1.7\log_{10}$ PFU/mL (Estripeaut et al., 2008; Torres et al., 2010). The same viral batch (A21102-1) was used for all the experiments. Eighty to ninety percent confluent Hep-2 cells were used to assess viral loads in fresh bronchoalveolar lavage (BAL) as previously described (Jafri et al., 2004; Mejias et al., 2004). The remaining supernatant was stored at −80 °C for viral load quantification using a single step real time PCR targeting the N gene, with lower limits of detection of 10 copies/mL (Estripeaut et al., 2008; Mella et al., 2013).

2.3. Experimental design and sample collection

Bovine Lactoferrin (FrieslandCampina Domo, Amersfoort, The Netherlands) was daily weighted and diluted in Dubelcco's phosphate-buffered saline (DPBS) and administered by oral gavage (PO) in a total volume of 200 μL/animal from 48 h before to 4 days after RSV or 10% EMEM inoculation once or twice a day for 7 consecutive days. We used 4 different experimental doses of bLF in separate experiments including: 2 mg, 3 mg, 6 mg and 10 mg per day. Five independent experiments were performed; each group included 7–15 mice per time point per experimental group: (a) RSV infected and treated with bLF, (b) RSV infected and sham treated with DPBS and (c) non-infected sham treated mice also with DPBS; for a total of 58 mice (Table 1A). Mice were evaluated daily from day −2 to day 5 of the protocol before bLF administration to assess weight loss, airway obstruction and disease severity using a clinical disease severity score (CDSS) developed by our group that included four parameters: (1) activity, (2) fur appearance, (3) weight loss, and (4) Penh values, as a measurement of airway obstruction. Each item of the score ranged from 0 (normal) to 3 (severe) with a higher score of 12 (Table 1B). The same operator evaluated all mice prior to any other procedure. We performed 4 additional experiments in which we used bLF at doses of 40, 50 and 100 mg/animal/day via oral gavage from 96 h, 72 h and 48 h before to 4 days, 6 days and 9 days after RSV inoculation respectively. Lastly we performed an additional experiment in which bLf was administered by intraperitoneal (IP) injection at 3 different doses (1 mg, 5 mg and 10 mg/animal/day) starting at 48 h before to 4 days post RSV inoculation with similar results (data not shown).

On day 5 post-RSV inoculation animals were anesthetized with carbon dioxide before cardiac puncture. Blood was collected and serum specimens were stored at −80 °C for further cytokine analysis. Bronchoalveolar lavage (BAL) was obtained as previously described for viral load quantification by culture and real-time PCR and whole-lung specimens were harvested and fixed in 10% buffered formalin for histologic evaluation. Formalin fixed lung sections were stained with H&E and further evaluated by a pathologist blinded to the study protocol to assess pulmonary inflammation using a standardized histopathologic score (HPS) that has been previously validated in the RSV mouse model (Estripeaut et al., 2008; Jafri et al., 2004; Mejias et al., 2004; Torres et al., 2010). The HPS system assigns values from 0 (no inflammation) to 17 (severe inflammation) based on 5 parameters: (1) peribronchiolar and bronchial infiltrates, (2) bronchiolar and bronchial luminal exudates, (3) perivascular infiltrate, (4) parenchymal monocytic infiltrate, and (5) parenchymal pneumonia (granulocytic infiltrates in the alveolar spaces).

2.4. Pulmonary function test

Unrestrained whole-body plethysmography (Buxco Research Systems, Troy, NY) was used to assess airway obstruction by measuring baseline enhanced pause (Penh) daily from day −2 to day 5 post RSV inoculation in all experimental groups. Previous studies showed that Penh values correlate with pulmonary airflow resistance or obstruction (Estripeaut et al., 2008; Jafri et al., 2004; Mejias et al., 2004; Rios et al., 2004; Torres et al., 2010). Respiratory rate (RR) was also measured in all experimental groups daily from day −2 to day 5 post-RSV inoculation.

2.5. Cytokine concentrations in serum samples

Concentrations of the following cytokines were measured in serum samples on day 5 post-RSV inoculation using a 20-plex Bead Immunoassay (Invitrogen Corporation, Carlsbad, CA): interferon (IFN)-γ, interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, interferon gamma-induced protein 10 (IP-10), KC (murine IL-8 homologous), monocyte chemotactic protein (MCP)-1, monokine induced interferon gamma (MIG), macrophage inflammatory protein (MIP)-1α, tumor necrosis factor (TNF)-α, fibroblast growth factor (FGF)-basic, granulocyte macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF). All samples were run in duplicate. The lowest limit of detection ranged from 4 to 76 pg/mL. For statistical

Table 1A
bLF dosing according to study group.

Group (dosing)	Number of animals per group
RSV infected bLF treated (2 mg/day)	$N = 7$
RSV infected bLF treated (3 mg/day)	$N = 7$
RSV infected bLF treated (6 mg/day)	$N = 8$
RSV infected bLF treated (10 mg/day)	$N = 12$
RSV infected untreated	$N = 15$
Uninfected control	$N = 9$

Table 1B
Clinical disease severity score.

	Normal (0)	Mild (1)	Moderate (2)	Severe (3)
Activity	Normal	Decreased	Inactive	Inactive and hunched
Ruffled Fur	Normal	Barely	Moderate	Severely
Weight loss	No	3–9%	10%	>10%
Penh	0.4–0.6	0.7–0.9	1–1.2	>1.3

analysis, samples with optical fluorescence intensity readings below the limit of the standard curve of the assay were assigned a value half of that of the detection level. Plates were read using Luminex[100] plate reader (Luminex Corporation, Austin, TX).

2.6. Statistical analysis

Differences between groups were tested using one-way analysis of variance (ANOVA) or non-parametric Kruskal–Wallis test. If these tests demonstrated a significant difference between groups ($p < 0.05$) Tukey or Dunn's test to correct for multiple comparisons was used. For all statistical analyses GraphPad Prism 5 and Sigma Plot (version 11.0) were used.

3. Results

3.1. Comparative effect of bLF on RSV loads determined by tissue culture and RT-PCR

On day 5 post-inoculation, the peak of RSV disease in this model, BAL samples were harvested and viral loads determined by plaque assay and real-time PCR. By quantitative culture, viral loads in RSV-infected mice treated with bLF at 2 mg, 3 mg, 6 mg and 10 mg/animal/day were not significantly different than those measured in RSV-infected untreated mice 3.31 [3.12–3.52] PFU/mL $\log_{10}$ but significantly higher compared with uninfected controls, which were below the limit of detection ($p < 0.0001$) (Fig. 1A). BAL RSV loads by RT PCR ranged from 4.82 [3.70–5.57] to 5.51 [5.24–5.83] on day 5 post-infection, and were not significantly different between RSV-infected bLF treated and untreated mice ($p > 0.05$). RSV RNA was not detected in any uninfected control (Fig. 1B).

3.2. Lack of effect of bovine Lactoferrin on RSV-induced clinical disease

To determine the effect of bLF on clinical disease severity, mice were weighted daily and disease severity assessed using a clinical disease severity score. Except for uninfected controls whose weight remained unchanged throughout the study (<5% of variation); all RSV infected animals lost from 9.25% to 16.40% (day 5) of total body weight independent of the administration of bLF (Table 2).

In addition to better assess disease severity we used a clinical disease severity (CDSS) score that included subjective [(1) level of activity and (2) fur appearance] and objective parameters [(3) weight loss, and (4) Penh values] for 8 consecutive days. CDSS before RSV inoculation were 0 [0–1] in all groups and remain unchanged in uninfected controls. However, CDSS in RSV-infected mice significantly increased after RSV inoculation (Fig. 2A). We did not find significant differences in CDSS between RSV-infected mice treated with bLF and RSV-infected untreated mice from day 1 to day 5 post RSV inoculation ($p > 0.05$). On day 5 post RSV inoculation, the mean CDSS ranged from 7.4 ± 1.2 to 9.2 ± 1.3 on RSV-infected mice treated with bLF while mean values for RSV-infected untreated mice was 9.2 ± 0.8 (Fig. 2B).

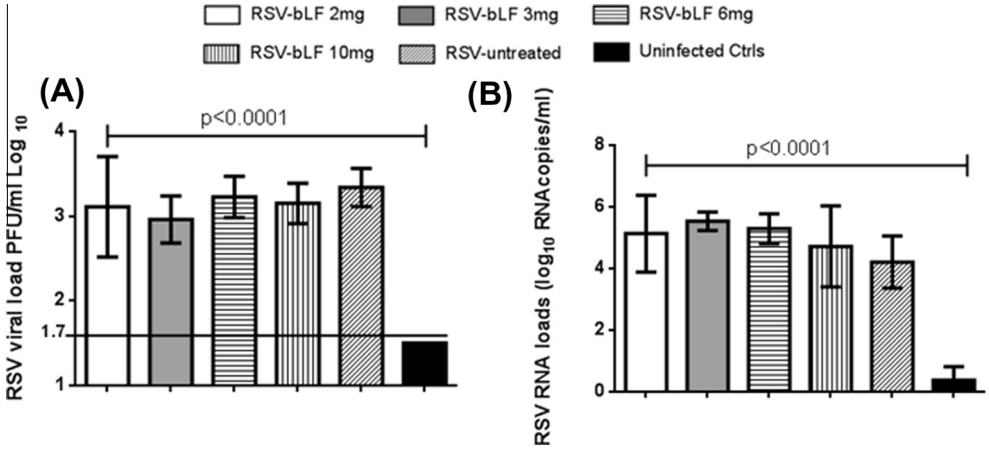

Fig. 1. Comparative effect of bLF on BAL RSV loads: (A) On day 5 post RSV inoculation, mice (7–15 per group) were euthanized and BAL RSV loads measured by plaque assay. Viral loads were significantly higher in RSV-infected mice compared to uninfected controls independent of bLF administration ($p < 0.0001$); (B) BAL RSV RNA loads were significantly higher in RSV-infected treated or untreated mice compared with uninfected mice ($p < 0.0001$). Comparisons made by one-way ANOVA with Tukey's test for multiple comparisons.

Table 2
Comparative weight loss of RSV-infected bLF treated mice, RSV-infected untreated mice and uninfected controls during acute RSV infection.

Group	Protocol days								Total weight loss (%)
	Day −2	Day −1	Day 0	Day 1	2	Day 3	Day 4	Day 5	
Uninfected controls	17.7 ± 1.22	17.5 ± 1.43	17.0 ± 2.15	17.0 ± 2.15	16.8 ± 2.09	16.9 ± 2.11	17.2 ± 1.87	17.1 ± 1.65	3.39
RSV untreated	18.5 ± 1.80	18.3 ± 2.09	18.2 ± 2.11	17.4 ± 2.00	17.2 ± 1.85	17.5 ± 1.95	16.9 ± 1.99	15.9 ± 1.90	14.05
RSV-bLF 2 mg	17.3 ± 1.32	17.6 ± 0.95	17.2 ± 1.94	16.5 ± 2.18	16.4 ± 2.32	16.1 ± 2.70	16.8 ± 0.78	15.7 ± 0.81	9.25
RSV-bLF3 mg	18.9 ± 0.77	18.9 ± 0.66	18.9 ± 0.74	18.2 ± 0.79	17.7 ± 0.84	17.3 ± 0.66	17.1 ± 1.17	15.8 ± 0.77	16.40
RSV-bLF 6 mg	18.2 ± 1.16	18.2 ± 1.31	17.7 ± 1.72	17.8 ± 1.26	17.0 ± 1.31	16.5 ± 0.99	16.6 ± 1.19	15.3 ± 1.01	15.93
RSV-bLF 10 mg	17.8 ± 1.40	17.4 ± 1.56	17.4 ± 1.64	16.5 ± 1.64	16.7 ± 1.29	16.6 ± 1.46	16.3 ± 1.20	15.3 ± 1.13	14.04
[a]*p* value	0.310	0.279	0.356	0.324	0.777	0.563	0.860	0.161	

Weight (in grams) is expressed as mean ± SD.
[a] One-way ANOVA with Tukey test for multiple comparisons was applied to identify differences between the groups ($p < 0.05$).

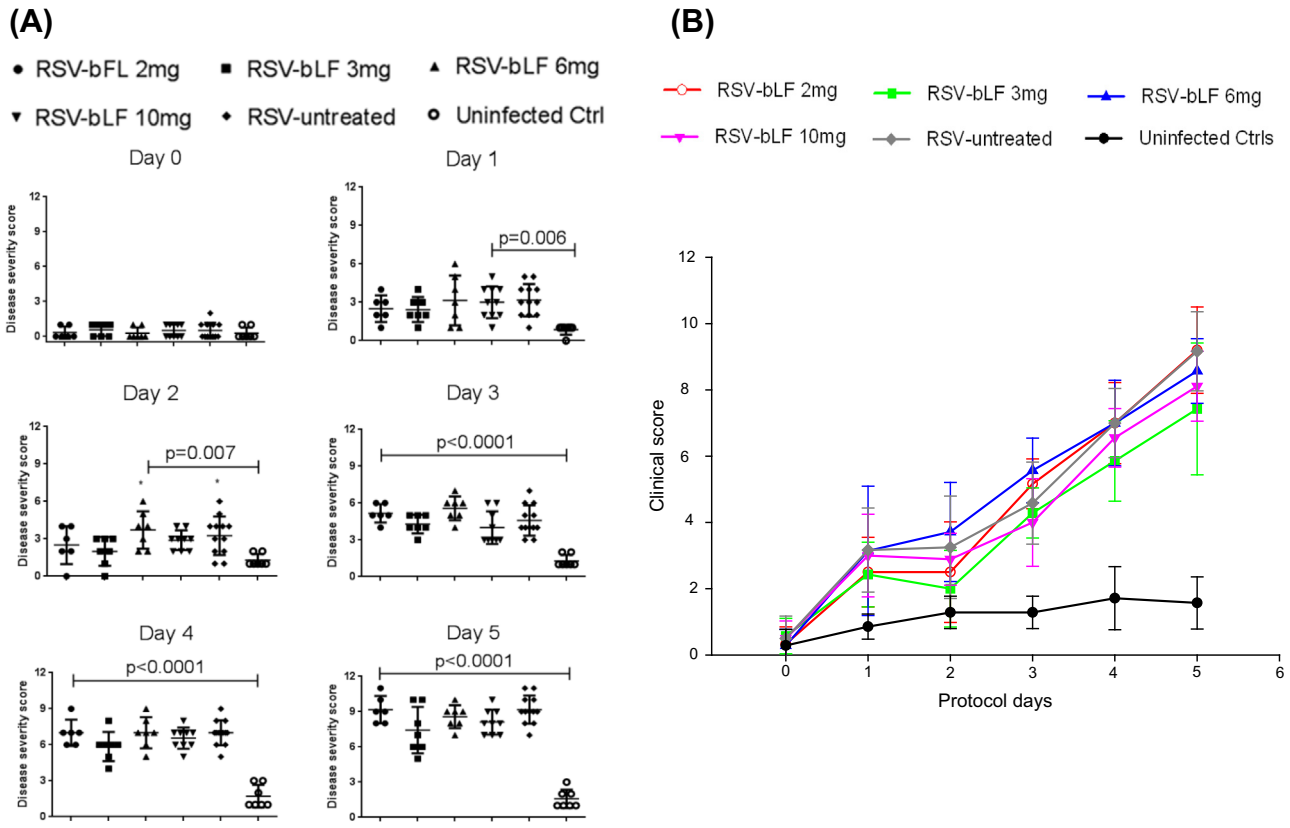

Fig. 2. Clinical disease severity scores (CDSS) in RSV-infected mice treated with bLf at different doses and RSV-infected untreated mice. (A) Disease severity scores were recorded daily before any intervention and comprised four parameters: level of activity, fur appearance, weight loss and airway obstruction (Penh). (B) Comparison between RSV-infected mice treated with bLF and untreated mice. Comparisons made using One-way ANOVA with Tukey's test for multiple comparisons.

Table 3
Serum cytokine concentrations (pg/mL) on Day 5 post-RSV inoculation.

	2 mg	3 mg	6 mg	10 mg	RSV-untreated	Uninfected	[a]*p* value	[b]*p* value
Th1 response								
IFN-γ	340.9 [259.6–548.6]	591.2 [549.2–958.6]	509.1 [435.8–528.8]	393.2 [337.2–577.4]	592.3 [521.5–711.9]	11	0.037[d]	0.005[d]
IL-2	38.1 [25.8–95.9]	51.9 [43.4–53.9]	37.5 [31.3–37.5]	35.1 [24.1–43.6]	38.8 [36.3–45.3]	36.0 [32.9–37.5]	0.061[d]	0.076[d]
IL-12	107.2 ± 44.3	122.4 ± 30.6	112.5 ± 48.7	124.8 ± 56.3	116.4 ± 21.4	54.0 ± 45.4	0.952[c]	0.480[c]
Th2 response								
IL-4	252.8 [148.7–382.9]	321.1 [304.7–597.1]	243.2 [195.4–380.6]	223.9 [174.1–286.8]	311.2 [259.2–337.3]	17	0.034[d]	0.005[d]
Proinflammatory response								
IL-1β	142.5 [107.6–217.4]	243.9 [202.9–354.3]	186.1 [153.6–200.8]	175.4 [142.5–223.9]	207.1 [194.5–271.8]	13.5	0.010[d]	0.002[d]
IL-6	65.0 ± 38.1	100.4 ± 35.9	72.2 ± 19.5	73.2 ± 23.7	89.1 ± 19.7	16.5	0.189[c]	0.004[c]
TNF-α	35.2 [10.3–90.0]	51.2 [25.0–78.9]	25.0 [18.7–25.0]	25.0 [10.1–41.6]	35.2 [30.1–54.7	35.2 [10.6–43.7]	0.136[d]	0.202[d]
Chemokines								
KC	341.6 [235.8–785.9]	553.8 [393.6–933.3]	356.9 [238.8–400.7]	329.1 [252.8–379.2]	474.5 [324.3–1092.4]	266.6 [257.5–386.5]	0.155[d]	0.164[d]
IP-10	426.1 ± 130.5	570.1 ± 88.7	490.7 ± 51.5	552.2 ± 89.5	525.6 ± 32.6	5.5	0.053[c]	<0.001[c]
MIG	683.7 [125.2–1541.7]	295.2 [201.5–848.3]	257.6 [173.8–277.4]	166.0 [109.0–215.8]	305.5 [207.2–338.6]	42.9 [40.8–49.0]	0.108[d]	0.014[d]
MCP-1	58.2 ± 43.7	97.6 ± 14.2	72.6 ± 13.9	85.5 ± 24.1	78.5 ± 13.8	23.5	0.078[c]	0.006[c]
MIP-1α	50.5 ± 42.2	60 ± 21.3	28.9 ± 11.1	34.4 ± 20.5	56.9 ± 22.3	56.2 ± 11.6	0.074[c]	0.083[c]
Growth factors								
FGF-basic	113.5 [56.3–144.2]	119.2 [66.8–148.4]	81.7 [44.9–155.8]	66.6 [27.7–91.8]	98.3 [88.5–107.6]	25	0.111[d]	0.017[d]

Data is expressed as mean ± SD or median [25–75 IQR] as appropriate. For uninfected controls we evaluated cytokine concentration in 4 mice while for RSV-infected groups treated with bLF or DPBS the number of mice ranged from 6 to 8 animals per group.
[a] Comparisons between RSV-infected-bLF treated and RSV-infected untreated groups.
[b] Comparisons between RSV-infected groups and uninfected controls.
[c] One-way ANOVA with Tukey test for multiple comparisons.
[d] Kruskal–Wallis with Dunn's multiple comparison test.

3.3. Effect of bovine Lactoferrrin on systemic cytokine concentrations

Twenty cytokines were measured in serum using a 20-plex bead immunoassay. Of those, IL-1α, IL-5, IL-10, IL-13, IL-17, GM-CSF and VEGF were below the limit of detection in all experimental groups. Of the Th1-related cytokines (IL-2, IL-12 and IFN-γ) only IFN-γ concentrations were significantly increased in RSV-infected mice compared with non-infected controls independent of the administration of bLF ($p = 0.005$). Similarly, concentrations of IL-4 (Th-2), the chemokines CXCL9/MIG, CXCL10/IP-10, CCL2/MCP-1

and the pro-inflammatory cytokines IL-1β and IL-6 were significantly increased in RSV infected mice compared with non-infected controls with no differences observed with the administration of bLF. Other cytokines and chemokines such as, KC (IL-8), TNF-α, or CCL3/MIP-1α were slightly elevated in RSV infected treated or untreated mice compared with non-infected controls (Table 3). Lastly, Th1:Th2 (IFN-γ:IL-4) ratios were comparable between untreated and bLF treated RSV-infected mice (data not shown).

3.4. Lung inflammation in RSV infected mice treated with bovine Lactoferrin

On day 5 post-inoculation, RSV-infected mice treated with bLF showed similar HPS scores compared with RSV-infected untreated mice (8.33 ± 0.78) independent of the bLF dose ($p > 0.05$) and demonstrated dense inflammatory infiltrates, acute pneumonia with a predominant neutrophilic infiltrate, and moderate number of macrophages in the alveolar space (Fig. 3A–C). In addition, there were no significant differences when analyzing each component of the score separately which included (1) peribronchiolar and bronchial infiltrates, (2) bronchiolar and bronchial luminal exudates, (3) perivascular infiltrate, (4) parenchymal monocytic infiltrate, and (5) parenchymal pneumonia (granulocytic infiltrates in the alveolar spaces). Non-infected controls did not show signs of inflammation with HPS scores consistently ranging between 0 and 1 (Fig. 3A).

3.5. Effect of bovine lactoferrin in Pulmonary Function during acute RSV infection

To assess airway obstruction (AO), baseline Penh values were measured daily in all study groups. Penh values increased transiently on day 1 after RSV inoculation, decreased by day 2 and increased again on day 3 reaching the peak on day 5 post-RSV inoculation. Daily administration of bLF did not modify AO, which was comparable between bLF treated mice and RSV-infected untreated mice ($p > 0.05$) (Fig. 4). Respiratory rate (RR) was also evaluated in all experimental groups. RR values ranged from 394.3 (±94.59) breaths per minute (bmp) to 475.6 (±46.40) bmp on day -2 of the protocol and from 376.4 (±74.50) to 403.0 (±26.57) bmp on day 5 post-RSV inoculation independent of bLF administration. As shown in Table 4, RR values were similar between groups ($p > 0.05$) (Table 4).

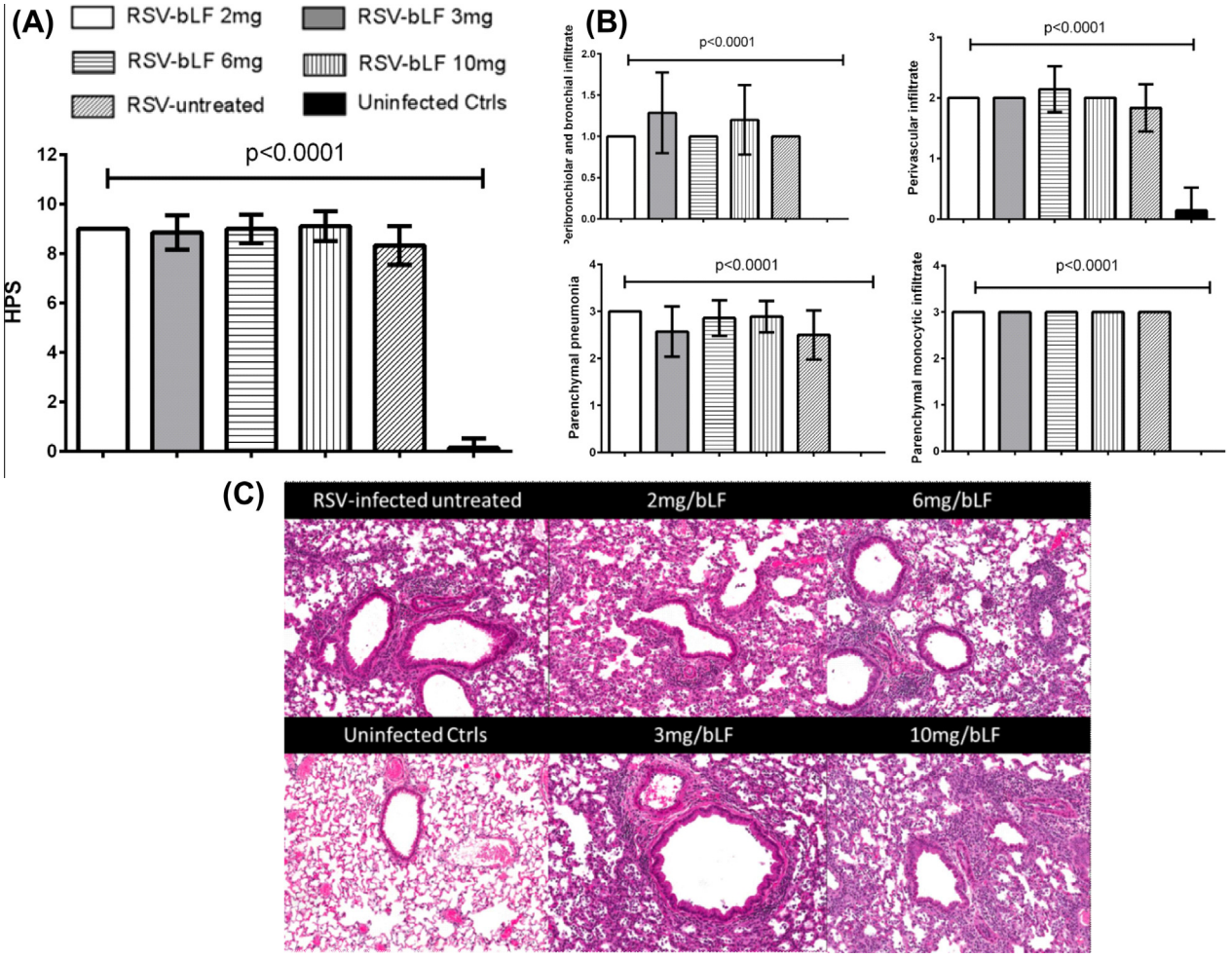

Fig. 3. Effect of bLF treatment on lung inflammation. (A) Lung HPS on day 5 post RSV inoculation ($n = 7–15$); (B) Evaluation of each component of the HPS individually. Luminal exudate scores were zero in all samples (not shown). Values are represented as means and SD, $p < 0.05$ by one-way ANOVA with Tukey's multiple comparison test. RSV-infected bLF treated mice showed similar HPS scores compared with RSV-infected untreated mice ($p > 0.05$), but significantly greater compared with uninfected controls ($p < 0.0001$) when assessing the total score and each individual parameter. (C) Lung sections from RSV infected mice showed activation of the vascular endothelium with binding of intravascular leukocytes to the endothelial cells and perivascular edema. In addition, acute pneumonia with neutrophils predominance and moderate numbers of macrophages in the alveolar spaces was observed. In some foci, the alveolar spaces were expanded by lymphocytic inflammation. No significant differences in the degree or type of inflammation were found between the different treatment groups. In contrast, lung sections from uninfected mice demonstrate no perivascular/peribronchial infiltrates or alveolar infiltrates.

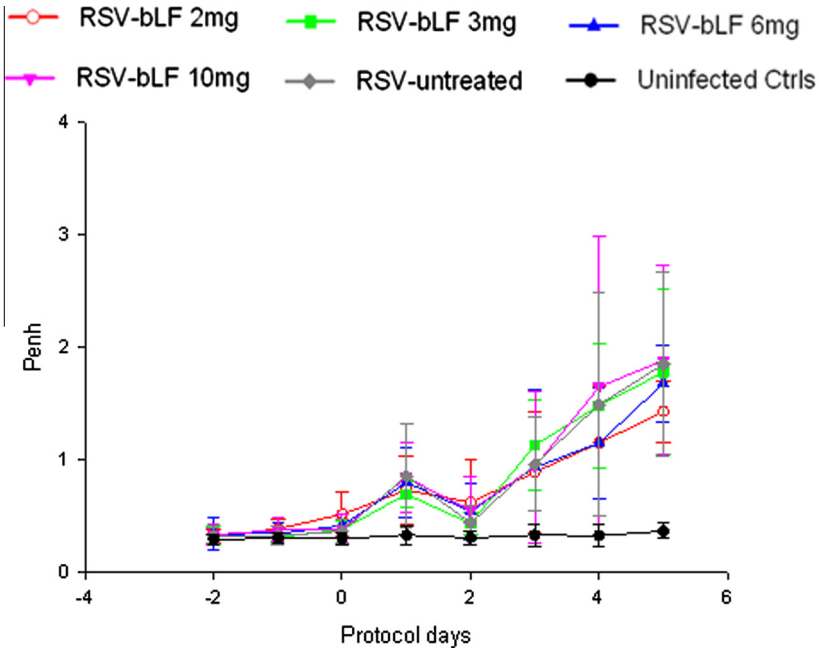

Fig. 4. Effect of bLF on airway obstruction. To assess AO Penh values were recorded daily before administration of bLF or DPBS. Y axis represents the baseline Pehn values and the X axis the study days. Comparisons made by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$).

Table 4
Respiratory rate of RSV-infected bLF treated mice, RSV-infected untreated mice and uninfected controls during acute RSV infection.

Groups	Protocol days							
	Day −2	Day −1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Uninfected ctrl	441.7 (±80.2)	384.0 (±45.4)	377.7 (±36.1)	395.3 (±37.3)	381.3 (±43.9)	389.9 (±31.8)	375.3 (±34.5)	381.3 (±29.9)
RSV-untreated	469.1 (±57.7)	380.6 (±61.1)	350.3 (±38.8)	397.8 (±34.9)	391.5 (±54.0)	379.5 (±80.2)	406.2 (±49.7)	394.1 (±48.4)
RSV-bLF 2 mg	394.3 (±94.6)	373.1 (±58.9)	316.0 (±60.3)	390.3 (±35.1)	390.0 (±74.8)	403.1 (±56.7)	400.8 (±35.2)	403.0 (±26.6)
RSV-bLF 3 mg	441.6 (±39.2)	395.1 (±52.3)	334.3 (±24.9)	415.3 (±27.9)	413.7 (±33.7)	411.3 (±32.6)	407.6 (±26.2)	389.7 (±32.7)
RSV-bLF 6 mg	475.6 (±46.4)	380.5 (±50.5)	348.6 (±31.6)	409.1 (±21.7)	400.0 (±26.1)	411.3 (±42.2)	408.9 (±50.2)	376.4 (±74.5)
RSV-bLF 10 mg	438.3 (±47.8)	366.5 (±80.7)	335.7 (±58.2)	387.1 (±43.9)	372.8 (±53.4)	409.9 (±62.9)	384.8 (±36.3)	401.5 (±45.7)
[a]*p* value	0.137	0.950	0.210	0.581	0.665	0.734	0.404	0.834

Respiratory Rate (in breaths per minute/BPM) is expressed as mean ± SD.
[a] One-way ANOVA followed by Tukey post hoc test for multiple corrections was applied to identify differences between the groups ($p < 0.05$).

4. Discussion

In this study we were unable to demonstrate a protective effect of lactoferrin on the response to RSV infection in the mouse model. RSV replication, the systemic cytokine responses, clinical disease severity scores, lung inflammation and lung function, did not differ following the administration of bovine lactoferrin by different routes and at different doses. To our knowledge this is the first study that has analyzed in depth the effect of bLF in an *in vivo* model of RSV infection. Studies, mostly performed in cell culture suggest that LF exhibits an antiviral effect against a broad range of RNA and DNA viruses that infect humans and animals (Andersen et al., 2001; Gonzalez-Chavez et al., 2009; Grover et al., 1997; Hasegawa et al., 1994; Puddu et al., 1998; Sano et al., 2003). *In vitro* LF exhibits inhibitory activity against HSV-1, HIV, HCV, poliovirus, rotavirus, CMV and also against RSV (Andersen et al., 2001; Fujihara and Hayashi 1995; Hasegawa et al., 1994; Ikeda et al., 1998, 2000; Portelli et al., 1998; Puddu et al., 1998; Sano et al., 2003; Superti et al., 1997). It has been suggested that the antiviral activity of LF lies in the early phase of infection by interacting with virus particles or blocking cellular receptors and therefore preventing the internalization of the virus (Berlutti et al., 2011).

The antiviral effect of LF on RSV has been demonstrated in different *in vitro* studies. Human LF inhibited RSV growth by culture even when used at lower concentrations than those present in human milk (Fujihara and Hayashi, 1995; Portelli et al., 1998). In addition to its antiviral effect *in vitro* which seems to be related to direct binding of hLF to the F1 protein subunit of RSV, human LF has shown an immunomodulatory effect as it significantly reduced the concentrations of IL-8 when added to Hep-2 cells before RSV infection (Sano et al., 2003). Despite the anti-RSV effect of LF *in vitro*, we could not document *in vivo* suppression of RSV replication or differences in lung or systemic inflammation. Similarly, a small study in mice that used intranasal inoculation of lactoferrin on days 2–5 following RSV infection, did not show a positive effect on lung function, lung inflammation or RSV loads on day 6 post infection (Welliver et al., 1999). In a mouse model of influenza infection a dose of 62.5 mg of bLF by gavage administered daily starting 24 h before the infection, did not show either antiviral, immunomodulatory or clinical effect, as influenza loads assessed by culture on day 6 post-inoculation, BAL concentrations of IL-6, IL-12 and IFN-γ, and weight loss were not modified compare to infected untreated controls. The lack of antiviral activity was attributed to a possible lack of absorption from the small bowel to the

systemic circulation and therefore to the lungs, which could partially explain our results. On the other hand, pre-treatment with LF seemed to be associated with reduction of lung consolidation assed by a lung consolidation score suggesting a beneficial effect of LF in the development of influenza-induced pneumonia (Shin et al., 2005). Similar results were found in a mouse model of HSV-2 infection where viral titers were not reduced with the administration of LF at doses of 200 μg/body before or after viral inoculation by oral gavage (Shestakov et al., 2012). In contrast, in a mouse model of LPS-induced lung injury the IP injection of LF (5 mg/mouse) was associated with significant reductions of total number of leukocytes in BAL samples, increased IL-10 and decreased TNF-α concentrations (Li et al., 2012). Studies have shown that lactoferrin is relatively resistant to digestion by gastric enzymes and is able to retain its antiviral properties against enteroviruses, which replicate primarily in the gut (Furlund et al., 2012). We suggest that the lack of effect of bLF in the lungs after oral administration could be explained in part by the fact that the primary site of RSV infection is the respiratory and not in the gastrointestinal tract where LF-virus interactions are more likely to occur. The immunomodulatory effect of lactoferrin has been previously described (Legrand et al., 2006). In mice oral administration of bovine lactoferrin stimulated intestine-associated immune functions including IL-18 dependent NK-cell activity, and local expression of type-I interferon (Kuhara et al., 2006). In patients with chronic HCV bLF monotherapy significantly reduced HCV viremia by inducing a Th1 cytokine environment, and in children with recurrent respiratory tract infections the administration of bLF and curcumin modified the lymphocyte population and cytokine responses (Zuccotti et al., 2009).

In summary our findings differ from other studies performed *in vitro* and *in vivo* systems showing a positive effect of LF on other viral infections. In our study oral administration of bLF did not exert antiviral activity on day 5, the peak of RSV replication in this model. In addition we did not observe differences in the concentrations of pro and anti-inflammatory cytokines, lung inflammation and function or clinical disease. Possible explanations could be related to timing, dosing and route of bLF administration, which we tested: (1) We performed different experiments with lower and higher doses [ranging from 0.75 mg/animal/day to 100 mg/animal/day (data not shown)] and found similar results; (2) we used different protocols of bLF administration starting 96 h prior RSV inoculation, again with no differences observed between study groups (data not shown) and, (3) we also tested an alternative route of administration (IP injection) with comparable results. The pharmacokinetic and pharmacodynamic properties of bLF in mice are not completely understood and it is possible that the doses used in this study did not achieve optimal lung tissue concentrations. The fact that bLF treatment was not able to reduce viral infectivity or to ameliorate clinical disease in the mouse model does not preclude that it may have beneficial effects in humans. The search for new nutritional strategies to support health of infants is a promising field. Clinical studies addressing the role of bLF in infants, the target population for RSV disease, are needed.

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