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ORIGINAL ARTICLE

Modulatory Effects of Oral Bovine Lactoferrin on the IgA Response at Inductor and Effector Sites of Distal Small Intestine from BALB/c Mice

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Abstract Bovine lactoferrin (bLf) up-modulates intestinal IgA that is essential for homeostasis and which might confer protection to the distal small intestine that is vulnerable to inflammation. This study analyzed the effects of bLf administered orally on the IgA response at inductive (Peyer's patches) and effector (lamina propria) sites of the distal small intestine in mice. Groups of five healthy male BALB/c mice were orally treated with 5 mg of bLf for 7, 14, 21, or 28 days. Then, mice were killed and the distal small intestine was dissected. Intestinal fluid samples were analyzed to determine IgA and IgM levels by enzyme-immuno assay. Peyer's patches and lamina propria were analyzed for IgA^+ or IgM^+ plasma cells, B, $CD4^+$ T and $CD8^+$ T cells as well as $CD4^+$ T cells positive for either pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, interferon-γ and interleukin (IL)-12] or for IgA-producing ILs (IL-4, -5, -10 and -6) by cytofluorometry. Antibodies, antibody-secreting cells, and B and T responses in both Peyer's patches and lamina propria were higher in bLf-treated than bLf-untreated mice. The generation of IL-10 and IL-6 $CD4^+$ T cells in Peyer's patches or TNF-α and IL-12 $CD4^+$ T cells in lamina propria showed similar response patterns. On days 14 and 28, cytokine/IL $CD4^+$ T cell responses were increased in Peyer's patches or decreased in lamina propria. The effect of bLf on the elicitation of IgA indicates a potential application of bLf as a nutraceutical to control inflammation in the distal small intestine.

Keywords Bovine lactoferrin · IgA · Intestinal modulation · Ileum · Peyer's patches · Intestinal lamina propria

Introduction

Immunoglobulin A (IgA) is a key component of mucosal immunity and is involved in mechanisms of protection and homeostasis in the intestine (Cerutti 2008). IgA production requires the activation and class switch recombination (CSR) of IgM^+ B cells to become IgA^+ B cells (Cerutti 2008). At the intestinal level, adaptive IgA^+ B cell generation occurs at the germinal centers of Peyer's patches and entails the participation of $CD4^+$ T cells previously primed by antigen-activated presenting cells such as dendritic cells. Surface ligation of the peptide-major histocompatibility complex (MHC)-II (on B cells) with an α/β T cell receptor (on T cells) and CD40 (B cell) with CD40L (T cell) is critical for B cell activation and CSR. Several $CD4^+$ T subpopulations contribute to T-dependent CSR including T regulatory (Treg) cells that secrete transforming growth factor (TGF)-β that has a pivotal role in CSR, and Th2 lymphocytes secreting interleukins (ILs) such as IL-2, -4, -5, -6 and -10 that induce IgA^+ B cell

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maturation into IgA^+ plasma cells. Furthermore, T follicular helper cells derived from Treg contribute to CSR by releasing TGF-β (Cerutti 2008). After priming, naive IgA^+ B and T lymphocytes differentiate to either effector or memory cells, traffic out from the Peyer's patches via the efferent lymph and migrate toward the mesenteric lymph nodes and the thoracic duct to reach the peripheral blood. Finally, lymphocytes home to the intestinal lamina propria where IgA^+ B cells mature into IgA^+ plasma cells, which release dimers and/or polymers of IgA joined by a J-chain (Cerutti 2008).

Bovine lactoferrin (bLf) is a diferric (Fe^{3+}) binding glycoprotein (80 kDa molecular weight) secreted in milk and other exocrine secretions, which belongs to the transferrin family. bLf has immune-modulatory effects on antigen presenting cells, Th1/Th2 lymphocyte balance and pro-inflammatory cytokines such as interferon (IFN)-γ, TNF-α and IL-12 (Fischer et al. 2006; Puddu et al. 2009). Several immuno-modulatory effects of exogenous bLf have been observed in the intestine following its oral administration to healthy mice. These include the up-modulation of intestinal IgA, serum IgM, IgA^+ and IgM^+ B cells from lamina propria, IL-4 and IL-5 from Th2 Peyer's patches cells, IL-10 from intraepithelial lymphocytes, IFN-γ from Th1 mesenteric lymph nodes and the proliferation of lymphocytes from Peyer's patches or $CD8^+$ T cells in the colon (Debbabi et al. 1998; Sfeir et al. 2004; Takakura et al. 2006; Wang et al. 2000). Moreover, bLf stimulates the generation of MHC-II^+ cells as well as $CD21^+$ MHC-II^+ B cells in ileal Peyer's patches in piglets (Comstock et al. 2014).

Altered relationships of IgA with microbiota might underlie inflammatory responses in the distal small intestine (Maynard et al. 2012). The effects of oral bLf on the production of IgA at inductive (Peyer's patches) and effector (lamina propria) compartments with a focus on the distal small intestine from healthy mice have not been reported. Information concerning this approach could provide knowledge about the influence of bLf on the generation of IgA involved in mechanisms for the control of homeostasis at the distal small intestine that is prone to inflammation (Maynard et al. 2012). The aim of this study was to analyze the effects of orally administered bLf on the IgA response and associated parameters in inductive and effector compartments of the distal small intestine in healthy BALB/c mice. Our results provide evidence supporting the up-modulating influence of oral bLf on IgA and IgM antibodies, IgA^+ and IgM^+ plasma cells, total B cells, $CD4^+$ and $CD8^+$ T lymphocytes either in the inductive or effector sites of the distal small intestine. The increased responses of IgA induced by bLf might help preserve homeostasis and physiological inflammatory reactions at the distal small intestine.

Materials and Methods

Animals

Male BALB/c mice (Harland Sprague–Dawley) at 8 weeks of age and 25–30 g of body weight were housed as groups of five in plastic cages with woodchip bedding. Food (5001 Laboratory Rodent Diet, LabDiet, St Louis, MO, USA) and purified drinking water were provided ad libitum. Animal handling was in accordance with the Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) and was approved by the Institutional Animal Care and Use Committee. Animals were held under an adaptation period to environmental conditions for 2 weeks before starting the interventions after 10 weeks of age.

Bovine Lactoferrin

Bovine lactoferrin (3 % iron saturated) was purchased from NutriScience Innovations (Trumbull, CT, USA) and the purity was confirmed as described previously (Drago-Serrano et al. 2010). A solution of bLf containing 5 mg per 100 μL of vehicle (sterile saline solution 0.85 % w/v NaCl) was prepared for the experimental treatments. This dose of bLf was optimal for eliciting modulatory effects that provide host resistance to intestinal infections caused by pathogenic bacteria (Drago-Serrano et al. 2010).

Experimental Design

Four groups of five mice were treated daily by buccal deposition with a micropipette containing 100 μL of sterile saline solution with 5 mg of bLf for 7, 14, 21 and 28 days. The control group consisted of mice receiving a standard diet with free access to water.

Collection of Samples

Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (100-mg/kg ketamine; 13-mg/kg xylazine) cocktail, and exsanguinated by cardiac puncture. The small intestine was dissected and the distal segment was flushed with 5 mL of sterile phosphate-buffered saline (PBS) with 0.02 % sodium azide. Intestinal washings were centrifuged at $10{,}000 \times g$ for 20 min at 4 °C and the intestinal fluids collected from the supernatants were mixed with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany) and stored at −70 °C until evaluation of total and specific antibodies (Resendiz-Albor et al. 2010). Then, Peyer's patches and lamina propria were collected for flow cytometry assays.

Enzyme-Immuno Assay

Levels of IgA and IgM in intestinal fluids were assessed by enzyme-immuno assay based on a protocol previously described (Drago-Serrano et al. 2010). Total IgA and IgM in μg/mL or levels of anti-bLf IgA or IgM as absorbance values at $\lambda = 490$ (A490 nm) from five mice per day are reported as the mean ± standard deviation (SD).

Flow Cytometry Assays

The isolation of lymphocytes from Peyer's patches and lamina propria from the distal small intestine was carried out as previously described (Resendiz-Albor et al. 2005, 2010). Cell suspensions of Peyer's patches and lamina propria from the distal small region were adjusted to 1×10^6 cells/mL in PBS for cytofluorometric analysis (Resendiz-Albor et al. 2005). The percentage of IgA^+ and IgM^+ plasma cells was detected by the addition of a cocktail containing anti-CD19 PE, anti-CD138 APC, anti-IgA FITC and anti-IgM FITC antibodies (all from BD Biosciences, San Jose, CA, USA). Plasma cells and B cells were fixed, permeabilized, and stained according to the BD Bioscience protocol for intracellular staining. The surface phenotype of T cells was detected using fluorescent-labeled monoclonal antibodies: anti-CD3 FITC, anti-CD4 PerCP, anti-CD8α PE and the B cell surface phenotype was detected using a cocktail containing anti-CD19 APC and anti-CD45/B220 PerCP antibodies (all from BD Biosciences). Cells were incubated for 30 min at room temperature. Then, the cells were washed with PBS and fixed in 2 % formaldehyde. To detect intracellular cytokine production, lymphocytes were stimulated with a mixture containing phorbol myristate acetate, ionomycin and Brefeldin A (Leukocyte Activation Cocktail Kit, BD Biosciences) and incubated for 4 h at 37 °C and 5 % CO_2. Then, antibodies to cell surface markers, anti-CD3 FITC and anti-CD4 PerCP were added and incubated as before. For intracellular staining of $CD4^+$ T cells, fixation and permeabilization were performed using Cytofix/Cytoperm Kits (BD Biosciences) according to the manufacturer's instructions. These cells were incubated with anti-IL-4 PE, anti-IFN-γ APC, anti-IL-5 PE, anti-IL-10 APC, anti-IL-6 PE, anti-TNF-α PE and anti-IL-12 PE antibodies. For all samples, the expression of CD69 was measured as an activation control.

The fluorescent signal intensity was recorded and analyzed by an FACScalibur flow cytometer (Becton–Dickinson). Events were collected from the lymphocyte gate on the FSC/SSC dot plot. Overall, 20,000 gated events were acquired from each sample using CellQuest research software (Becton–Dickinson). Data were analyzed using Summit software v4.3 (Dako, Colorado Inc.). Data from five mice per group are reported as the mean ± SD.

Statistical Analysis

Data are expressed as the mean ± SD of five mice per group from three independent experiments. Multiple comparisons of immunological data between treated with bLf versus untreated groups were analyzed by one-way ANOVA. If a significant main effect was identified ($p < 0.05$), the means of the respective groups were compared using the post hoc Holm-Sidak method. All analyses were performed using the statistical program SigmaPlot for Windows Version 11.0 software (Systat Software Incorporated, San Jose, CA, USA).

Results

bLf Increases the Total and Specific Levels of IgA and IgM at the Distal Small Intestine

The analysis of antibody production at the distal small intestine revealed that mice treated with bLf displayed higher ($p < 0.001$) levels of total IgA (days 7–28) and IgM (days 7, 14 and 21) compared with the control group (Table 1). Regarding the specific antibody response, the bLf-treated groups showed higher levels of IgA and IgM anti-bLf ($p < 0.001$) at all days of assessment compared with the control group (Table 1).

bLf Modulates the IgA^+ and IgM^+ Plasma Cell Responses at the Distal Small Intestine

The plasma cell percentage (%) in the distal small gut was analyzed at inductive (Peyer's patches) and effector (lamina propria) sites. Groups of mice treated with bLf had a significantly higher percentage ($p < 0.001$) of IgA^+ plasma cells at Peyer's patches (days 7 and 28) and lamina propria (days 7, 14, 21 and 28) compared with the control group (Table 2). Analysis of the IgM^+ plasma cell isotype showed that mice treated with bLf had a higher percentage of IgM^+ plasma cells at Peyer's patches ($p < 0.05$, days 7 and 14, $p < 0.001$, days 21 and 28) while at the lamina propria, the percentage of IgM^+ plasma cells was higher ($p < 0.001$, days 7–21) or lower ($p < 0.001$, day 28) compared with the control group (Table 2).

bLf Modulates the Percentage of T and B Populations in Peyer's Patches and Lamina Propria in the Distal Small Intestine

Analysis of T and B lymphocyte populations indicated that the percentage of B cells at Peyer's patches in mice treated with bLf was higher ($p < 0.05$, day 7) or lower ($p < 0.01$, day 14) while the percentage of B cells at the effector site

Table 1 Antibody levels in intestinal secretions of mice orally treated with bLf

	Control	Day 7	Day 14	Day 21	Day 28
Total IgA (μg/mL)	3.40 ± 0.90	32.00 ± 1.99[a]	14.02 ± 0.48[a]	15.10 ± 1.28[a]	28.10 ± 1.35[a]
Total IgM (μg/mL)	2.23 ± 0.19	4.45 ± 0.41[a]	4.48 ± 0.41[a]	6.40 ± 0.60[a]	2.56 ± 0.29
IgA anti-bLf (A_{490nm})	0.05 ± 0.01	0.47 ± 0.01[a]	0.83 ± 0.02[a]	0.95 ± 0.11[a]	0.77 ± 0.11[a]
IgM anti-bLf (A_{490nm})	0.08 ± 0.01	0.35 ± 0.01[a]	0.38 ± 0.01[a]	0.30 ± 0.05[a]	0.21 ± 0.02[a]

Total IgA or IgM in μg/mL or specific IgA or IgM as absorbance values at $\lambda = 490$ nm (A_{490nm}) is expressed as mean ± SD of five mice per group on days of bLf treatment from three independent experiments

Significant differences: [a] $p < 0.001$ compared with the control group

Table 2 Effects of bLf on the levels of IgA^+ and IgM^+ plasma cells in the distal small intestine of mice

	Mean percentage of plasma cells				
	Control	Day 7	Day 14	Day 21	Day 28
Peyer's patches					
IgA^+	2.20 ± 0.14	12.40 ± 0.67[b]	2.28 ± 0.04	3.17 ± 0.73	5.36 ± 0.87[b]
IgM^+	2.10 ± 0.18	3.49 ± 0.49[a]	3.20 ± 0.30[a]	4.86 ± 0.50[b]	5.68 ± 0.73[b]
Lamina propria					
IgA^+	1.24 ± 0.14	10.22 ± 0.23[b]	2.73 ± 0.41[b]	2.63 ± 0.26[b]	2.77 ± 0.09[b]
IgM^+	0.83 ± 0.04	3.14 ± 0.22[b]	1.29 ± 0.03[b]	1.29 ± 0.10[b]	0.44 ± 0.04[b]

Percentage of IgA^+ or IgM^+ plasma cells in Peyer's patches or lamina propria is expressed as mean ± SD of five mice per group on days of bLf treatment

Significant differences: [a] $p < 0.05$, [b] $p < 0.001$ compared with the control group

or lamina propria was higher at all days of bLf treatment ($p < 0.001$, days 7, 14 and 28, $p < 0.01$ day 21; Table 3) compared with the control group. Given the up-modulating effects of bLf on the generation of $CD4^+$ T and $CD8^+$ T cells and their role in the secretion of ILs, both T subpopulations were evaluated (Sfeir et al. 2004; Wang et al. 2000). Comparisons of the percentage of T cell subpopulations (Table 3) showed that compared with the control group, mice treated with bLf had a higher percentage of $CD3^+/CD4^+$ T cells at Peyer's patches ($p < 0.001$, day 7, $p < 0.01$, day 21 and $p < 0.05$, day 28) and at the lamina propria ($p < 0.001$, on all days of bLf treatment) as well as a higher percentage of $CD3^+/CD8^+$ T cells at Peyer's patches ($p < 0.001$, days 7, 21 and 28, $p < 0.01$, day 14) or at the lamina propria ($p < 0.001$, all days; Table 3).

bLf Modulates the Cytokine/IL $CD3^+/CD4^+$ T cell response in Peyer's Patches and Lamina Propria in the Distal Small Intestine

The modulatory influence of bLf on pro-inflammatory (IFN-γ, TNF-α and IL-12) or IgA-producing (IL-4, -5, -10 and -6) $CD3^+/CD4^+$ T cell responses in Peyer's patches and lamina propria of the distal small intestine were assessed (Table 4). Analysis of Peyer's patches indicated that in mice treated with bLf the percentage of $CD3^+/CD4^+$ T cells was higher ($p < 0.001$) for IFN-γ (days 7 and 14), TNF-α (day 28) or IL-12 (days 14–28) or was lower for TNF-α (days 7–21) compared with the control group. Moreover, in the Peyer's patches from mice treated with bLf, a significantly higher percentage of $CD3^+/CD4^+$ T cells producing IL-4 ($p < 0.001$ days 7 and 28), IL-5 ($p < 0.01$ days 14 and 28, $p < 0.001$ days 7 and 21), IL-10 and IL-6 ($p < 0.001$ days 7,14 and 28) or a significantly lower percentage producing IL-10 and IL-6 ($p < 0.001$ day 21) were observed compared with the control group.

Evaluation of the cytokine/IL T cell profile in the intestinal lamina propria revealed that in mice treated with bLf the percentage of pro-inflammatory cytokine-producing $CD3^+/CD4^+$ T cells (Table 4) was significantly higher ($p < 0.001$) for IFN-γ (day 7), TNF-α and IL-12 (days 7 and 21) or lower for IFN-γ (days 14–28), TNF-α and IL-12 (days 14 and 28) compared with the control group. Moreover, for mice treated with bLf the percentage of $CD3^+/CD4^+$ T cells (Table 4) was significantly higher for those secreting IL-4 ($p < 0.001$, days 7 and 14), IL-5 ($p < 0.01$, day 7) and IL-10 ($p < 0.001$, days 7 and 21) or lower for those secreting IL-4 ($p < 0.05$, day 21), IL-5 ($p < 0.001$, days 14–28), IL-10 ($p < 0.05$, day 14, $p < 0.001$, day 28) and IL-6 ($p < 0.001$, days 14 and 21, $p < 0.01$ day 28) compared with controls.

Table 3 Effects of the bLf oral administration on B and T cells populations in Peyer's patches and lamina propria from the distal small intestine of mice

	Mean percentage of cells				
	Control	Day 7	Day 14	Day 21	Day 28
Peyer's patches					
$CD19^+/B220^+$	60.09 ± 2.52	64.88 ± 0.93[a]	53.95 ± 2.76[b]	59.11 ± 1.90	59.24 ± 1.84
$CD3^+/CD4^+$T	24.09 ± 1.03	37.73 ± 1.53[c]	23.21 ± 2.28	28.20 ± 1.03[b]	27.92 ± 1.55[a]
$CD3^+/CD8^+$T	2.50 ± 0.60	5.41 ± 0.58[c]	4.22 ± 0.75[b]	4.87 ± 0.41[c]	5.95 ± 0.47[c]
Lamina propria					
$CD19^+/B220^+$	20 ± 1.24	29.16 ± 1.62[c]	39.38 ± 2.82[c]	25.54 ± 0.33[b]	33.53 ± 1.32[c]
$CD3^+/CD4^+$T	16 ± 1.79	46.63 ± 0.62[c]	26.38 ± 0.92[c]	51.49 ± 0.7[c]	39.81 ± 1.02[c]
$CD3^+/CD8^+$T	6.7 ± 0.5	15.36 ± 0.17[c]	22.59 ± 2.49[c]	18.19 ± 1.51[c]	16.09 ± 0.48[c]

Percentage values of $CD19^+/B220^+$ B lymphocytes, $CD3^+/CD4^+$ T and $CD3^+/CD8^+$ T cell populations in Peyer's patches or lamina propria from distal small intestine is expressed as mean ± SD of five mice on days of bLf treatment from three independent experiments

Significant differences: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared with the control group

Table 4 Cytokine/interleukin $CD4^+$ T cell responses in the distal small intestine after the oral administration of bLf

	Mean percentage of $CD3^+/CD4^+$ T cells				
	Control	Day 7	Day 14	Day 21	Day 28
Peyer's patches					
IFN-γ	3.43 ± 0.15	4.69 ± 0.13[c]	4.15 ± 0.16[c]	3.61 ± 0.14	3.41 ± 0.16
TNF-α	3.76 ± 0.30	1.88 ± 0.20[c]	1.69 ± 0.17[c]	1.69 ± 0.18[c]	6.80 ± 0.70[c]
IL-12	2.41 ± 0.24	2.11 ± 0.22	7.26 ± 0.74[c]	3.51 ± 0.40[c]	4.23 ± 0.50[c]
IL-4	2.76 ± 0.11	8.38 ± 0.90[c]	2.29 ± 0.03	3.26 ± 0.05	4.18 ± 0.14[c]
IL-5	2.03 ± 0.20	4.90 ± 0.50[c]	2.71 ± 0.30[b]	5.22 ± 0.60[c]	2.75 ± 0.30[b]
IL-10	3.01 ± 0.07	3.39 ± 0.08[c]	4.61 ± 0.13[c]	2.56 ± 0.09[c]	3.37 ± 0.07[c]
IL-6	3.52 ± 0.08	4.64 ± 0.15[c]	6.03 ± 0.17[c]	2.29 ± 0.23[c]	4.16 ± 0.42[c]
Lamina propria					
IFN-γ	1.79 ± 0.05	2.26 ± 0.13[c]	0.94 ± 0.13[c]	1.47 ± 0.06[c]	0.39 ± 0.06[c]
TNF-α	1.32 ± 0.13	2.47 ± 0.25[c]	0.51 ± 0.05[c]	1.50 ± 0.15[c]	0.82 ± 0.09[c]
IL-12	1.27 ± 0.30	4.85 ± 0.50[c]	0.69 ± 0.09[c]	2.47 ± 0.25[c]	0.87 ± 0.09[c]
IL-4	0.98 ± 0.09	1.53 ± 0.14[c]	1.45 ± 0.13[c]	0.83 ± 0.05[a]	1.08 ± 0.01
IL-5	1.60 ± 0.08	1.77 ± 0.07[b]	0.77 ± 0.06[c]	1.46 ± 0.08[c]	0.87 ± 0.09[c]
IL-10	1.05 ± 0.10	2.79 ± 0.24[c]	0.83 ± 0.13[a]	1.52 ± 0.22[c]	0.47 ± 0.05[c]
IL-6	1.11 ± 0.10	1.14 ± 0.13	0.59 ± 0.06[c]	0.49 ± 0.05[c]	0.95 ± 0.09[b]

Percentage of pro- (IFN-γ, TNF-α and IL-12) or anti-inflammatory (IL-4, -5 -10 and -6) cytokine/interleukin $CD3^+/CD4^+$ T cells from Peyer's patches and lamina propria is expressed as mean ± SD of five mice per group from three independent experiments

Significant differences: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared with the control group

Discussion

The current study demonstrated for the first time the influence of bLf on the IgA response both at the inductive (Peyer's patches) and effector (lamina propria) sites of the distal small intestine from healthy BALB/c mice. bLf increased the levels of total IgA and IgM as well as specific IgA and IgM on all days evaluated. In addition, the influence of bLf on the response of IgA^+/IgM^+ plasma cells, as well as B and T cells, was differentially regulated in Peyer's patches and lamina propria. Events that might underlie the divergent up-modulating influence of bLf on antibody production and the cellular response at inductor and effector sites include: (1) extent of absorption, accumulation and/or luminal elimination during the chronic administration of oral bLf (Fischer et al. 2007); (2) differential receptor mediated

binding for eventual sub-epithelial transport of bLf across Peyer's patches via M cells or through lamina propria by epithelial columnar cells (Hu et al. 1990; Talukder et al. 2003); (3) differential cellularity, i.e., Peyer's patches are enriched in immature immunocompetent cells while the lamina propria is populated by mature terminally differentiated cells (Cerutti 2008).

These findings are in line with the elicitation of humoral and cellular immune responses as described in Peyer's patches or lamina propria from the whole small intestine of mice orally treated with bLf (Debbabi et al. 1998; Sfeir et al. 2004; Wang et al. 2000). Lactoferrin has direct effects on B cells and potentially modulates their function as APCs to promote T cell responses (Hwang et al. 2011). Elicitation of intestinal immunity might result from direct stimulation via the bLf receptor expressed on B and T cells (Debbabi et al. 1998). The adaptive mechanisms involved in the effects of bLf reported here might include the enhanced expression of CD4 antigen on T cells (Dhennin-Duthille et al. 2000), increased maturation of B (Zimecki et al. 1995) and T lymphocytes as observed with human lactoferrin (Bi et al. 1997). In addition or alternatively, the effects of bLf on IgA responses might derive from its ability to trigger $CD4^+$ T priming by enhancing MHC-II expression on APCs as described for bLf-treated macrophages or the effect of human lactoferrin on dendritic cells (Spadaro et al. 2008; Wilk et al. 2007).

In this study, waves of cytokine/ILs $CD4^+$ T cell responses induced by the oral administration of bLf merged. For example, the same response profile was found between IL-6 and IL-10 $CD4^+$ T cells in Peyer's patches or TNF-α and IL-12 $CD4^+$ T cells in the lamina propria. Interestingly, increased (14 and 28 days) or decreased (day 21) responses of IL-6/IL-10 $CD4^+$ T cells in Peyer's patches corresponded with a decreased (14 and 28 days) or increased (day 21) response of TNF-α and IL-12 $CD4^+$ T cells in the lamina propria. The underlying mechanisms of these patterns are not fully known but might involve signals (either adaptive or innate) on $CD4^+$ T cells through which bLf modulates the opposite generation of pro-inflammatory cytokines and IgA-producing ILs, both of which are implicated in IgA generation. In addition, to IL-4, IL-5 and IL-6, IL-10 is a pleiotropic Th2 cytokine with anti-inflammatory properties that is involved in IgA production (Cerutti 2008). Paradoxically, IL-10 elicited by bLf, acts as a Th1-like cytokine that favors pro-inflammatory responses as observed in intraepithelial lymphocytes from jejunal and ileum of mouse (Takakura et al. 2006). In contrast, TNF-α and IL-12 are components of intestinal immunity that enhance the killing activity of T and NK cells against tumor cells as observed in healthy mice treated with bLf (Iigo et al. 2004). Furthermore, both nitric oxide and TNF-α derived from a dendritic cell subset are involved in $CD4^+$ T cell priming for intestinal IgA production (Tezuka et al. 2007).

Additional patterns including the response of pro-inflammatory cytokines (IL-12, IFN-γ, TNF-α) as well as IgA-directing ILs (IL-4,-5,-10,-6) $CD4^+$ T cells were generally increased in Peyer's patches or were decreased in the lamina propria on days 14 and 28. Despite the down-modulation of cytokine/ILs $CD4^+$ T cell responses on days 14 and 28, increased levels of IgA^+ plasma cells as well as B and T lymphocyte populations were found on all days of bLf treatment in the lamina propria as previously described. These cytokine/ILs $CD4^+$ T cell responses modulated by bLf might control physiological inflammation in the lamina propria. Unlike Peyer's patches, the lamina propria is regarded as a compartment in a permanent state of physiological inflammation caused by the presence of mononuclear cells and granulocytes (Pabst 1987). bLf is a lipopolysaccharide-binding protein that modulates the secretion of pro-inflammatory cytokines (IFN-γ, TNF-α) via Toll-like receptor (TLR)-4 signaling (Curran et al. 2006; Haversen et al. 2002; Na et al. 2004; Puddu et al. 2007). In the lamina propria, ligation of TLRs expressed on dendritic and epithelial cells by microbial products derived from gut microbiota provides signals for innate IgA generation (Cerutti 2008). The ileum is highly enriched in TLR-4 expression as well as microbiota involved in the generation of IgA (Ortega-Cava et al. 2003; Maynard et al. 2012; Ohashi et al. 2006).

In this study, we did not evaluate TGF-β^+ $CD4^+$ T cells that are essential for CSR. Despite this limitation, we conclude that bLf increases the levels of IgA and most immuno-competent cells involved in IgA generation in both in the Peyer's patches and lamina propria from the distal small intestine. The effects of bLf on IgA elicitation were associated with the up- or down-modulation of cytokine/ILs $CD4^+$ T cell responses in Peyer's patches and lamina propria, respectively, on days 14 and 28.

The use of recombinant mouse lactoferrin (Ward et al. 1997) to explore the modulatory properties of species-specific conditions in future studies might provide substantive evidence of the modulatory effects of lactoferrin at the intestinal level. Although the results of this study in BALB/c mice cannot be directly extrapolated to humans, they indicate the potential application of bLf for human use as a nutraceutical (Korhonen and Pihlanto 2007) to strengthen homeostasis at the distal level that under some conditions is prone to inflammation. Moreover, given that the depletion of luminal iron prevents inflammation in the ileum (Werner et al. 2011), future studies might provide insights regarding the clinical implications for control of inflammation in the distal small intestine by taking advantage the IgA up-modulatory and iron-binding properties of bLf.

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