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Lactoferrin immunomodulation of DTH response in mice

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Abstract

Improved nontoxic adjuvants, especially adjuvants capable of inducing cell-mediated immunity (CMI), are needed for research in immunology and for development of human and veterinary vaccines. Bovine Lactoferrin, an effector molecule shown to directly participate in host defense, was assessed at various concentrations as an adjuvant component for induction of DTH responses to sheep red blood cells (SRBC). Subcutaneous immunization with Lactoferrin enhanced delayed type hypersensitivity (DTH) in CBA mice in a dose-dependent fashion; DTH responses were most significantly increased when sensitization was accomplished using Lactoferrin at 50 μg/dose and 250 μg/dose. Furthermore, Lactoferrin admixed with suboptimal dose of SRBC enhanced DTH responses by over 17-fold. Peritoneal cells collected from mice intraperitoneally injected with a 100 μg/dose of Lactoferrin demonstrated modest, but significant, production of TNF-α, IL-12 and MIP-1α when cultured in vitro, compared to saline-injected controls. J774A.1 murine macrophages stimulated with Lactoferrin resulted in increased TNF-α protein production, and upregulated IL-12 and IL-15 mRNA. Levels of message for chemokines MIP-1α and MIP-2 were also increased in a dose-dependent way. Taken together, these results indicate that Lactoferrin as an adjuvant may stimulate macrophages to generate a local environment likely to push immune responses towards development and maintenance of CMI.

Keywords: Lactoferrin; DTH; RT-PCR; Adjuvant

1. Introduction

The successful development of new vaccines is contingent upon the availability of adjuvants that are not only safe for the host, but also induce immune responses complementary to those generated during natural infection. Unfortunately, many non-toxic adjuvants produce a high degree of humoral immunity, with concurrent lack of CMI [1,2]. Currently, the only adjuvants approved for human use are the aluminium salts [3]. Alum has shown efficacy for vaccines, but in many instances has limited capacity for responses other than those which are humoral-mediated [2]. The prototype adjuvant for inducing cell mediated and DTH responses is Freund's Complete Adjuvant (CFA), consisting of a water-in-oil emulsion containing approximately 50% mineral oil, emulsifying agent Arlacel A, and killed mycobacterium. CFA is an effective adju-

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vant for generation of cell-mediated response; however, it is undesirable in animals and unacceptable for human use due to its severe side effects, including pain, abscess formation, local necrosis, and fever [4].

Lactoferrin, an 80-kDa glycoprotein involved in iron metabolism [5], may be a preferable adjuvant component. Lactoferrin has been shown to activate macrophages [6,7] and natural killer cells [8], as well as promote maturation of T [9] and B [10] cells. Lactoferrin also exhibits antimicrobial [11,12], antifungal [13], antiviral [14] and antitumor [15,16] properties. Cell surface receptors for Lactoferrin are found on monocytes [17] and activated lymphocytes [18]. Lactoferrin has demonstrated the ability to induce activation of intracellular signals [19] and upregulate adhesion molecule expression [20]. Although the regulatory action of Lactoferrin on immune system cells remains uncertain, many positive traits warrant its potential use as an adjuvant for veterinary vaccinations.

Lactoferrin was initially described as inhibitory to effector phases of cellular immune responses in presensitized mice [21]. However, Lactoferrin administered as an adjuvant component augmented cell-mediated DTH response to the same antigens [22]. The discrepancy between these observations has not yet been reconciled. Therefore, the goal of these studies was to examine Lactoferrin as an adjuvant to elicit in vivo DTH responses to antigen, and to further investigate molecular mechanisms involved in this process.

2. Materials and methods

2.1. Mice

CBA male mice (8–12 weeks old) were used for generation of DTH responses to SRBC, similar to previous experimentation [22]. Outbred Swiss Webster males (8 weeks old) were used to examine monocytic response in vivo to Lactoferrin. All mice were allowed continuous access to commercial, pelleted food and water ad libitum.

2.2. Antigens, adjuvants and reagents

Preparation of SRBC antigen was accomplished as described previously [22]. SRBC were kept in Alsever's medium and washed three times with PBS before use. Low endotoxin bovine milk lactoferrin (< 1 EU/mg by Limulus Amebocyte Lysate assay, < 25% iron saturated,), complete Freund's adjuvant (CFA) lot F-4258, incomplete Freund's adjuvant (IFA) lot F 5506, and lipopolysaccharide (LPS, *E. coli* k-235) were purchased from Sigma, MO, USA.

2.3. Generation of DTH response to SRBC

CBA mice were treated with antigen-adjuvant formulation via subcutaneous (s.c.) injection at the base of the tail in a 0.1 ml volume (naive and non-Lactoferrin treated mice served as control). After 4 days, the delayed type hypersensitivity reaction was elicited by s.c. administration of 10^8 SRBC in 0.05 ml PBS in both hind footpads [22,23]. After 24 h, the footpad swelling was measured with a caliper (accuracy 0.05 mm). The antigen specific reaction to SRBC was calculated by subtracting SRBC-elicited reaction in nonsensitized mice from SRBC-elicited reaction of sensitized animals. The results were calculated as a mean value from at least 6 mice ± standard error (SE), expressed in DTH units (1 unit = 0.1 mm).

2.4. In vivo stimulation

Peritoneal cells were collected from mice 48 h following intraperitoneal injection with either 100 μg Lactoferrin in 1 ml saline, or with 1 ml saline alone. Cells were suspended in culture medium consisting of RPMI 1640, L-glutamine, sodium pyruvate, and 10% fetal calf serum, and then distributed into 96-well flat bottom plates (5×10^5 cells/0.2 ml/well). Supernatants were collected after 2 days, and assessed for cytokine production by ELISA. Four mice per group were assessed in triplicate, and each supernatant collected was assayed twice.

2.5. Mitogenic activity

Activation of cells obtained from draining popliteal lymph nodes was determined by the MTT colorimetric method [24]. Similarly, J774A.1 murine monocytic cells (macrophages) [ATCC # TIB-67] cultured in the presence of Lactoferrin (0.5 μg/ml to 5 mg/ml) were examined for mitogenic response. A cell suspension was prepared at a density of 5×10^6 cells/ml in culture medium consisting of RPMI 1640, L-glutamine,

sodium pyruvate, antibiotics and 10% fetal calf serum. The cells were distributed into 96-well flat bottom plates (5×10^5 cells/0.2 ml/well) and cultured for 3–4 days without additional stimulation. MTT (tetrazolium bromide salt) was added to 1 mg/ml, and the absorbance at 450 nm read after 4 h by an ELISA plate reader (Molecular Devices, Sunnyvale, CA). This absorbance is linearly proportional to the number of live cells with active mitochondria.

2.6. ELISA evaluation

Supernatants collected from cells stimulated in vivo were examined for TNF-α, IL-1β, IL-10, IL-12 and MIP-1α using commercially available enzyme linked immunosorbent assay (ELISA) DuoSet kits (R&D Systems, Minneapolis, MN) [25]. Likewise, J774A.1 macrophages cultured in the presence of Lactoferrin (1 μg/ml to 125 μg/ml) for 18 h were examined for TNF-α protein production. Briefly, Costar 96-Well Vinyl Assay Plates were coated overnight with capture antibody. Plates were washed three times with wash buffer (0.05% Tween-20 in PBS). Blocking buffer (1% BSA, 5% sucrose, 0.05% NaN_3 in PBS) was added for 3 h. After three washings, 100 μl cell supernatants were added for 2-h incubation. Biotin-conjugated secondary antibodies were added after washing, incubated for 2 h, washed, and then developed using streptavidin–horseradish peroxidase (Sigma) and TMB Microwell Peroxidase Substrate (Kierkegaard and Perry). Absorbance was read at 570 and 450 nm on an ELISA plate reader (Molecular Devices). A mean of triplicate wells was calculated based on a standard curve constructed for each assay, using recombinant murine cytokines (R&D Systems). Limit of sensitivity for these assays ranged between 5 and 10 pg/ml.

2.7. Quantitative bioluminescent RT-PCR

Evaluation of relative changes in mRNA for multiple cytokines and chemokines was accomplished using the Bioluminescent Quantitative Reverse Transcriptase–Polymerase Chain Reaction (BL QRT-PCR) method [26–28]. J774A.1 cells (5×10^5 cells) were cultured with Lactoferrin (1 μg/ml to 1000 μg/ml) for 4 or 24 h. Briefly, 1 μg total RNA was isolated and reverse-transcribed to cDNA [27,29]. The cDNA was diluted 1:8, and amplified using a sense primer biotinylated on the 5′ terminal nucleotide to facilitate later capture on Streptavidin-Microtiterplates® (MicroCoat, Penzberg, Germany). The PCR primer pair was chosen to span at least one intron, thus precluding amplification of genomic DNA. Detection used a digoxigenin labeled probe and AquaLite®/ChemFlash anti-DIG (Chemicon, Temecula, CA) [30]. Products are reported as β-actin normalized RLU values. Primers and probes are listed in Table 1, or have been listed elsewhere [29].

Table 1
Oligonucleotide Primers and Probes

Protein	Primers		Product size (bp)	No. of cycles	Probe
β-actin	s[a]	biotin-ATGGATGACGATATCGCT	185	23	GAGCACCCTGTGCTGCTC-dig
	as	ATGAGGTAGTCTGTCAGGT			
IL-10	s	biotin-GACCAGCTGGACAACATAC	401	28	CATTTCCGATAAGGCTTGG-dig
	as	CTGGAGTCCAGCAGACTC			
IL-12 (p40)	s	biotin-AGCACCAAATTACTCCGGAC	121	30	TCTTTGCACCAGCCATGAG-dig
	as	ATTCCACATGTCACTGCCC			
IL-15	s	Biotin-AGAGGCAGCACAGCTCCAT	121	30	GAAGCTTAGTTTGCCCAGCA-dig
	as	TAAGTCTGAGACGAGCTCTTT			
MIP-1α	s	biotin-GCAGCAGCGAGTACCAGTC	345	23	CTGTAGGAGAAGCAGCAG-dig
	as	GGCATTCAGTTCCAGGTCA			
MIP-2	s	biotin-TCCCACCTGCCGGCT	252	30	TCCTGGGGGCGTCACA-dig
	as	TGAACCAGGGGGGCTT			

[a] s, sense; as, antisense; biotin, 5′ -biotinylated; dig, 3′ -digoxygenin; bp, base pairs.

2.8. Statistics

All data are expressed in DTH units (1 unit = 0.1 mm) as mean values from up to 10 determinations ± SE. ELISA evaluation shows mean values with standard deviation (SD). Differences between groups were analyzed by the Student unpaired *t*-test when two groups were analyzed and analysis of variance (ANOVA) when more than two groups were analyzed.

3. Results

3.1. Effect of lactoferrin on DTH to SRBC in mice

Bovine Lactoferrin augmented DTH response in CBA mice, immunized s.c. at the base of the tail with 10^8 SRBC in Complete Freund's Adjuvant. A sensitizing dose of SRBC antigen was administered together with either Lactoferrin and CFA, or CFA alone. Four days later the DTH reaction was elicited in hind footpads by injection of 10^8 SRBC in PBS and the footpad swelling was measured after 24 h (Table 2). An antigen-specific response was calculated by subtracting the nonspecific reaction caused by injection of the eliciting dose of antigen into the footpads of naive mice. Sensitization without Lactoferrin elicited a response of 11.8 ± 0.57 units. Elicited DTH responses were significantly increased ($P < 0.001$) when sensitization was accomplished using Lactoferrin at both 50 μg/dose (22.4 ± 0.93 units) and 250 μg/dose (21.9 ± 0.81 units), but not at 10 μg/dose (12.6 ± 0.38 units).

Table 2
Co-stimulatory effects of Lactoferrin on SRBC-induced DTH

Treatment of mice		DTH units ± SE
CFA alone	–	11.8 ± 0.57
CFA +	10 μg LF	12.6 ± 0.038
	50 μg LF	22.4 ± 0.93*
	250 μg LF	21.9 ± 0.81*

Mice were immunized s.c. at tail base with 10^8 SRBC in CFA. Lactoferrin was admixed together with CFA and the sensitizing dose of antigen, and injected s.c. Four days later, DTH reaction was elicited in hind footpads by injection of 10^8 SRBC in PBS. The footpad swelling was measured after 24 h and expressed in DTH units (1 unit = 0.1 mm), ± SE. Antigen-specific response was calculated by subtracting nonspecific reaction caused by injection of the eliciting dose of antigen into footpads of naive mice. Values significantly different from CFA alone, $*P < 0.001$. LF = Lactoferrin.

Table 3
Co-stimulatory effects of Lactoferrin on SRBC-induced delayed type hypersensitivity using suboptimal dose of SRBC

Treatment of mice		DTH units ± SE
IFA alone	SRBC 10^6	1.1 ± 0.57
	SRBC 10^7	7.8 ± 0.60
	SRBC 10^8	18.3 ± 0.93
IFA + LF 250 μg	SRBC 10^6	17.6 ± 0.55*
	SRBC 10^7	20.3 ± 0.99*
	SRBC 10^8	20.9 ± 0.82

Mice were immunized s.c. with SRBC (10^6, 10^7 or 10^8) in IFA, or in IFA and Lactoferrin (250 μg). Four days later the DTH reaction was elicited by s.c. administration of 10^8 SRBC into hind footpad. Footpad swelling was measured after 24 h and expressed in DTH units (1 unit = 0.1 mm), ± standard error. Values significantly different from IFA alone control with same SRBC dose, $*P < 0.001$.

Our objective was to eliminate the need for many of the immunostimulatory components (e.g. mycobacterial cell wall) found in CFA. Therefore, further sensitization was accomplished using Lactoferrin (250 μg/dose) admixed with the eliciting dose of 10^6, 10^7 or 10^8 SRBC antigen and given s.c. with incomplete Freund's (IFA) (vehicle only). Again, the magnitude of DTH reaction was determined by the footpad test. When Lactoferrin was admixed with IFA and SRBC, a relatively low level of sensitizing antigen could be used to achieve production of DTH response (Table 3). Use of Lactoferrin admixed with suboptimal dose of antigen greatly enhanced DTH response. The most spectacular effects were demonstrated when using 100-fold reduced antigen dose (10^6 SRBC), normally non-immunogenic (1.1 ± 0.57 units) to mice. In this case, DTH responses were 17-fold augmented by Lactoferrin. Lactoferrin given to mice with 10-fold reduced dose of antigen (10^7 SRBC) also significantly enhanced DTH response. In these experiments, Lactoferrin given to mice with 10^8 SRBC did not significantly increase footpad swelling (20.9 ± 0.82 units vs. 18.3 ± 0.93 units for control); however, it is important to note that DTH responses were not suppressed in this reaction.

3.2. Stimulation of cellular immune response to SRBC

The effect of Lactoferrin on mitogenic activity in draining lymph nodes was examined. Mice were trea-

ted with increasing doses of Lactoferrin with sensitizing dose of SRBC in IFA. DTH responses were induced, and 24 h later popliteal lymph nodes were isolated. As illustrated in Table 4, proliferation of lymph node cells from mice treated with Lactoferrin was significantly augmented ($P<0.01$) in a dose–response manner, compared to mice sensitized in the absence of Lactoferrin.

3.3. In vivo stimulation by Lactoferrin

We next addressed whether Lactoferrin could directly activate cells involved in antigen presentation. Mice were intraperitoneal primed with a single bolus injection of 100 μg Lactoferrin, or given a saline as a control. Cells were harvested 48 h later, counted, and placed into culture without further stimulation to assess in vivo activation due to Lactoferrin. Administration of Lactoferrin caused a modest, but non-significant, increase in total number of cells recovered from the peritoneal cavity (Table 5). Cells recovered from both groups were primarily mononuclear, with a high predominance of larger macrophages (approximately 70%) present in both groups.

Assessment of supernatants indicated a modest, but significant, activation of cells obtained from the Lactoferrin-treated mice (Table 6). There was significant increase in production of IL-12 and macrophage inflammatory protein 1 alpha (MIP-1α) in the Lactoferrin treated group, compared to the saline treated controls ($P<0.05$). In the Lactoferrin group, IL-12 was significantly elevated, with production of 1.08 ng/ml compared to 0.83 ng/ml from the saline control group ($P=0.044$). MIP-1α production was also significantly increased, with 0.84 ng/ml produced from the Lactoferrin treated mice compared to 0.72 ng/ml by the saline control derived cells ($P=0.047$). A slight and non-significant increase in TNF-α production was also present compared to saline controls (1.42 vs. 1.24 ng/ml; $P=0.06$). There was no significant production of IL-1β or IL-10 in either group.

Table 4
Activation response following Lactoferrin sensitization

Treatment of mice		Mean OD ± SE
IFA alone	SRBC 10^8	0.259 ± 0.007
IFA + 10 μg LF	SRBC 10^8	$0.318 \pm 0.010^+$
IFA + 50 μg LF	SRBC 10^8	$0.319 \pm 0.005^+$
IFA + 250 μg LF	SRBC 10^8	0.470 ± 0.016*

Mice were sensitized with SRBC in IFA s.c. into tail base (control), or admixed with 10–250 μg/dose Lactoferrin. Four days later, DTH responses were induced with administration of 10^8 SRBC into hind footpads. After 24 h the draining, popliteal lymph nodes were isolated, cell suspensions prepared and incubated for 4 days (5×10^5 cells/well) in the absence of antigen. Mitogenic activity was determined by the MTT colorimetric method (Mean $OD_{450\ nm}$ ± standard error). Values significantly different from IFA alone control, $+P<0.01$, $*P<0.001$.

Table 5
Intraperitoneal injection of Lactoferrin increases peritoneal cell numbers

Group	Cells recovered ($\times 10^6$)	Range ($\times 10^6$)
Saline	2.4 ± 0.1	1.62–3.84
Lactoferrin	3.9 ± 1.0*	3.15–5.20

Mice were injected i.p. with a bolus dose of 100 μg Lactoferrin in saline, or saline alone. Chart represents total cells recovered from the peritoneal cavity 48 h later. *T*-test analysis indicating modest increase in cells recovered compared to saline control group, $*P<0.072$.

3.4. Lactoferrin stimulation of murine monocytic J774A.1 cells

Direct effects of Lactoferrin on purified culture J774A.1 murine monocytic cells (macrophages) were analyzed. J774A.1 cells were cultured in the presence of Lactoferrin (1 μg/ml to 5 mg/ml) and examined for general activation, protein production, and changes in mRNA synthesis. Lactoferrin stimulated proliferation of J774A.1 cells in a dose-dependent manner, with maximal activation response upon culture with 50 μg/ml to 500 μg/ml Lactoferrin (Fig. 1). In a similar protocol, supernatants were analyzed for secreted TNF-α protein at 18 h post incubation with Lactoferrin (1 μg/ml to 125 μg/ml). TNF-α was produced by 18 h in a dose-dependent manner (Fig. 2), with levels elevated through 48 h post incubation (not shown). ELISA analysis also revealed that no IL-1β, IL-6 or IL-10 protein was detected above background at any time point examined (significant amounts were detected for incubation with LPS positive control).

3.5. Lactoferrin induction of cytokine mRNAs

J774A.1 cells were cultured in increasing doses of Lactoferrin (1 to 500 μg/ml), or 5 ng/ml LPS positive control, and examined for changes in multiple immu-

Table 6
In vivo stimulation following i.p. injection of Lactoferrin

Cytokine	Saline			Lactoferrin			*P* value
	Mean value [ng/ml]	SD	Range	Mean value [ng/ml]	SD	Range	
IL-1β	0.160	0.06	0.096–0.308	0.194	0.12	0.096–0.508	0.210
IL-10	0.306	0.11	0.084–0.524	0.303	0.09	0.148–0.488	0.920
IL-12	0.836	0.31	0.448–1.530	1.080	0.48	0.392–2.430	0.044
TNF-α	1.240	0.26	0.733–1.720	1.420	0.39	0.974–2.350	0.060
MIP-1α	0.721	0.16	0.387–1.050	0.843	0.24	0.529–1.540	0.047

Peritoneal cells recovered from mice intraperitoneally injected with 100 μg Lactoferrin were cultured without further stimulation, and supernatants assessed by ELISA for production of listed immuno-mediator. Table represents mean value of protein [ng/ml] with standard deviation, at 48 h post culture, with range of responses indicated for four mice per group assessed twice in triplicate. *P* value for *t*-test indicates relative differences between groups.

noregulatory messages using quantitative bioluminescent reverse transcriptase methods (BL QRT-PCR). mRNA was isolated at 4 or 24 h post incubation with Lactoferrin, reverse transcribed to cDNA and quantitatively analyzed.

Lactoferrin induced production of IL-12(p40) mRNA from J774A.1 cells, in a biphasic manner (Fig. 3). Four hours post incubation, substantial increase was seen using both low (1 μg/ml) and higher doses (100–500 μg/ml), in the absence of additional antigen. Responses were similar to those seen at 24 h post incubation with Lactoferrin. Dramatic increases in mRNA were demonstrated for IL-15 (Fig. 4). IL-15 levels were increased by 4 h post incubation, with

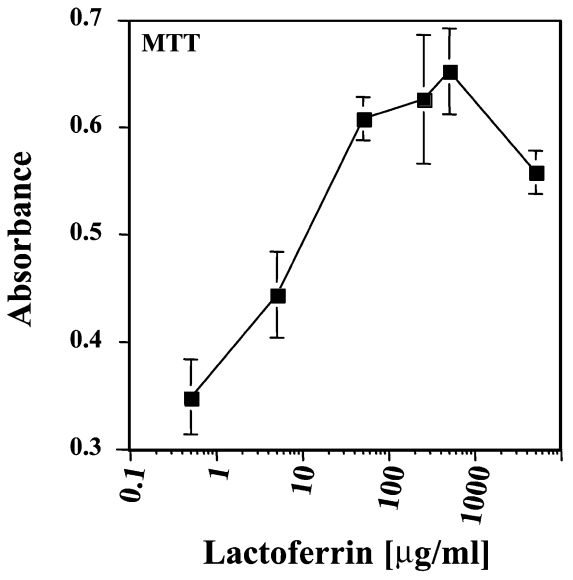

Fig. 1. Lactoferrin-induced proliferation of cultured macrophages. J774A.1 cells were stimulated for 72 h with Lactoferrin (0.5 to 5000 μg/ml), after which a tetrazolium bromide salt (MTT) was added (1 mg/ml). After 4 h, absorbance was read at 450 nm on an ELISA plate reader. Average values for three wells with standard deviation are shown.

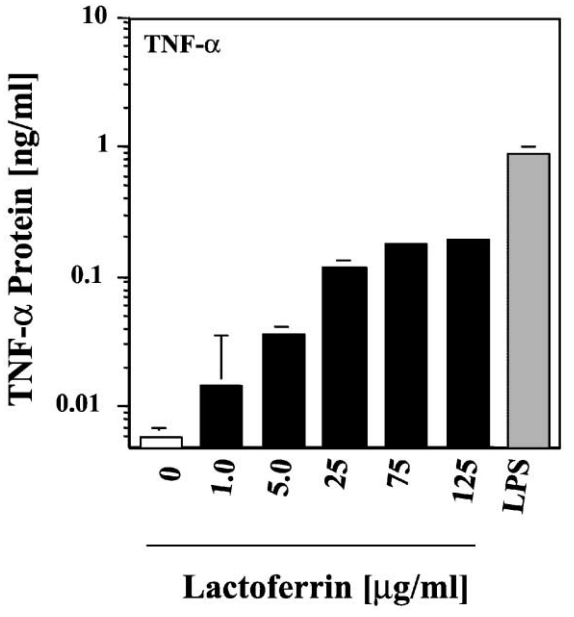

Fig. 2. Lactoferrin-induced TNF-α protein production. J774A.1 cells (5×10^5/well) were stimulated with bovine Lactoferrin (1.0 to 125 μg/ml) or LPS (5 ng/ml; hatched bar) for 18 h. TNF-α production was quantified by ELISA analysis. Lactoferrin induced production of TNF-α protein in a dose-dependent manner. Average values (ng/ml) for three wells with standard deviation are shown. No Lactoferrin (open bar).

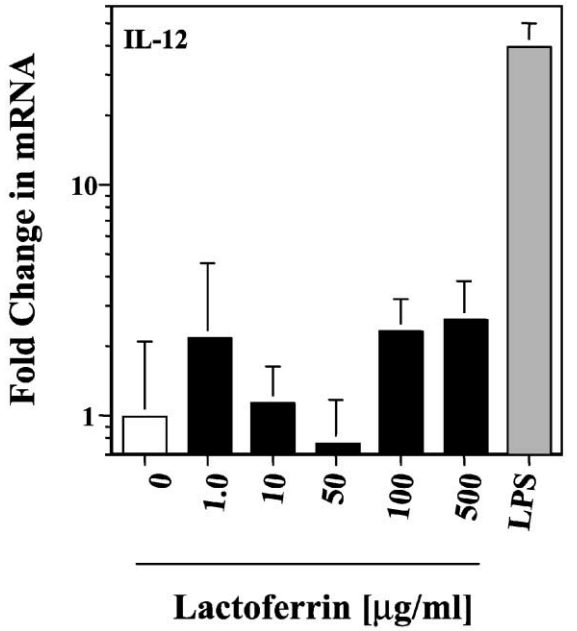

Fig. 3. Lactoferrin-induced IL-12(p40) mRNA from macrophages. Biphasic expression of IL-12 mRNA in J774A.1 cells was determined following incubation with Lactoferrin (1.0 to 500 μg/ml). Message was reverse-transcribed to cDNA, and evaluated by QRT-PCR and bioluminescent hybridization immunoassay. cDNA from cultured cells were amplified using IL-12(p40) specific primers; product was quantitated as β-actin normalized values. The fold increase compared values obtained for untreated cells (open bar). Positive increase in IL-12 mRNA shown for LPS (5 ng/ml; hatched bar). Data expressed as mean values for three wells over two experiments, standard errors shown.

levels reaching higher than 10-fold at all levels examined except the lowest dose of 1 μg/ml. In fact, incubation with Lactoferrin at 5 μg/ml increased mRNA levels greater than 120-fold above the constitutive level. In contrast, LPS stimulation was only 17.2-fold greater than controls.

IL-10 mRNA was dramatically reduced by 4 h post stimulation (Fig. 5). As levels of Lactoferrin increased, the message for IL-10 completely disappeared (calculated quantitatively at a greater than 10-fold decrease vs. constitutive levels) at 500 and 1000 μg/ml. This decrease was maintained through 24 h post incubation. LPS positive control readily induced IL-10 mRNA in these cells.

Although protein levels for TNF-α were elevated following Lactoferrin stimulation, no change in TNF-α mRNA was detected. Levels of mRNA for IL-3, Nitric Oxide Synthase, and IL-6 remained at constitutive levels at 4 and 24 h post incubation with Lactoferrin, at all concentrations tested (not shown).

3.6. Lactoferrin-induced chemokine mRNA production

BL QRT-PCR analysis also revealed changes in chemokine mRNA production in response to Lactoferrin. By 4 h post incubation, mRNA levels in J774A.1 cells had significantly elevated message (>10-fold) for macrophage inflammatory protein 2 (MIP-2) (not shown); at higher doses (50 μg/ml to 1 mg/ml) changes were seen after 24 h incubation (Fig. 6). Also at 24 h post incubation, J774A.1 cells produced considerable MIP-1α mRNA in a dose dependent manner, with greater than 30-fold induction using 50 and 100 μg/ml Lactoferrin. The relative changes in mRNA were

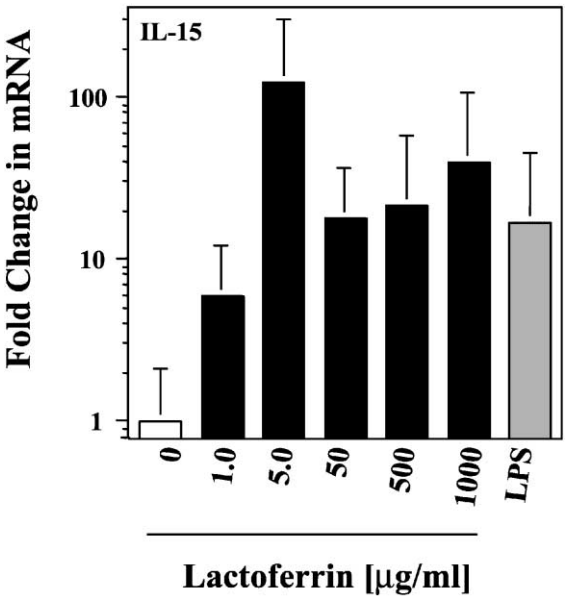

Fig. 4. Lactoferrin-induced IL-15 mRNA from monocyte/macrophages. Expression of IL-15 mRNA in the J774A.1 cells was examined following incubation with Lactoferrin (1.0 to 1000 μg/ml) or LPS (5 ng/ml; hatched bar) for 4 h. Message was evaluated by quantitative RT-PCR and bioluminescent hybridization immunoassay. Samples were quantitated as β-actin normalized RLU values, expressed as relative change compared to values obtained for untreated cells (open bar). Data are expressed as mean values for three wells with standard errors shown.

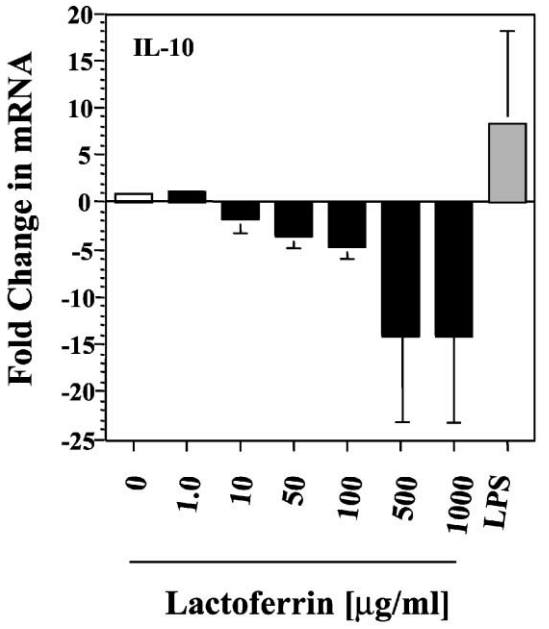

Fig. 5. Decreased IL-10 mRNA following incubation with Lactoferrin. Expression of IL-10 mRNA in the J774A.1 cells was examined following incubation with Lactoferrin (1.0 to 1000 μg/ml) or LPS (5 ng/ml; hatched bar). Message was evaluated by BL QRT-PCR, and compared to values obtained for untreated cells (open bar). Data are expressed as mean values for three or four wells with standard errors shown.

specific for a subset of chemokines; no changes were demonstrated for macrophage chemotactic factor-1 (MCP-1) or IP-10.

4. Discussion

We demonstrate that Lactoferrin given to mice subcutaneously at the time of immunization with antigen augments DTH responses in mice in a dose-dependent manner. The response due to Lactoferrin appears to be in part via direct effects upon antigen presenting cells; intraperitoneal administration of Lactoferrin led to stimulation of primary peritoneal exudate cells to produce TNF-α, IL-12 and MIP-1α. We build on these findings to show that Lactoferrin can stimulate production of immune mediators important in induction of cell mediated responses from immortalized murine macrophages.

Macrophages contribute to the inflammatory response following administration of adjuvant materials, readily secreting cytokines and chemokines to recruit other immune system cells. The J774A.1 cells were used here as an investigative model for possible macrophage responses in vivo. As such, they may not truly represent skin resident macrophages and dendritic cells

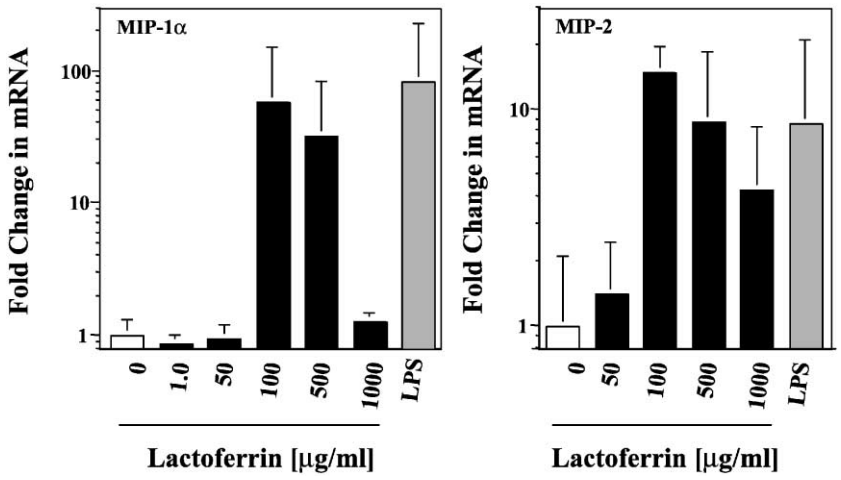

Fig. 6. Lactoferrin-induced chemokine mRNA from monocyte/macrophages. Expression of MIP-1α and MIP-2 chemokine mRNA in the J774A.1 cells was examined following incubation with Lactoferrin (1.0 to 1000 μg/ml) or LPS (5 ng/ml; hatched bar) for 24 h. Message was evaluated by quantitative RT-PCR and bioluminescent hybridization immunoassay. Samples were quantitated as β-actin normalized RLU values, expressed as relative change compared to values obtained for untreated cells (open bar). Data are expressed as mean values for three wells with standard errors shown.

responding to adjuvant during subcutaneous administration of adjuvanted antigens. However, these cells are a well characterized culture line that can readily respond to stimulation with adjuvant material [31,32], and can prime T cell responses to entrapped antigen through upregulation of B7.1 and B7.2 on their surface after adjuvant administration [31]. The J774A.1 macrophages behaved in a similar manner to the peritoneal cells stimulated with Lactoferrin in vivo. In the studies described here, Lactoferrin stimulated the production of TNF-α protein from cultured J774A.1 cells. Although protein levels for TNF-α were elevated following Lactoferrin stimulation, no change in TNF-α mRNA was detected. This is not surprising due to regulation of TNF-α protein at the post-transcriptional level [33]. Lactoferrin also increased mRNA for IL-12, IL-15, and chemotactic factors. Conversely, IL-10 mRNA decreased considerably from constitutive levels at high doses.

It is interesting to consider the mechanisms under which Lactoferrin, an important component of the host defense system, can serve as an adjuvant/accessory immunostimulant [34,35]. Both systemic and local co-administration of Lactoferrin with sensitizing dose of antigen enhances delayed type hypersensitivity in mice [22]. Lactoferrin is an iron binding protein with multifunctional immunoregulatory properties [7,8,34–37]. Initial vascular inflammation response to Gram negative bacteria coincides with a burst of pro-inflammatory cytokines TNF-α, IL-1β, and colony stimulating factor (CSF), all derived from activated monocytes–macrophages. Subsequent to the release of cytokines, neutrophils acutely increase, invade the tissues and attract these cells. Degranulation of neutrophils is linked to a massive release of Lactoferrin [38]. The signals by which Lactoferrin is released from neutrophils are different depending on the type, location and severity of insult [38,39]. Furthermore, when released from neutrophils, Lactoferrin can modify the immune status of cells to develop adequate responses to pathogens or clinical insults [39,40]. Taken together, the released Lactoferrin may prime innate phagocytic cells as they respond to local damage during acute infection.

In examination of draining lymph nodes (popliteal), we noticed increased activation events due to Lactoferrin following induction of DTH, in a dose-dependent manner. This suggests involvement or activation of Th1 cells. We cannot rule out that this response may be in part due to specific T cells responding to heterologous epitopes between bovine Lactoferrin and murine Lactoferrin. However, non-murine Lactoferrin has been shown in mice to promote maturation of T cell precursors into immunocompetent helper cells [7] and differentiation of immature B cells to become efficient antigen-presenting cells [7]. Lactoferrin can also increase antigen-presenting cell activity [8], as well as directly stimulate production of TNF-α from human PBMCs [40]. Responses are dose-dependent; levels 10–100 fold greater than those used in these studies can cause inhibition of TNF-α in the presence of LPS [41]. Cellular receptors specific for Lactoferrin are present on immune cells. Receptors for Lactoferrin have been identified and characterized on monocytes [17], B and T cells [42]. Once bound to the cell surface, Lactoferrin mediates up-regulation of adhesion molecule expression, such as that seen for leukocyte function associated-1 (LFA-1) antigen on human peripheral blood lymphocytes [43], an effect mediated by TNF-α. We show here the potential for Lactoferrin-mediated induction of both C–X–C (MIP-2) and C–C (MIP-1α) chemokines. Taken together, these described phenomenon may result in augmentation of the immune response since LFA-1 antigen is involved in cell to cell cooperation between T cells (LFA-1) and antigen-presenting cells (ICAM-1) during antigen presentation.

Lactoferrin therefore appears to be an ideal adjuvant component candidate for the production of cell-mediated responses. In healthy individuals, Lactoferrin does not elicit antibody production to itself. Also, there is no reported toxicity to Lactoferrin. To date there is no evidence for undesirable effects associated with subcutaneous, intramuscular, or oral administration of Lactoferrin (e.g. inflammation, necrosis, granuloma formation), with little to no overall toxicity [44,45]. Adjuvants that directly regulate immunomodulators of immune responses should be most efficacious, both qualitatively and quantitatively. Dose of adjuvant administered is important. J774A.1 cells produced IL-12 mRNA in a biphasic manner, the reasons for which remain unknown at this time. This suggests involvement of a high and low affinity receptor for Lactoferrin on these cells. More important is the presence of IL-12 message, even at low concentrations of Lactoferrin. IL-12 augments the production of IFNγ, which favors the production of cell-mediated responses [46]. The primary cascade of cytokine production at the site of

antigen and adjuvant injection is critical to further development of immune responses. The absence of IL-10, and the reduction in IL-10 mRNA from the J774A.1 cells, may be significant because of this molecule's ability to inhibit Th1 function [47]. Recent experiments indicate that in addition to initial immunomodulators that drive T helper cell development (e.g. IL-12, IL-4), it is also necessary to produce secondary molecules such as IL-15 to maintain T cell memory responses. IL-15 is involved in protection against infections through activation of innate immunity and via mounting further adaptive immunity [48]. IL-15 stimulates T cells and B cells to proliferate and secrete cytokines. IL-15 also exerts unique functions such as stimulation of phagocytes and migration of activated/memory T cells [49], with evidence for an additional role in development of memory cells to further dictate cell-mediated responses [50,51]. Although IL-15 may not play a direct role in our 4-day sensitization studies, the stimulation of phagocytes may be relevant in our model of DTH using specific antigen.

There is a genuine need for an adjuvant to replace CFA for generation of DTH and CMI. Clearly, CFA is an effective adjuvant for generation of cell-mediated response; however, it is undesirable even as a research reagent due to granuloma formation at the site of injection and local ulceration when delivered subcutaneously [4]. A specific niche for Lactoferrin-based adjuvants could be in the research market, as a replacement for CFA. Towards that goal, we have undertaken some basic experimental protocols to determine mechanisms of action in induction of the CMI response by Lactoferrin. The studies suggest that Lactoferrin may assist development of Th1 cell-mediated immunity, particularly in the absence of a pre-established adaptive response. Adjuvants must be targeted according to several criteria, such as target species, antigens, type of immune response, route of inoculation, or duration of immunity desired. Lactoferrin containing adjuvants may offer a unique opportunity to exploit a natural immune regulatory molecule to improve efficacy of vaccines.

References

[1] Gupta Jr RK, Rost BE, Relyveld E, Siber GR. Adjuvant properties of aluminum and calcium compounds. In: Powell MF, Newman MJ, editors. Vaccine design subunit and adjuvant approach. New York: Plenum; 1995. p. 229–48.

[2] Wijburg OL, van den Dobbelsteen GP, Vadolas J, Sanders A, Strugnell RA, van Rooijen N. The role of macrophages in the induction and regulation of immunity elicited by exogenous antigens. Eur J Immunol 1998;28:479–87.

[3] Linblad EB. Aluminium adjuvants. In: Stewart-Tull DES, editor. The Theory and Practical Application of Adjuvants. New York: Wiley; 1995. p. 21–35.

[4] Stewart-Tull DES. The use of adjuvants in experimental vaccines: II. Water-in-oil emulsions: Freund's complete and incomplete adjuvants. In: Robinson A, Farrar GH, Wiblin CN, editors. Methods in Molecular Medicine: Vaccine Protocols. Totowa: Humana Press; 1996. p. 141–5.

[5] Byrd TF, Horwitz MA. Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in nonactivated and interferon gamma-activated human monocytes depending upon its degree of iron saturation. Iron–lactoferrin and nonphysiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. J Clin Invest 1991;88:1103–12.

[6] Sorimachi K, Akimoto K, Hattori V, Ieri T, Niwa A. Activation of macrophages by lactoferrin: secretion of TNF-α, IL-8 and NO. Biochem Med Biol Int 1997;43:79–87.

[7] Lima MF, Kierszenbaum F. Lactoferrin effects on phagocytic cell function: I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. J Immunol 1985;134:4176–83.

[8] Ambruso DR, Johnston Jr RB. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J Clin Invest 1981;67:352–60.

[9] Zimecki M, Mazurier J, Machnicki M, Wieczorek Z, Montreuil J, Spik G. Immunostimulatory activity of lactotransferrin and maturation of CD4–CD8-murine thymocyte. Immunol Lett 1991;30:119–24.

[10] Zimecki M, Mazurier J, Spik G, Kapp JA. Human lactoferrin induces phenotypic and functional changes in splenic mouse B cells. Immunology 1995;86:112–27.

[11] Bhimani RS, Vendrov Y, Furmañski PJ. Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice. Appl Microb 1999;86:135–44.

[12] Tomita M, Bellamy W, Takese M, Yamamuchi K, Wakabayashi H, Kawase KJ. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. Dairy Sci 1991;74:4137–42.

[13] Wakabayashi H, Abe S, Okutomi T, Tansho S, Kawase K, Yamaguchi H. Cooperative anti-*Candida* effects of lactoferrin or its peptides in combination with azole antifungal agents. Microbiol Immunol 1996;40:821–5.

[14] Sato R, Inanami O, Tanaka Y, Takase M, Naito Y. Oral administration of bovine lactoferrin for treatment of intractable stomatitis in feline immunodeficiency virus (FIV)-positive and FIV-negative cats. Am J Vet Res 1996;57:1443–6.

[15] Yoo YC, Watanabe S, Watanabe R, Hata K, Shimazaki K, Azuma I. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. Jpn J Cancer Res 1997;88:184–90.

[16] Bezault J, Bhimani R, Wiprovnick J, Furmanski P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res 1994;54: 2310–2.
[17] Birgens HS, Hansen NE, Karle H, Kristensen LO. Receptor binding of lactoferrin by human monocytes. Br J Haematol 1983;54:383–91.
[18] Mazurier J, Legrand D, Hu WL, Montreuil J, Spik G. Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography. Eur J Biochem 1989;179:481–7.
[19] Duthille I, Masson M, Spik G, Mazurier J. Lactoferrin stimulates the mitogen-activated protein kinase in the human lymphoblastic T Jurkat cell line. Adv Exp Med Biol 1998;443: 257–60.
[20] Zimecki M, Miedzybrodzki R, Mazurier J, Spik G. Regulatory effects of lactoferrin and lipopolysaccharide on LFA-1 expression on human peripheral blood mononuclear cells. Arch Immunol Ther Exp 1999;47:257–64.
[21] Zimecki M, Machnicki M. Lactoferrin inhibits the effector phase of the delayed type hypersensitivity to sheep erythrocytes and inflammatory reactions to *M. bovis* (BCG). Arch Immunol Ther Exp 1994;42:171–7.
[22] Zimecki M, Kruzel ML. Systemic or local co-administration of Lactoferrin with sensitizing dose of antigen enhances delayed type hypersensitivity in mice. Immunol Lett 2000;74:183–8.
[23] Millet P, Kalish ML, Collins WE, Hunter RL. Effect of adjuvant formulation on the selection of B-cell epitopes expressed by a malaria peptide vaccine. Vaccine 1992;10:547–50.
[24] Seder RA, Paul WE, Davis MM, Fazekas J, Groth B. The presence of interleukin-4 during in vitro priming determines the lymphokine producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med 1992;176:1091–7.
[25] Perez RL, Roman J, Roser S, Little C, Olsen M, Indrigo J, et al. Cytokine message and protein expression during lung granuloma formation and resolution induced by the mycobacterial cord factor trehalose-6,6'-dimycolate. J Interferon Cytokine Res 2000;20:795–804.
[26] Actor JK, Kuffner T, Dezzutti CS, Hunter RL, McNicholl J. A flash-type bioluminescent immunoassay that is more sensitive than radioimaging: quantitative detection of cytokine cDNA in activated and resting human cells. J Immunol Methods 1998; 211:65–77.
[27] Actor JK. Quantitation of cytokine mRNA by flash-type bioluminescence. In: O'Neill L, editor. Methods in Molecular Medicine: Interleukin Protocols. Totowa, NJ: Human Press; 2001. p. 83–97.
[28] Siddiqi A, Jennings VM, Kidd MR, Actor JK, Hunter RL. Evaluation of electrochemiluminescence and bioluminescence based assays for quantitating specific DNA. J Clin Lab Anal 1996;10:423–31.
[29] Actor JK, Breij E, Wetsel RA, Hoffmann H, Hunter Jr, RL, Jaganath C. A role for complement C5 in organism containment and granulomatous response during murine tuberculosis. Scand J Immunol 2001;53:464–74.
[30] Stults NL, Rivera HN, Ball RT, Smith DF. Bioluminescent hybridization immunoassays for digoxigenin-labeled PCR products based on AquaLite, a calcium-activated photoprotein. J NIH Res 1995;7:74.
[31] Krishnan L, Sad S, Patel GB, Sprott GD. The potent adjuvant activity of archaeosomes correlates to the recruitment and activation of macrophages and dendritic cells in vivo. J Immunol 2001;166:1885–93.
[32] Makabi-Panzu B, Sprott GD, Patel GB. Coenzyme Q10 in vesicles composed of archaeal ether lipids or conventional lipids enhances the immuno-adjuvanticity to encapsulated protein. Vaccine 1998;16:1504–10.
[33] Clark A. Post-transcriptional regulation of pro-inflammatory gene expression. Arthritis Res 2000;2(3):172–4.
[34] Lonnerdal B, Iyer S. Lactoferrin: molecular structure and biological function. Annu Rev Nutr 1995;15:93–110.
[35] Sanchez L, Calvo M, Brock JH. Biological role of lactoferrin. Arch Dis Child 1992;67:657–61.
[36] Vorland LH. Lactoferrin: a multifunctional glycoprotein. APMIS 1999;107:971–81.
[37] Zimecki M, Pierce A, Spik G, Wieczorek Z. Immunoregulatory properties of the proteins present in human milk. Arch Immunol Ther Exp 1987;35:351–6.
[38] Kruzel M, Zagulski T, Zimecki M. Lactoferrin and insult-induced metabolic imbalance. In: Shimazaki K, Tsuda H, Tomita M, Kuwata T, Perraudin JP, editors. Proceedings, 4th International Conference on Lactoferrin: Structure, Function and Applications. ICS. New York: Elsevier; 2000. p. 301–11.
[39] Zimecki M, Spiegel K, Wlaszczyk A, Kubler A, Kruzel M. Lactoferrin increases the output of neutrophil precursors and attenuates the spontaneous production of TNF-α and IL-6 by peripheral blood cells. Arch Immunol Ther Exp 1999;47: 113–8.
[40] Adamik B, Zimecki M, Wlaszczyk A, Berezowicz P, Kubler A. Lactoferrin effects on the in vitro immune response in critically ill patients. Arch Immunol Ther Exp 1998;46:169–76.
[41] Choe YH, Lee SW. Effect of lactoferrin on the production of tumor necrosis factor-alpha and nitric oxide. J Cell Biochem 1999;76:30–6.
[42] Bi BY, Leveugle B, Liu JL, Collard A, Coppe P, Roche AC, et al. Immunolocalization of the lactotransferrin receptor on the human T lymphoblastic cell line Jurkat. Eur J Cell Biol 1994; 65:164–71.
[43] Zimecki M, Miedzybrodzki R, Mazurier J, Spik G. Regulatory effects of lactoferrin and lipopolysaccharide on LFA-1 expression on human peripheral blood mononuclear cells. Arch Immunol Ther Exp 1999;47:351–64.
[44] Yamauchi K, Toida T, Kawai A, Nishimura S, Teraguchi S, Hayasawa H. Mutagenicity of bovine lactoferrin in reverse mutation test. J Toxicol Sci 2000;25:63–6.
[45] Yamauchi K, Toida T, Nishimura S, Nagano E, Kusuoka O, Teraguchi S, et al. 13-week oral repeated administration toxicity study of bovine lactoferrin in rats. Food Chem Toxicol 2000; 38:503–12.
[46] Germann T, Rude E, Schmitt E. The influence of IL-12 on the development of Th1 and Th2 cells and its adjuvant effect for humoral immune responses. Res Immunol 1995;146: 481–6.

[47] Mosmann TR, Moore KW. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol Today 1991;12: A49–53.
[48] Yoshikai Y, Nishimura H. The role of interleukin 15 in mounting an immune response against microbial infections. Microb Infect 2000;2:381–9.
[49] Naora H, Gougeon ML. Activation, survival and apoptosis of CD45RO+ and CD45RO-T cells of human immunodeficiency virus-infected individuals: effects of interleukin-15 and comparison with interleukin-2. Immunology 1999;97:181–7.
[50] Ishimitsu R, Nishimura H, Yajima T, Watase T, Kawauchi H, Yoshikai Y. Overexpression of IL-15 in vivo enhances Tc1 response, which inhibits allergic inflammation in a murine model of asthma. J Immunol 2001;166:1991–2001.
[51] Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000;191:771–80.