Short communication

# THE EFFECT OF CARBOHYDRATE MOIETY STRUCTURE ON THE IMMUNOREGULATORY ACTIVITY OF LACTOFERRIN IN VITRO

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**Abstract:** The aim of this study was to evaluate the immunoregulatory effects of recombinant human lactoferrin (rhLF) in two in vitro models: (1) the secondary humoral immune response to sheep erythrocytes (SRBC); and (2) the mixed lymphocyte reaction (MLR). We compared the non-sialylated glycoform of rhLF as expressed by glycoengineered *Pichia pastoris* with one that was further chemically sialylated. In an earlier study, we showed that sialylated rhLF could reverse methotrexate-induced suppression of the secondary immune response of mouse splenocytes to SRBC, and that the phenomenon is dependent on the interaction of lactoferrin (LF) with sialoadhesin (CD169). We found that the immunorestorative activity of sialylated rhLF is also dependent on its interaction with the CD22 antigen, a member of the immunoglobulin superfamily that is expressed by B lymphocytes. We also demonstrated that only sialylated rhLF was able to inhibit the MLR reaction. MLR was inhibited by bovine lactoferrin (bLF), a glycoform that has a more complex glycan structure. Desialylated bLF and lactoferricin, a bLF-derived peptide devoid of carbohydrates, did not express such inhibitory activity. We showed that the interaction of LF with sialic acid receptors is essential for at least some of the immunoregulatory activity of this glycoprotein.

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## INTRODUCTION

Lactoferrin (LF) is an iron-binding protein contained in the exocrine fluids of mammals. With other protective proteins, such as lysozyme and IgA, it acts in the first line defense system against pathogens [1, 2]. LF is involved in maintaining homeostasis [3]; antioxidant [4] and antitumor [5] activities; maintaining the equilibrium state for intestinal microflora [6]; iron transport [7]; proteolysis [8]; lipopolysaccharide binding [9]; the induction of T and B lymphocyte maturation [10, 11]; the regulation of myelopoiesis [12]; and the immune response [13, 14]. The immunoregulatory activity of LF was also shown in mitogen-induced proliferation and the mixed lymphocyte reaction [15].

It is plausible that mammals have not developed specific receptors for LF because it is capable of interacting with its host cell receptors through various peptide or carbohydrate regions [16]. LF uses low- and high-affinity receptors that evolved to recognize structures on viruses, bacteria, and fungi: toll-like receptors [17, 18], heparan sulfate-containing proteoglycans [19, 20], CD14 [21], nucleolin [22], intelectin [23], C-type lectins [24], and sialic acid-binding immunoglobulin superfamily lectins (siglecs) [25]. In the C-type lectin family, LF uses mannose receptor [26] and DC-SIGN [27]. It was recently determined that it uses CD169 (sialoadhesin) from the siglec family [28].

LF, as a cationic molecule, interacts with lipopolysaccharide (LPS) in the serum. It effectively competes with LPS-binding protein (LBP), preventing the binding of the LPS–LBP complex to the CD14 receptor [29]. This function is independent of the sugar component, and is associated with the binding of LPS by the highly cationic portion of LF.

Interestingly, the function of LF as an adjuvant in the promotion of immunity to *Mycobacterium tuberculosis* in mice was associated with the presence of sialic acid [30]. In our earlier studies, we demonstrated that LF can reverse methotrexate-mediated suppression in the model of the humoral secondary immune response in vitro [31]. Using that model, we identified the receptor responsible for the upregulatory action of hLF as the CD169 antigen (also known as sialoadhesin or siglec-1) [28], which has an N-terminal domain that contains the sialic acid-binding site, mainly found on macrophages [32] acting as professional accessory cells [33] and antigen-presenting cells [34].

To further explore LF–cell receptor interactions, we evaluated the role of the CD22 molecule, another siglec family lectin that is present on B cells [35], in the in vitro immunorestorative activity of rhLF in methotrexate-induced suppression of the humoral immune response. We also attempt to assess the importance of sialic acid in human and bovine milk-derived LFs in the suppression of the human and mouse two-way mixed lymphocyte reaction.

## MATERIALS AND METHODS

### Mice
CBA and BALB/c mice (8 weeks old) were obtained from the Institute of Laboratory Medicine in Łódź, Poland. The mice were kept in standard conditions (air conditioned with a 12 h/12 h light/dark cycle, and commercial pelleted food and water ad libitum). The local ethics committee approved the study.

### Reagents and antibodies
We obtained sheep red blood cells (SRBC) from the University of Life and Environmental Sciences of Wrocław in Poland. Fetal calf serum (FCS) was from Gibco, and methotrexate (MTX) from Lachema. Rat anti-mouse anti-CD169 antibodies were purchased from AbD Serotec, and anti-CD22 antibodies from R&D Systems. The lectins *Sambucus nigra*-biotinylated (SNA) and *Griffonia simplicifolia* II (GSLII) were from Vector Laboratories, Inc., concanavalin A (ConA) was from Pharmacia, Fine Chemicals, and *Ricinus communis* agglutinin I (RCA I) and the monosaccharides were from Sigma-Aldrich. ConA, RCA and GSLII were biotinylated using biotinamidocaproate-N-hydroxysuccinimide ester from Sigma-Aldrich [36]. All of the other reagents were from Sigma-Aldrich.

### Lactoferrins
Non-sialylated ($Gal_2GlcNAc_2Man_3GlcNAc_2$; rhLF A) and chemically sialylated ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$; rhLF B) glycoengineered *Pichia pastoris*-derived human LFs were provided by PharmaReview Corporation. Both glycoforms were highly purified with endotoxin levels less than 4 EU/mg [28]. Bovine milk-derived LF (bLF), with an endotoxin level of 0.16 EU/mg, and bovine lactoferricin (bLFcin) were donated by Morinaga Co.

### Partial deglycolysation of bovine lactoferrin
bLF was treated with a mixture of *Diplococcus pneumoniae* glycosidases containing exo- and endoglycosidases including: α-neuraminidase, β-D-galactosidase, N-acetyl-β-D glucosaminidase, endo-N-acetyl-β-D-glucosaminidase and endo-N-acetyl-α-D-galactosaminidase but not α or β-D mannosidase [37]. The mixture of *D. pneumoniae* glycosidases was prepared as described [38]. bLF was treated with a mixture of the glycosidases in 0.1 M citrate-phosphate buffer (pH 5.5), and incubated for 6 days at 37ºC under toluene. The products of the enzymatic degradation were fractionated by gel filtration on a Sephadex G-100 column in 0.1 M acetate buffer (pH 5.8). The fraction containing deglycosylated protein (separated from the glycosidases and released sugars) was dialyzed against water and lyophilized. The total neutral sugar levels were determined using the phenol-sulfuric acid method for untreated and deglycosylated bLF (DegbLF). They were 6.6 and 4.7%, respectively. Binding of biotinylated lectins (reactive with bLF) to ELISA 96-well flat-bottom plates coated with untreated and enzymatically deglycosylated bLF was compared. SNA (specificity for α 2-6 sialic acid) and RCA (specificity for

β-D galactose) did not bind to enzyme-treated bLF, and GSLII (N-acetyloglucosamine specific lectin) showed about 80% lower binding than native bLF. No differences were found in the binding of concanavalin A lectin (which binds to the α-D-mannosyl and α-D-glucosyl groups) for native or deglycosylated bLF. These results showed significant, but not complete deglycosylation of bLF. The integrity of the deglycosylated bLF was confirmed by SDS-PAGE.

**The secondary humoral immune response to SRBC in vitro**
BALB/c mice were sensitized intraperitoneally with 0.2 ml of 5% SRBC suspension. After four days, the spleens from eight mice were isolated and a pooled cell suspension was prepared and suspended in the culture medium (RPMI-1640, supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics) at a density of $5 \times 10^6$ cells/ml. The cells were distributed to 24-well plates in 1 ml aliquots and 0.05 ml of 0.005% SRBC was added. MTX (0.5 mM) was added to the cultures at 24 h following initiation of the cell culture. The antibodies (anti-CD169, final dilution 1:250; and anti-CD22, final dilution 1:500), mannose (0.25 mM), galactose (0.25 mM) and sialic acid (0.25 mM) were added at the beginning of the culture. rhLF B (1 µg/ml) was added to the cultures 30 min after the inclusion of antibodies and monosaccharides. The number of antibody-forming cells (AFC) in the cultures was determined using local hemolysis in agar gel [39].

**Isolation of human PBMC**
Venous blood was withdrawn from healthy donors into heparinized syringes and diluted twice with PBS. PBMC (peripheral blood mononuclear cells) were isolated by centrifugation on a Ficoll-uropoline gradient (density 1.077 g/ml) and centrifuged at $800 \times g$ for 20 min at 4ºC. The interphase cells were then washed three times with Hanks' medium and re-suspended in the culture medium at density of $2 \times 10^6$ cells/ml.

**Mixed lymphocyte reaction**
Two models were used: a two-way human model and a one-way mouse model. For the two-way human model, PBMC from two donors ($2 \times 10^5$/100 µl each) in culture medium were mixed and placed in flat-bottom 96-well culture plates. LFs were added to the cultures at a concentration of 5 µg/ml. After a 5-day incubation in a cell culture incubator, the degree of cell proliferation was determined using the MTT colorimetric method [40].
For the one-way mouse model, splenocytes were isolated as described above. BALB/c splenocytes were incubated for 45 min at 37ºC in the presence of mitomycin c (50 µg/ml), followed by $3 \times$ wash with Hanks' medium. For the assay, $4 \times 10^5$ BALB/c cells/100 µl were mixed with $2 \times 10^5$ CBA cells/100 µl of the culture medium, and incubated in flat-bottom 96-well plates for 6 days in a cell culture incubator. LFs were used at a concentration of 25 µg/ml. The degree of cell proliferation was determined using the MTT colorimetric method [40].

**Statistics**

The results are presented as the mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post-hoc comparison with Tukey's test to estimate the significance of the difference between the groups. The significance was determined at $p < 0.05$. The statistical analysis was performed using STATISTICA 6.1 for Windows.

## RESULTS

**LF reverses MTX-induced suppression of the humoral secondary immune response to SRBC by interaction with CD22 and CD169 antigens**

The results in Table 1 show that the CD22 molecule is one of the targets for LF action in the reversal of MTX-induced suppression of the secondary humoral immune response in vitro to SRBC by sialylated rhLF. In the same experiment, we verified the involvement of the CD169 antigen in the reconstituting action of rhLF. The optimal concentrations of the LFs, MTX, antibodies, and sugars in this model were established in our previous studies [28, 31]. These results

Table 1. The number of antibody-forming cells (AFC) and the involvement of the CD22 and CD169 antigens in restoration of the MTX-suppressed secondary humoral immune response to SRBC by recombinant human lactoferrin. The results are the mean values of AFC/$10^6$ viable cells from quadruplicate wells ± S.E.

| Experimental system | AFC/$10^6$ cells | |
|---|---|---|
| | Mean | SE |
| Control | 1195 | 43.20 |
| MTX | 655 | 40.82 |
| rhLF B | 1585 | 66.08 |
| Anti-CD169 | 1235 | 74.83 |
| Anti-CD22 | 1245 | 75.50 |
| Sialic acid | 1275 | 32.66 |
| Mannose | 1560 | 55.60 |
| Galactose | 1355 | 28.28 |
| rhLF B + MTX | 1070 | 20.62 |
| rhLF B + anti-CD169 + MTX | 180 | 32.02 |
| rhLF B + anti-CD22 + MTX | 105 | 31.09 |
| rhLF B + sialic acid + MTX | 270 | 101.45 |
| rhLF B + mannose + MTX | 1235 | 40.82 |
| rhLF B + galactose + MTX | 1145 | 66.08 |

Statistics: control vs. rhLF B p = 0.0009; control vs. MTX p = 0.0001; control vs. mannose p = 0.0023; control vs. rhLF B + anti-CD169 + MTX p = 0.0001; control vs. rhLF B + anti-CD22 + MTX p = 0.0001; control vs. rhLF B + sialic acid + MTX p = 0.0001; MTX vs. rhLF B + MTX p = 0.0004; MTX vs. rhLF B + anti-CD169 + MTX p = 0.0001; MTX vs. rhLF B + anti-CD22 + MTX p = 0.0001; MTX vs. rhLF B + sialic acid + MTX p = 0.0011; MTX vs. rhLF B + mannose + MTX p = 0.0001; MTX vs. rhLF B + galactose + MTX p = 0.0001; rhLF B + MTX vs. rhLF B + anti-CD169 + MTX p = 0.0001; rhLF B + MTX vs. rhLF B + anti-CD22+ MTX p = 0.0001; rhLF B + MTX vs. rhLF B + sialic acid + MTX p = 0.0001 (ANOVA).

showed that the antibodies to CD169 and CD22 alone or monosaccharides (mannose, galactose and sialic acid) did not change the magnitude of the immune response measured as the number of antibody-forming cells. The addition of rhLF to the control cultures was stimulatory, but when added to MTX-treated cells, rhLF significantly ($p = 0.0004$) reduced MTX-induced suppression of the immune response. As demonstrated previously [28], the inclusion of sialic acid but not mannose or galactose in the cultures prevented rhLF-mediated upregulation of the immune response suppressed by MTX. Lastly and more importantly, the immunorestorative action of rhLF was blocked when anti-CD22 or anti-CD169 antibodies were used. The experiment was run in duplicate showing the same effects of added LFs, antibodies and monosaccharides. The results of one experiment are shown.

**The effects of rhLFs on the mixed lymphocyte reaction of human PBMC**
Sialylated and non-sialylated forms of rhLF showed differential effects in the model of human two-way MLR. Fig. 1 presents results from four representative blood donor combinations where only sialylated lactoferrin (rhLF B) used at concentrations of 5 µg/ml inhibited cell proliferation.
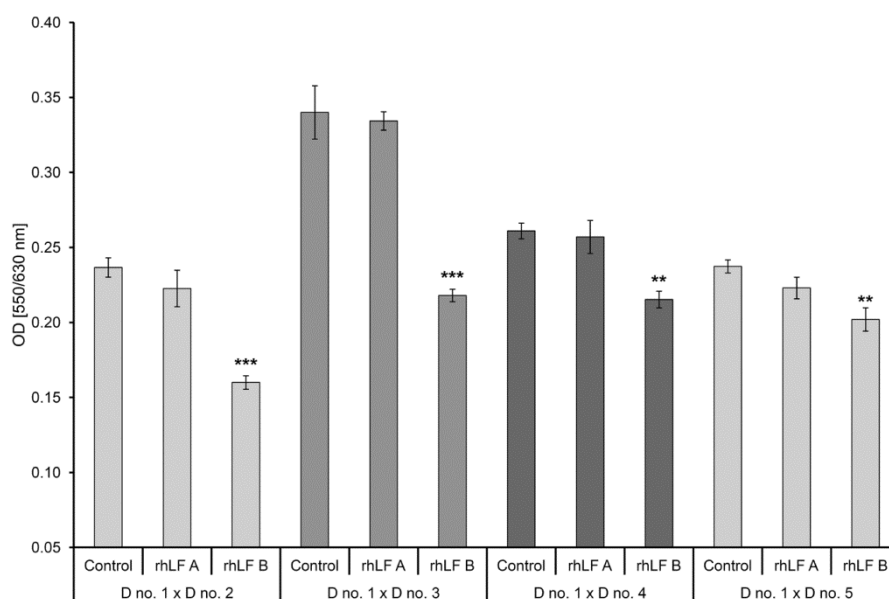


Fig. 1. The effects of sialylated (rhLF B) and non-sialylated (rhLF A) recombinant human LFs on the two-way mixed lymphocyte reaction of human PBMC. PMBC from two donors (D) each ($2 \times 10^5$/well) were mixed and placed in 96-well culture plates for a 5-day incubation. The LFs were used at a concentration of 5 µg/ml. The degree of cell proliferation was measured using the MTT colorimetric method. The results are presented as mean OD values from 4 wells ± SE. Only the effect of sialylated LF was statistically significant (***$p < 0.001$; **$p < 0.05$) compared to the control culture.

**Effects of deglycosylation of LF on mixed lymphocyte reaction in mice**
The importance of sugar residues in LF molecules has also been demonstrated in one-way MLR using CBA/BALB/c mice and milk-derived bovine LF. The results indicate that only the intact form of LF inhibited MLR (Fig. 2). There was no such effect for LF with a partially removed carbohydrate moiety (see the Materials and Methods section). Similarly, lactoferricin, a non-glycosylated LF-derived peptide [41], showed no inhibitory effect on MLR.
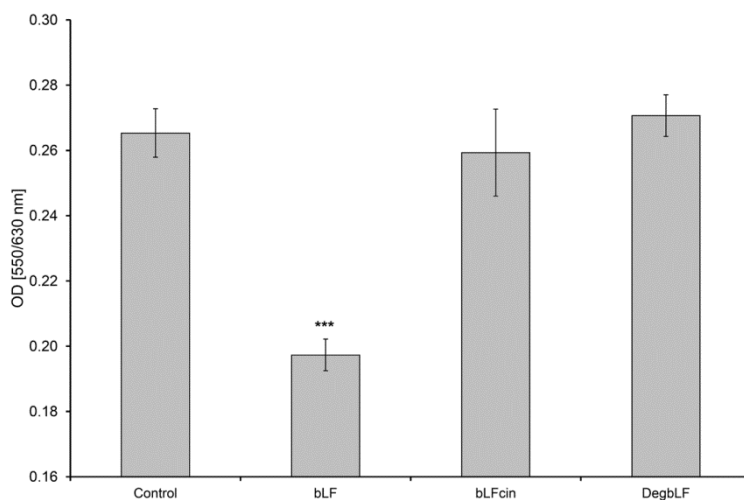


Fig. 2. The carbohydrate moiety in bovine LF is required for the inhibition of the one-way mixed lymphocyte reaction in mice. Splenocytes from BALB/c mice were preincubated with mitomycin c, followed with 3 washes. In the assay, $4 \times 10^5$ BALB/c cells were mixed with $2 \times 10^5$ CBA splenocytes (total volume 200 μl/well) in 96-well plates and incubated for 6 days. Lactoferrins were used at a concentration of 25 μg/ml. The proliferation rate was determined using the MTT method. The results are presented as mean OD values from four wells ± SE. Only bLF showed a statistically significant inhibitory effect (***$p < 0.001$) when compared with the control cultures.

## DISCUSSION

This report provides further insight into the mechanisms of the immunoregulatory activities of LF via interaction with the CD22 receptor, which has some affinity to sialic acid. The involvement of the CD22 antigen on B cells in the regulation of the immune response has yet to be fully elucidated. It is generally regarded as a negative signal receptor for B cell division and antibody response [35]. However, CD22 has a cytoplasmic domain possessing motifs potentially able to provide both inhibitory and stimulatory signals [42]. The occupation of CD22 by a specific antibody or sialic acid did not result in the inhibition or stimulation of the immune response, suggesting that under normal conditions, CD22 does not affect the magnitude of the immune response (Table 1). However, in the immunosuppressed immune response mediated in vitro by

MTX, which acts as a proapoptotic agent [43, 44], B cells may require triggering of a stimulatory signal via CD22 to develop the optimal immune response. Such a signal could be delivered by binding LF to CD22 and result in the protection of B cells against the suppressive action of MTX. Such a presumption is supported by the fact that the stimulatory signal delivered by LF was prevented by occupation of the receptor by a specific antibody or sialic acid. Although CD22 receptors on most B cells are occupied by endogenous sialylated ligands mediating the inhibitory signals, a proportion of B cells also exist with an activated phenotype with unmasked CD22 [45], and are thus available for rhLF binding. Therefore, it is possible that rhLF can trigger the stimulatory signals in these cells [42].

In addition to the proposed mechanism, rhLF may serve as a B-cell differentiation factor for immature B cells in the splenocyte population [11] and enlarge a pool of antigen-responding cells. Such action of LF could also explain the higher antibody response in control, unsuppressed cultures. Interestingly, rhLF amplified MTX-induced suppression when the lactoferrin receptors were blocked by the appropriate antibodies or sialic acid. At present, the explanation of this phenomenon may be purely speculative. It is possible that rhLF may bind to another type of cell receptor on B lymphocytes, such as that described by Kawasaki [46], which interacts with the basic amino acid residues of human LF. Such receptors [20] are involved in binding heparan sulfate and other acidic groups present in DNA or lysozyme.

Based on the results of the secondary humoral immune response experiments, we propose that both CD169 on accessory cells (presumably macrophages) and CD22 on B cells are involved in the mediation of the lactoferrin upregulatory actions observed in the models of immune suppression [31, 47] or in the application of suboptimal doses of antigen [48]. Based on the model used in this study, we also established that blocking only one of the LF receptors (on B lymphocytes or accessory cells) is sufficient to abolish the restorative effect of LF on the suppressed immune response, supporting the importance of accessory cell–B cell collaboration in the generation of the immune response [49, 50].

The inhibition of the mixed lymphocyte reaction by rhLF, using human PBMC, was also dependent on the presence of sialic acid residues. It is conceivable that in that model, rhLF used the same type of receptor as in the immune response to SRBC in vitro. However, in this case, the consequences of these interactions are inhibitory with respect to cell proliferation. A possible explanation for this phenomenon is that LF inhibits IL-1 production [51], which provides a necessary signal for monocyte-dependent anti-CD3-induced T-cell proliferation [52]. However, it should be stressed that the effects of LF on human MLR may be different depending on the combination of blood donors [15].

The importance of sialic acid in the LF molecule in MLR inhibition was also confirmed in the experiment employing bovine LF devoid of sialic acid and β-D-galactose (Fig. 2). The results of this experiment support the concept that the inhibitory action of LF in this model is dependent on the interaction of this

glycoprotein with a receptor bearing specificity for sialic acid and not, for example a specificity for the mannose receptor [53] (mannose and a part of N-acetyloglucosamine were not removed from the bLF preparation). Therefore, it was obvious that lactoferricin, a non-glycosylated lactoferrin-derived peptide sharing many properties with LF [41], would not suppress MLR.

In summary, here we demonstrated that LF is capable of reversing the immunosuppressive action of MTX by interaction with the CD22 receptor on B cells. We also found that in two experimental models, the immunoregulatory activity of LF was dependent on the interaction of this glycoprotein with a receptor specific for sialic acid. Furthermore, revealing the immunorestorative properties of lactoferrin in the system of the immunosuppressed immune response strengthens the concept that rhLF may be useful in augmenting efficacy of vaccination, particularly in cases of immune dysfunction or immunosuppression.

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