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Recombinant human lactoferrin expressed in glycoengineered *Pichia pastoris*: effect of terminal *N*-acetylneuraminic acid on *in vitro* secondary humoral immune response

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Abstract

Traditional production of therapeutic glycoproteins relies on mammalian cell culture technology. Glycoproteins produced by mammalian cells invariably display N-glycan heterogeneity resulting in a mixture of glycoforms the composition of which varies from production batch to production batch. However, extent and type of N-glycosylation has a profound impact on the therapeutic properties of many commercially relevant therapeutic proteins making control of N-glycosylation an emerging field of high importance. We have employed a combinatorial library approach to generate glycoengineered *Pichia pastoris* strains capable of displaying defined human-like N-linked glycans at high uniformity. The availability of these strains allows us to elucidate the relationship between specific N-linked glycans and the function of glycoproteins. The aim of this study was to utilize this novel technology platform and produce two human-like N-linked glycoforms of recombinant human lactoferrin (rhLF), sialylated and non-sialylated, and to evaluate the effects of terminal N-glycan structures on *in vitro* secondary humoral immune responses. Lactoferrin is considered an important first line defense protein involved in protection against various microbial infections. Here, it is established that glycoengineered P. *pastoris* strains are bioprocess compatible. Analytical protein and glycan data are presented to demonstrate the capability of glycoengineered P. *pastoris* to produce fully humanized, active and immunologically compatible rhLF. In addition, the biological activity of the rhLF glycoforms produced was tested *in vitro* revealing the importance of *N*-acetylneuraminic (sialic) acid as a terminal sugar in propagation of proper immune responses.

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Keywords

Recombinant human lactoferrin; *Pichia pastoris* expression system; Humanized N-linked glycoforms; Humoral immune responses

Introduction

Compared to the mammalian cell culture technology currently in use for the production of most therapeutic glycoproteins, the methylotrophic yeast *Pichia pastoris* has the capability of yielding up to 20 times more product. However, glycoproteins derived from *P. pastoris* as well as other fungal and yeast expression systems display fungal-type high mannose N-linked glycans. These glycans are believed to contribute to reduce the half-life of the glycoprotein *in vivo* and may be immunogenic, thus limiting the potential therapeutic value of fungal-derived glycoproteins [15].

Human N-glycosylation is a multi-step process localized to the secretory pathway of cells. The complex metabolic engineering endeavor of replicating the mammalian glycosylation machinery in yeast requires the cloning and functional expression of a large number of foreign glycosylation pathway enzymes in the host strains. Each enzyme catalyzes a reaction yielding the substrate for the subsequent enzyme. Thus, each enzyme must be properly targeted and must function at high efficiency in its respective location in the secretory pathway. The application of a combinatorial library approach has been essential to generate *P. pastoris* strains harboring combinations of mannosidases, glycosyltransferases, GlcNAc/Gal transporters, and Gal epimerase [24]. Over the past years we have created a library of glycoengineered *P. pastoris* strains each capable of displaying defined N-linked glycans at high uniformity [5,8, 12,17,24]. This growing library of strains has the potential to allow elucidation of the relationship between specific N-linked glycans and the function of glycoproteins.

Lactoferrin (LF), an iron-binding glycoprotein is found in most mammalian exocrine secretions, including milk, tears, saliva, bronchial and intestinal secretions and also in the secondary granules of neutrophils. It is considered a first line defense protein involved in protection against microbial infections [26,31] and prevention of systemic inflammation [2, 3,21]. More recently, lactoferrin has been implicated in immunoregulatory functions [19,42, 44,46], as a modulator of vaccine function [18], and containing chemoprotective activity [1]. The primary structure of human LF is characterized by a single polypeptide chain containing 692 amino acids organized in two highly homologous lobes, designated the N- and C-lobe, each capable of binding one ferric ion (Fe^{+++}). The low-density lipoprotein receptor-related protein-1 and -2 (LRP1 and LRP2) are considered primary LF receptors. Although members of the LRP family are generally considered as endocytic receptors, LRP1 can also function as a signaling receptor [29]. The key to understanding the molecular basis of LF various activities is thought to reside in part according to patterns of glycosylation [37]. There are three possible N-linked glycosylation sites in hLF, one at Asn138, a second site at Asn479, and a third site at Asn624; differential utilization of these sites results in distinct glycosylation variants [30, 35]. Human LF glycans are the *N*-acetyllactosaminic type, α1-3-fucosylated on the *N*-acetylglucosamine residue linked to the peptide chain. Unlike the milk-derived LF, the neutrophilic form is not fucosylated, and the difference in molecular structure and function of the two forms is not fully understood [23,32].

The aim of this study was to produce two glycoforms of recombinant human lactoferrin (rhLF): sialylated and non-sialylated, and evaluate the effects of terminal N-glycan structures on *in vitro* secondary humoral immune responses suppressed by methotrexate (MXT). To this end glycoengineered *P. pastoris* capable of producing a highly uniform N-glycan structure with

terminal galactose ($Gal_2GlcNAc_2Man_3GlcNAc_2$) was selected and employed for *in vitro* sialylation to create terminally sialylated N-glycan ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$) of rhLF. Here, it is demonstrated that LF with specific human N-glycan structures can be produced in glycoengineered lines of the yeast *P. pastoris* and the two major LF glycoforms produced in this expression system exhibit a significantly different ability to overcome the suppressive action of methotrexate in the secondary, humoral immune response to sheep erythrocytes in mice.

Experimental procedures

Strains, culture conditions and reagents

Escherichia coli strain TOP10 was used for recombinant DNA work. *P. pastoris* yAS309 [24] was used for generation of rhLF producing strains. Protein expression studies were done at room temperature in a 96-well plate format with buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol as a growth medium. The induction media were buffered methanol-complex medium (BMMY) consisting of 0.5% methanol and buffered dextrose-complex medium (BMDY) consisting of 2% dextrose, respectively. hLF standard purified from human milk was purchased from Sigma (St. Louis, MO). Restriction and modification enzymes were from New England BioLabs (Beverly, MA). All chemicals were ACS-grade, and CMP-sialic acid and trypsin were purchased from Sigma (St. Louis, MO), recombinant rat α 2,6-(*N*)-sialyltransferase from Calbiochem (San Diego, CA), 2-aminobenzamide (2-AB) dye from Aldrich (St. Louis, MO). A polyclonal antibody against *P. pastoris*-derived host cell proteins (anti-HCP) was generated as follows; the culture supernatant from *P. pastoris* lacking hLF construct was purified in a similar fashion as described in the capturing step of rhLF. The purified proteins in PBS were used for polyclonal antibody generation in rabbits (Rockland Immunochemical Inc., Boyertown, PA).

Expression constructs and generation of production strains

For the signal sequence study, pPICZA (Invitrogen, Carlsbad, CA) was digested with *Eco*RI and *Kpn*I, and the resulting pPICZA was ligated with 11 different signal sequences (*Eco*RI and blunt ended) and the codon-optimized hLF cDNA (blunt and *Kpn*I ended) (provided by PharmaReview Corporation, Houston, TX). For the promoter study, PpAOX1 promoter was replaced with the PpGAPDH promoter in pBK422 at *Bgl*II and *Eco*RI sites. All expression constructs were sequence verified. For the generation of hLF production strains, *Pme*I-digested DNAs were transformed into yAS309 by electroporation, according to the *Pichia* expression kit handbook from Invitrogen. See Table 1 for designated plasmids and strains.

Detection of rhLF

Proteins were separated by 4–20% gradient SDS-PAGE and then electroblotted onto nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was probed with rabbit anti-human LF antibody (anti-hLF) (1:1000) (Sigma, St. Louis, MO) and followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:4,000) (Pierce, Rockford, IL). The results were visualized using an ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL).

Fermentation

A seed culture was prepared by adding 1 ml of thawed cells to a 2 L baffled flask containing 400 ml of 4% BMGY medium. When an OD600 of 20±5 was reached, the seed culture was transferred to the production fermenter. The systems was controlled by Applikon 1030 Bio-controller with closed loop control of pH, temperature, dissolved oxygen concentration and

foam control as described earlier [24]. The pH was maintained at 6.0 throughout the fermentation. Fermentation runs were carried out in 15 L (12 L working) autoclavable glass bioreactors from Applikon (Foster City, CA).

Protein purification

Primary clarification was performed at 4°C for 15 min at 13,000×*g* by centrifugation in a Sorvall Evolution RC (Kendo, Asheville, NC) followed by the microfiltration and diafiltration steps using a 0.1 μm cut-off 3600 cm^2 PES hollow fibre cartridge (CFP-1-E-8A) (GE Amersham, Pittsburgh, PA) and 5×0.1 m^2 Pellicon 2 Mini 10 kDa NMWCO regenerated cellulose ultrafiltration cassettes (A screen) (Millipore, Billerica, MA), respectively. Protease inhibitors pepstatin A and chymostatin (Sigma) were added to the supernatant after filtration steps in a concentration of 5 and 3 μg/ml respectively. After the ultrafiltration/diafiltration/filtration steps, *P. pastoris*-derived rhLF was purified by two chromatographic steps; cation exchange chromatography using SP Sepharose Fast Flow followed by Heparin Sepharose 6 Fast Flow chromatography (GE Healthcare, Piscataway, NJ). Briefly, SP Sepharose resin was equilibrated with 50 mM Tris–HCl, pH 8.0 while the supernatant media was adjusted at the same pH and conductivity around 5 mS/cm by the diafiltration procedure. The elution was done with 10 column volume (CV) of a gradient of 0–1 M NaCl in the same buffer. rhLF containing fractions were pooled and dialyzed against 50 mM Tris–HCl, pH 7.5 overnight. As the final purification step the affinity resin, Heparin Sepharose 6 Fast Flow, was used and the column was equilibrated with 50 mM Tris–HCl (pH 7.5). The pooled and dialyzed protein from SP Sepharose was loaded on Heparin Sepharose and washed in three steps. The first wash was done with 2 CV of the same buffer, followed by the second wash with 10 CV of a detergent buffer (10 mM CHAPS, 10 mM EDTA in 50 mM Tris–HCl, pH 7.5) to decrease endotoxin levels. The last wash was carried out with a 10 CV of 50 mM Tris–HCl (pH 7.5). The protein was eluted with a 10 CV of a gradient of 0–1 M NaCl. A fraction of the pooled protein was dialyzed against PBS (pH 7.2) and stored at 4°C as a final product of nonsialylated hLF. The other fraction was dialyzed against 50 mM MES, pH 6.5 for the preparation of the *in vitro* sialylation. The *in vitro* sialylated rhLF was purified using Heparin Sepharose 6 Fast Flow and dialyzed against PBS (pH 7.2), and stored at 4°C.

Size exclusion chromatography

Proteins were separated with a BioRad BioSil SEC250 column (Hercules, CA) using a mobile phase of 100 mM sodium phosphate, pH 6.8, 150 mM NaCl and 0.05% sodium azide at 0.5 ml/min and detected at 280 nm.

Reverse-phase HPLC

Proteins were separated with a Phenomenex Jupiter 5 μ C4 300 Å column (Torrance, CA) using a 1.0 ml/min 39-min linear gradient from 95% to 30% buffer A (0.1% TFA in water). Buffer B was 0.08% TFA in acetonitrile. Temperature was maintained at 80°C using a column oven. A Hitachi diode array detector monitoring at 220 nm was used for detection.

MALDI-TOF analysis of glycans

N-glycans were released and separated from hLF as described earlier [8,12]. Molecular weight was determined by using a Voyager linear matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems) with delayed extraction.

N-glycan structure analysis

2-Aminobenzamide (2-AB) labeling was used to quantify N-glycan structures. A solution of 5% 2-AB dye and 6.3% sodium cyanoborohydride was prepared in 1:4 glacial acetic acid/DMSO. Five microliters of this solution was added to dried glycan samples, mixed, and

incubated for 2–3 h at 65°C. Each sample was applied to wells of a 96-well lysate plate (Promega Cat# A2241, Madison, WI) and then washed and pre-wetted with acetonitrile and adsorbed for 10–15 min; wells were then washed with 1 ml acetonitrile followed by three 1 ml 96% acetonitrile/4% water washes. Glycans were eluted three times with 0.4 ml water and dried in a centrifugal vacuum for 24 h. Labeled glycans were then separated by HPLC using a flow rate of 1.0 ml/min with a Prevail CHO ES 5-micron bead, amino-bound column using a 50-min linear gradient of 80% to 40% buffer A (100% acetonitrile). Buffer B consisted of 50 mM ammonium formate pH 4.4. Sialylated glycans were separated using a 30-min 80–40% Buffer A linear gradient with an additional 30-min gradient bringing buffer A from 40% to 0%. Labeled glycans were detected and quantified against standards using a fluorescence detector with an excitation of 330 nm and an emission at 420 nm.

Trypsin peptide mapping

Two milligrams of 0.25 mg/ml rhLF was diluted 1:2 with 10 M guanidine HCl pH 7.8. To this mixture, dithiothreitol (DTT) was added to a final concentration of 10 mM and incubated at 37°C for 1 h. Iodoacetic acid in Tris base neutralized with NH_4OH was added to a final concentration of 40 mM and incubated in the dark for 1 h. The sample was then buffer-exchanged and concentrated with a Vivaspin 50,000 MWCO concentrator to roughly 1 mg/ml in 100 mM Tris–HCl, pH 7.4. The sample was subsequently split into four aliquots with each receiving 2.6 μg of trypsin in 10 mM HCl. Samples were incubated overnight at 37°C then boiled for 5 min. Two aliquots were deglycosylated with 5 μl of PNGase F for 30 min at 37° C. Peptides were resolved with a Phenomenex Jupiter 4 μ Proteo 90 Å column using an 87-min linear gradient of 95% to 55% Buffer A (0.1% TFA in water) at 1.0 ml/min. Buffer B was 0.08% TFA in acetonitrile. Temperature was maintained at 30°C using a column oven. A Hitachi diode array detector monitoring at 220 nm was used for detection. One minute fractions were collected from the column into a 96-well plate and dried.

Nanospray mass spectrometry

Digested peptides were dissolved in 50 μl 30% acetonitrile, 0.1% formic acid. Fractions with peptides that appeared on the HPLC trace after PNGase F digest (RT=55, 56, 63, 64, 65, 66 min) were transferred to a 96-well Abgene sample plate and sealed with adhesive foil. A Thermo Finnigan LTQ mass spectrometer was set to data-dependent triple play mode to analyze the top five most intense peaks for 2.9 min through Advion Triversa Nanomate. Data were analyzed using SEQUEST (Thermo Finnigan, Waltham, MA).

In vitro sialylation

Two hundred microunits sialyltransferase and 3.875 mg CMP-sialic acid (final concentration; 52.5 μM) was added to 31 mg of hLF in 50 mM MES pH 6.5, 10 mM $MnCl_2$, 0.73 μM chymostatin, and 52 ng/l pepstatin and incubated overnight at 25°C. Sialylated rhLF was dialyzed against 20 mM Tris (pH 8.0) overnight followed by purification using heparin Sepharose Fast Flow 6.

Endotoxin assay

Endotoxin levels were assayed using a limulus amebocyte lysate endochrome assay kit from Charles River Laboratories according to the manufacturer's instructions (Charleston, SC).

Enzyme-linked immunosorbent assay

A high-protein binding 96-well plate (Costar) was coated with 100 μl/well of rabbit anti-human LF antibody (Sigma) diluted 1:5,000 in PBS (EMD Biosciences, San Diego, CA) overnight at 4°C. Antibody was aspirated and the plate was blocked for 1 h at room temperature with 200 μl of 3% BSA in PBS. Blocking solution was aspirated and replaced with 100 μl of serial

dilutions of commercially available human colostrum hLF (Sigma) and samples to be assayed. Standard hLF was diluted two-fold serially in PBS from 100 to 0.1 ng/ml; fermenter samples were typically diluted 1:100 and then two-fold serially to 1:100,000. Standards and samples were incubated for 1 h at room temperature then aspirated and washed three times with 300 μl/well of 0.05% Tween 20 in PBS using a manifold plate washer. Wash buffer was aspirated and then 100 μl/well of HRP-conjugated anti-hLF (Jackson Immunoresearch, West Grove, PA) diluted 1:5,000 in PBS was added and incubated for 1 h at room temperature. Wells were washed, 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added and the final reaction terminated with 1 MH_2SO_4 after which absorbance at 450 nm was measured.

Protein assays

Protein concentration was estimated using the method of Bradford as described [6]. Protein assay reagents were from Pierce Biotechnology (Rockford, IL). Bovine serum albumin (Pierce Biotechnology) and human milk lactoferrin (Sigma) were used as standards.

Mice

Twelve-week-old CBA male and female mice were used for the studies. All *in vivo* experiments were conducted under animal ethics committee approved guidelines.

The secondary humoral immune response in vitro

Mice were primed with intraperitoneal administration of 0.2 ml 1% sheep red blood cells (SRBC) suspension. Splenocytes were isolated after 4 days and single cell suspensions were prepared in culture medium consisting of RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The cells were incubated in 24-well culture plates (5×10^6/ml/well) with 50 μl 0.005% SRBC. Lactoferrins were added to the cells cultures in concentration of 1 μg/ml at the initiation of culture, MTX at concentration of 0.25–0.5 mM, after 24 h, and sialic acid (0.5 mM) at 30 min before addition of LF. The antibody against mouse sialoadhesin (Serotec, rat anti-mouse CD169, clone 3D6.112, final dilution 1:250) was added to the cell cultures 1 h before LF. After 4 days the number of AFC was determined by the method of local hemolysis in agar [27]. The results are shown as mean values of AFC number from five wells±SE, calculated per 10^6 viable cells.

Statistics

The differences across groups were determined by analysis of variance after testing homogeneity of variance by Levene's test. Individual grades were then compared using the Tukey's test for multiple comparisons. The data are expressed as: mean, mean±SE (standard error) and mean±SD (standard deviation). Differences were considered significant when $p<0.05$. The statistical analysis was performed using STATISTICA 6.0 for Windows.

Results

Optimization of recombinant human lactoferrin expression in glycoengineered P. pastoris

The expression of rhLF was optimized by such factors as codon usage, signal sequence, promoter, pH, $FeCl_3$, and induction time. Previously, DNA codon optimization has successfully been applied to improve expression of heterologous proteins in yeast. In order to improve the translational efficiency of rhLF in *P. pastoris*, a codon-optimized nucleotide sequence encoding hLF was synthesized based on the original sequence (Fig. 1). Amino acid codons were selected based on a *P. pastoris* codon usage table (GlycoFi™ proprietary).

A codon-optimized hLF cDNA was fused to 11 different signal sequences (pBK833–pBK850 in Table 1) to identify the optimal secretion sequence facilitating translocation of rhLF into the

secretory pathway and ultimately the culture medium. rhLF-producing strains representing 11 signal sequences were generated by transforming eleven expression constructs into yAS309. The efficiency of each signal sequence was evaluated by Western blot of the culture supernatants. Out of the signal sequences tested, *S. cerevesiae* alpha mating factor prepro (ScαMFppKR: pBK842) was selected in which ScαMFppKR was engineered to contain a Kex2p cleavage site (KR) at C-terminal of the signal sequence to facilitate processing of the protein prior to secretion of mature rhLF. The resulting strain was designated BK422. BK422 was further optimized to maximize rhLF production.

The pH of medium can have an impact on the overall production of the protein of interest by increasing the activity of specific proteases secreted from the host strain as well as influencing protein stability. In order to minimize proteolysis and to enhance protein stability, protein production was tested at different pH values in the induction medium (BMMY) ranging from 6.0 to 7.5. The pH of growth medium (BMGY) was maintained at 6.0. At pH over 6.5, proteolytic degradation of rhLF was observed. The optimal pH of the induction medium was found to be 6.0.

hLF is an iron-binding glycoprotein and is structurally organized into two lobes. Each lobe binds one Fe^{3+} ion. In some cases the expression levels of hLF have been influenced by supplementation of $FeCl_3$ [36]. In order to identify the optimal concentration of $FeCl_3$, the induction medium was supplemented with $FeCl_3$ in the range of 0.05–2.0 mM final concentration. In our study, $FeCl_3$ supplementation had little effect on the product yield in the range from 0.05 to 0.5 mM whereas concentrations above 0.5 mM displayed a negative impact. Thus, $FeCl_3$ supplementation was not required in our induction medium at pH 6.0.

Two different *P. pastoris* (Pp) promoters were evaluated for their ability to drive rhLF expression. An inducible promoter from *P. pastoris*: alcohol oxidase 1 (PpAOX1) and one constitutive promoter derived from the glyceraldehyde-3-phosphate dehydrogenase gene of *P. pastoris* (PpGAPDH) were evaluated for their ability to drive hLF expression. The AOX1 promoter is tightly regulated at the transcription level, that is, it is repressed in the presence of glucose, whereas it is strongly induced in the methanol medium. rhLF was induced at 0.5% methanol under AOX1 promoter and at 2% dextrose under GAPDH promoter. The proteolytic degradation of rhLF was more pronounced under GAPDH promoter (BK427) than AOX1 promoter-driven protein expression (BK422). The AOX1 promoter-driven expression displayed the least amount of proteolysis and it was used to produce rhLF in a bioreactor.

A time course study was performed to determine the optimal induction time. Methanol was added to the culture every 20 h to maintain rhLF induction during 4-day fermentation. Samples were taken at different time-points during fermentation. Over the course of 4 days of induction, product yield continued to increase. However, an increased amount of proteolysis was also observed. An induction time of about 2 days was determined as an optimum.

Production of recombinant lactoferrin in a bioreactor

rhLF production run was carried out with BK422 in a 15 L bioreactor described in the Experimental procedures section. rhLF was induced for 38 h and the product yield was 99.8 mg/l as measured by ELISA using hLF standard (Sigma). Proteolytic degradation was further reduced by supplementing the fermentation media with the protease inhibitors pepstatin A and chymostatin during induction. A total 841 mg rhLF was subjected to microfiltration followed by ultrafiltration/diafiltration steps (see the “Experimental procedures” section). After a series of filtration steps, the product recovery was 183 mg of rhLF (21% recovery).

Purification of recombinant lactoferrin

rhLF was purified using SP Sepharose and Heparine Sepharose 6. SP Sepharose captured rhLF effectively and was easily scalable from 300 μl to 160 ml of resin. Heparin Sepharose 6 was able to separate isoforms of hLF and chosen for the final purification step to further improve the protein purity.

Figure 2 shows the SDS-PAGE and Western blot analysis of rhLF purified from the fermentation supernatant. rhLF was successfully purified by SP Sepharose (Fig. 2a,b and c). Western blot analysis using an anti-HCP antibody demonstrated that SP Sepharose is a good capturing step to obtain a highly pure protein free from host cell contaminants (Fig. 2c). As an advantage, passage over Heparin Sepharose significantly reduced endotoxin levels.

Generation of sialylated bi-antennary rhLF by in vitro sialylation

In vitro sialylation of rhLF was carried out as described in the Experimental procedures section, with purity reconfirmed by SDS-PAGE of the non-sialylated and sialylated rhLF. The protein N-terminal sequence was directly compared, indicating that the *in vitro* sialylation process did not affect the integrity of the N-terminus of the protein. The endotoxin levels were measured and determined to be 4 EU/mg (sialylated) and 3 EU/mg (non-sialylated), respectively.

The purity of sialylated and non-sialylated rhLF samples was confirmed by reverse phase HPLC (data not shown). Size exclusion chromatography was also performed, demonstrating similarity in molecular weights of both molecules. Prominent peaks at *m/z* of 79,373.5 and 81,250.86 were observed by MALDI-TOF (Fig. 3a). A second pair of peaks at 39,731.4 and 40,646.1 suggests doubly-charged species of 79.3 and 81.2 kDa respectively, correlating with the masses of doubly- and triply-glycosylated rhLF (79.6 and 81.2 kDa, respectively). MALDI-TOF analysis of sialylated rhLF (Fig. 3b) showed a broader peak at about 81,000 *m/z* with the doubly charged spectra having a *m/z* of ~40,000. The greater *m/z* ratio is most likely due to the presence of sialic acid in this sample.

MALDI-TOF analysis was used to assess the N-linked glycan profile after enzymatic release from rhLF. Analysis of N-linked glycans released from non-sialylated rhLF in the positive ion mode showed a predominant mass of 1,666.33 indicative of an afucosylated biantennary complex glycan with terminal galactose or $Gal_2GlcNAc_2Man_3GlcNAc_2$ (Fig. 4a). Additionally, the human glycoforms $GalGlcNAc_2Man_3GlcNAc_2$ (1504.08) and $Man_5GlcNAc_2$ (1,258.82) were also observed, although less prominent. N-glycan of sialylated rhLF was analyzed by MALDI-TOF in the negative ion mode and showed a dominant cluster of ions in the range of 2,229.03–2,444.43 consistent with a mass describing $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$ and associated cation adducts (Fig. 4b) Since MALDI-TOF provides a qualitative/semi-quantitative assessment of N-glycosylation, we employed a normal phase-HPLC analysis to quantify the relative abundance of specific N-linked glycan structures. Non-sialylated rhLF was determined to contain 50.99% $Gal_2GlcNAc_2Man_3GlcNAc_2$ (GS5.0) with other human-type glycans comprising $GlcNAc_2Man_3GlcNAc_2$ (GS4.0), $GalGlcNAc_2Man_3GlcNAc_2$ (GS4.5), $GalGlcNAcMan_5$ and $GlcNAcMan_5$ hybrids, and $Man_5GlcNAc_2$ (GS2.0). N-glycosylation analysis of the sialylated rhLF sample contained 46.2% terminally bisialylated N-linked glycans and 15.9% monosialylated N-linked glycans, where most of $GalGlcNAc_2Man_3GlcNAc_2$ (GS4.5) and $Gal_2GlcNAc_2Man_3GlcNAc_2$ (GS5.0) glycans were converted into $SiaGal_2GlcNAc_2Man_3GlcNAc_2$ (GS5.5) and $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$ (GS6.0). However, $GlcNAc_2Man_3GlcNAc_2$ (GS4.0) and high mannose containing structures content from sialylated rhLF remained similar to non-sialylated rhLF because they are not substrates for rat α 2,6-(*N*)-sialyltransferase.

Recently, it has been described that glycosylation of hLF derived from mammalian cells occurs predominantly at two sites (N138 and N479) in approximately 85% of all hLF molecules. Glycosylation at a single site (N479) or at all three sites (N138, N479, and N624) occurs in only approximately 5% and 9% of hLF, respectively [35]. In order to determine N-glycosylation occupancy of each site, rhLF was digested with trypsin. One half of the sample was deglycosylated with PNGase F with the other one-half remaining untreated. Both samples were subjected to HPLC analysis. Whole chromatograms for each sample were overlaid in order to identify glycopeptides and the same peptides lacking glycan. Figure 5 shows that the N-glycan occupancy was identified on chromatogram between 52 and 67 min of retention time. To confirm the peptide sequences, HPLC fractions were collected and subjected to MS/MS analysis. For fractions from the untreated sample, PNGase F treatment was performed prior to MS/MS analysis in order to elucidate the glycopeptide sequences. The mixture of glycopeptides (N138 and N479) was identified, but the glycopeptide containing N629 was not resolved well. It is likely that the N629 glycopeptide's signal was buried because of co-elution with other peptides. However, based on the peptide mapping, it was confirmed that all three N-linked sites of rhLF were predominantly occupied with N-glycans, whereas it has been shown that only 9% of hLF was N-glycosylated at all three sites when expressed in mammalian cell-derived systems.

Reconstituting action of recombinant lactoferrin in the secondary humoral immune response in mice suppressed by methotrexate

It was previously demonstrated that bovine lactoferrin (bLF) can reduce methotrexate (MTX)-induced suppression of secondary humoral immune responses *in vitro* to sheep erythrocytes (SRBC) [1]. Here both sialylated and non-sialylated rhLF were tested *in vitro* to demonstrate effects on the secondary humoral immune response. Human, milk-derived LF was used as a reference protein. The results (Fig. 6) demonstrated that sialylated rhLF was able to restore the number of antibody-forming cells in cultures treated with MTX. The non-sialylated rhLF was ineffective in the reduction of MTX-induced suppression of the secondary humoral immune response. Milk-derived LF was either moderately active or not active at all (data not shown). Since the results indirectly indicate the importance of sialic acid in mediation of the biological activity, a moderate concentration of free sialic acid was added to the cell cultures to block rhLF interaction with receptor (Fig. 7a). The results demonstrate that the addition of sialic acid significantly reduced the ability of sialylated rhLF to restore the MTX-mediated suppression. In addition, when monoclonal antibody to CD169, directed against the murine sialoadhesin, was added to the culture, the sialylated rhLF was unable to reverse the MTX-mediated suppression (Fig. 7b). This demonstrates a requirement of rhLF to bind to sialoadhesin for functional activity.

Discussion

GlycoFi™ has developed glycoengineered *P. pastoris* strains capable of producing glycoproteins with humanized N-glycans [5,16,17]. In contrast to mammalian cells, glycoengineered yeasts allow the production of glycoproteins with highly homogenous N-linked glycans. The use of glycoengineered yeast permits the exploration of the structure-function relationships and can improve efficacy of therapeutic glycoproteins. In this study, rhLF was produced at a high level in glycoengineered *P. pastoris* that predominantly consists of a humanized glycan, $Gal_2GlcNAc_2Man_3GlcNAc_2$. A subsequent *in vitro* sialylation of rhLF (glycoform: $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$) was further explored for reconstitution activity in a splenocyte model of immune-mediated recovery of suppressed responses.

Optimization of culture conditions and genetic improvements allowed for high-level expression of rhLF. Signal sequences play an important role in translocating proteins into the

secretory pathway and they have shown their different preferences for proteins. Based on the screening of signal sequence library, the signal sequences from *A. oryzae* (glucoamylase), *K. maxianus* (inulinase), and *P. pastoris* (KAR2), and *S. cerevisiae* (αMFppKR) demonstrated higher rhLF levels secreted in the culture supernatants as well as increased intracellular rhLF accumulation. The optimal signal sequence (ScαMFppKR) was found not only to facilitate its secretion, but also to minimize intracellular accumulation. In addition, N-terminal sequencing of rhLF confirmed the correct processing of the signal sequence (ScαMFppKR).

Several promoters have been developed for heterologous protein expression using the *Pichia* system. The AOX1 and GAPDH promoters are widely used for protein production. Under optimized culture conditions, the PpAOX1 promoter performed better than the PpGAPDH promoter since more degradation of rhLF was observed under the PpGAPDH promoter. This indicates that the inducible promoter (PpAOX1) provides better control, most likely due to the shorter production phase, than the PpGAPDH process.

The purification of rhLF was performed using two chromatographic steps after filtration. The SP Sepharose step proved to be an excellent capture and purification step as shown in Fig. 2. In addition, the removal of host cell proteins was very efficient as determined by Western blot analysis. The second step proved to be crucial for the reduction of endotoxin levels to a value <5 EU/mg, equally successful with sialylated and non-sialylated protein. The combination of SP Sepharose and Heparin Sepharose enabled greater than 95% purification of rhLF with no isoforms observed as seen with the commercial standard. Analytical data support that the purified rhLF should be sufficient and acceptable as a homogenous molecule to proceed with defined *in vivo* testing.

The sequence of hLF contains three putative N-linked glycosylation sites at positions Asn138, Asn479 and Asn624. It has been demonstrated that Asn138 and Asn479 are preferentially N-glycosylated in LF produced in human kidney-derived 293(S) cell lines, indicating site-specific heterogeneity [35]. However, all three N-linked sites of rhLF from our glycoengineered *P. pastoris* are predominantly occupied as characterized by peptide mapping and LC-MS (Fig. 5). This indicates that a significant portion of Asn624 located at the C-lobe is glycosylated in *P. pastoris* compared with that of mammalian cells. It has been reported that Asn624 is not glycosylated in human milk and leukocyte LFs [13,32]. In addition, glycoengineered *P. pastoris* do not display core-fucosylation of N-linked glycans. Non-fucosylated hLF is a characteristic of human neutrophilic leukocytes whereas human milk-derived LF contains fucose residues at the *N*-acetylglucosamine residue [13]. Sialylated rhLF derived from our glycoengineered *P. pastoris* demonstrates that the glycoform (afucosylated biantennary: $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$) is structurally the same as one of the glycoforms typically found in leucocytes.

Lactoferrin in humans is considered a first-line defense protein involved in a multitude of innate and adaptive immune responses. There are two primary forms of human LF, one contained in exocrine secretions including milk, tears, saliva, bronchial and intestinal secretions. The other form is present in the secondary granules of neutrophils. While the secreted form is thought to be involved in the host defense against microbial infection at mucosal sites, granulocytic lactoferrin has notable immunomodulatory function [22]. Also, the two forms of human LF are identical in their amino acid sequence, but different in sugar moiety. The granulocytic LF is not fucosylated and this seemingly predisposes this form to transduce certain signals that do not require fucose-specific receptors such as mannose receptor exhibiting equal affinity to fucose and mannose [38]. Here, it was demonstrated that the activity of rhLF depends on the terminal *N*-acetylneuraminic acid. This would suggest that reconstruction of MTX-inhibited secondary humoral immune response requires interaction of LF with cells expressing neuraminic dependent receptors. The prime candidate for such a receptor is sialoadhesin, a

sialic acid-binding receptor, found on murine tissue macrophages [10,14,28] and dendritic cells [4], and perhaps the CD22 marker on B cells that bears sequence similarity to sialoadhesin [20]. Nevertheless, the use of specific anti-sialoadhesin antibody, with no apparent cross-reactivity with CD22, makes such a possibility very unlikely. The reconstructive effect of LF on the secondary humoral response in MTX-suppressed splenocytes [1] was further explored. MTX, an antagonist of folic acid synthesis, causes apoptosis of activated cells primarily in the G1-and S-cycle phases [25]. Interestingly, LF could partially reconstitute the cellular immune response in MTX-treated mice, but not the primary humoral immune response *in vivo* [1]. It can further be hypothesized that memory T helper cells, responsible for development of the secondary immune response are less prone to apoptosis [40], coinciding well with lactoferrin's anti-apoptotic properties [9,34,45]. LF could prevent MTX-induced suppression in several ways, including the induction of specific cytokines in sialoadhesin-positive splenocytes. Mediators such as IL-6 [33], IL-12 [39], and TGF-beta [7] induce anti-apoptotic responses and LF indeed increases the production of such mediators [11,19,41]. Because LF has the ability to accelerate the function of the immune system cells (reviewed in 43), it opens new perspectives for the systemic application of recombinant human LF in many immune disorders. Furthermore, both the protein and sugar moiety are compatible with their natural counterparts, thus potentially limiting immunogenicity inherent in most biological therapeutics.

In conclusion, glycoengineered *P. pastoris* was used to produce fully humanized and immunologically compatible rhLF. The primary immune characteristic of rhLF showed differential effects of sialylated and non-sialylated rhLF on restoration of the secondary humoral immune response to SRBC in mouse splenocytes. This suggests that the terminal *N*-acetylneuraminic acid plays an important role in propagation of proper immune responses. Further studies are underway to demonstrate additional actions of recombinant human LF, *in vitro* and *in vivo*, and elucidate the function of terminal sugars in defined innate and adaptive immune functions.

Acknowledgments

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Abbreviations

Man, mannose
Gal, galactose
GlcNAc, *N*-acetylglucosamine
Sia, sialic acid
hLF, human lactoferrin
rhLF, recombinant human lactoferrin
anti-hLF, anti-human LF antibody
anti-HCP, anti-host cell protein antibody
CV, column volume
AFC, antibody forming colonies
MALDI-, matrix-assisted laser desorption/ionization
TOF, time of flight
MS/MS, tandem mass spectrometry

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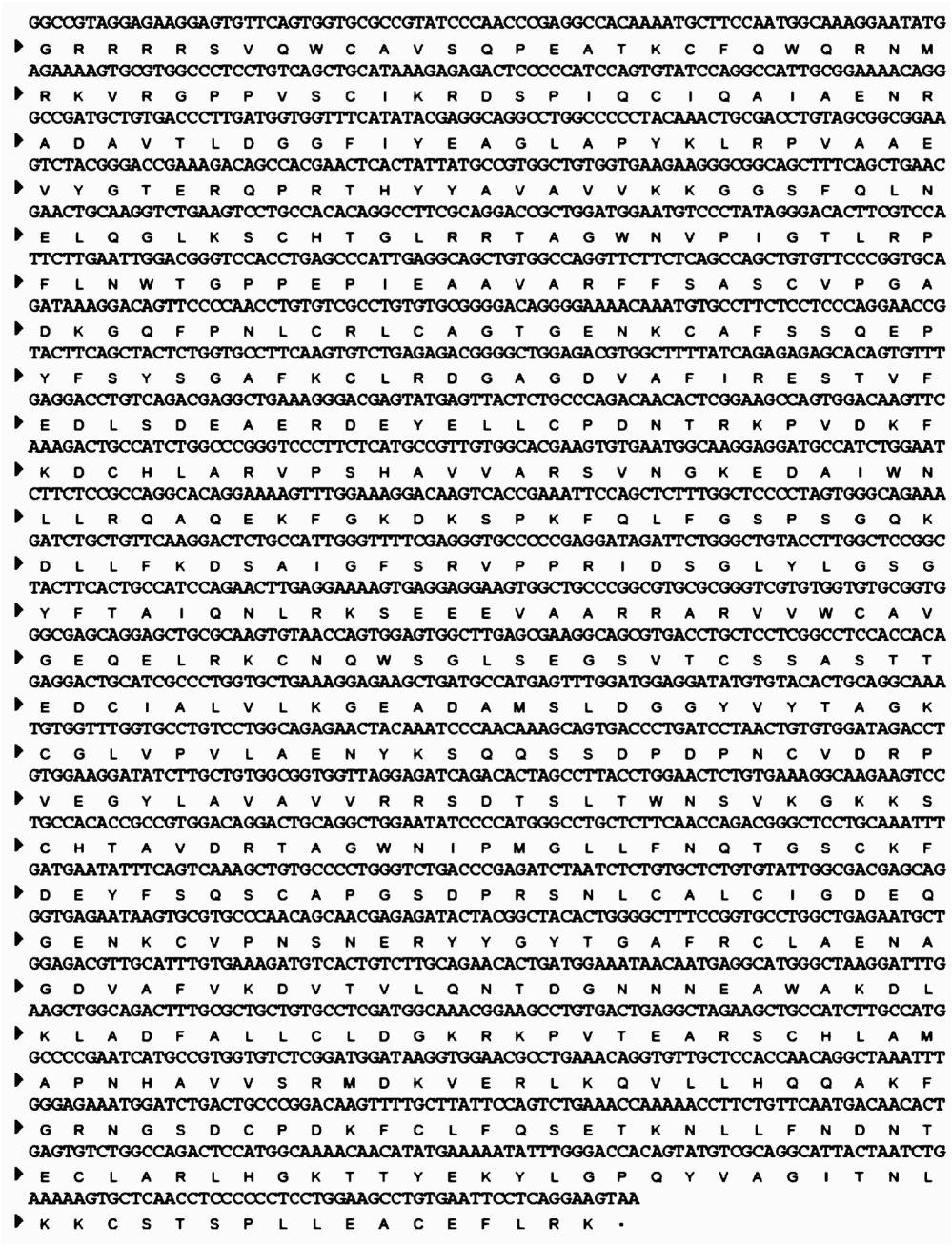

Fig. 1.
DNA sequence encoding the mature human lactoferrin

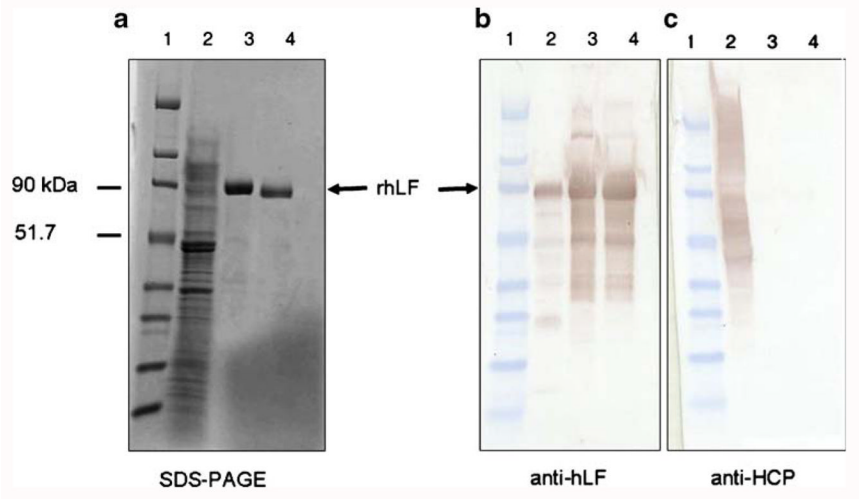

Fig. 2.

SDS-PAGE with Coomassie blue stain (**a**) and Western blot with antibodies *α*hLF (**b**) and *α*HCP (**c**) of samples at different purification steps. *Lane 1* broad range molecular weight standards, *lane 2* rhLF fermentation supernatant, *lane 3* SP Sepharose Fast Flow eluant, *lane 4* Heparin Sepharose 6 Fast Flow eluant. *αHCP* antibody against *Pichia* host cell proteins

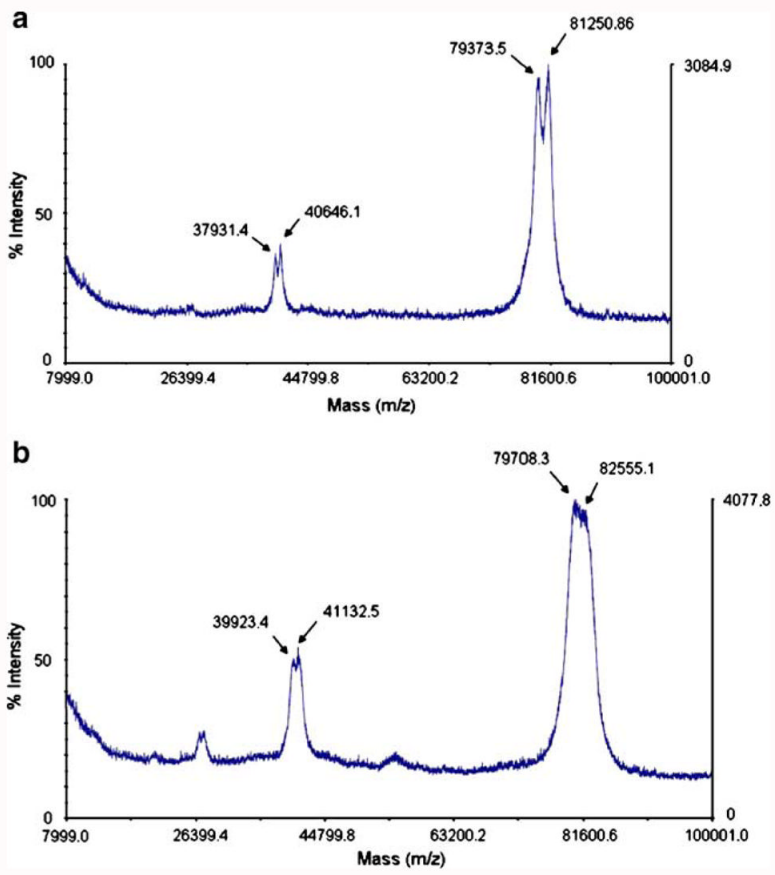

Fig. 3.

MALDI-TOF spectra of non-sialylated (**a**) and sialylated (**b**) rhLFs. Protein samples were diluted 1:1 with sinapinic acid in 0.1% trifluoroacetic acid and 50% acetonitrile, then spotted on a MALDI plate and examined using positive mode on an Applied Biosystems Bioanalyzer MALDI-TOF instrument

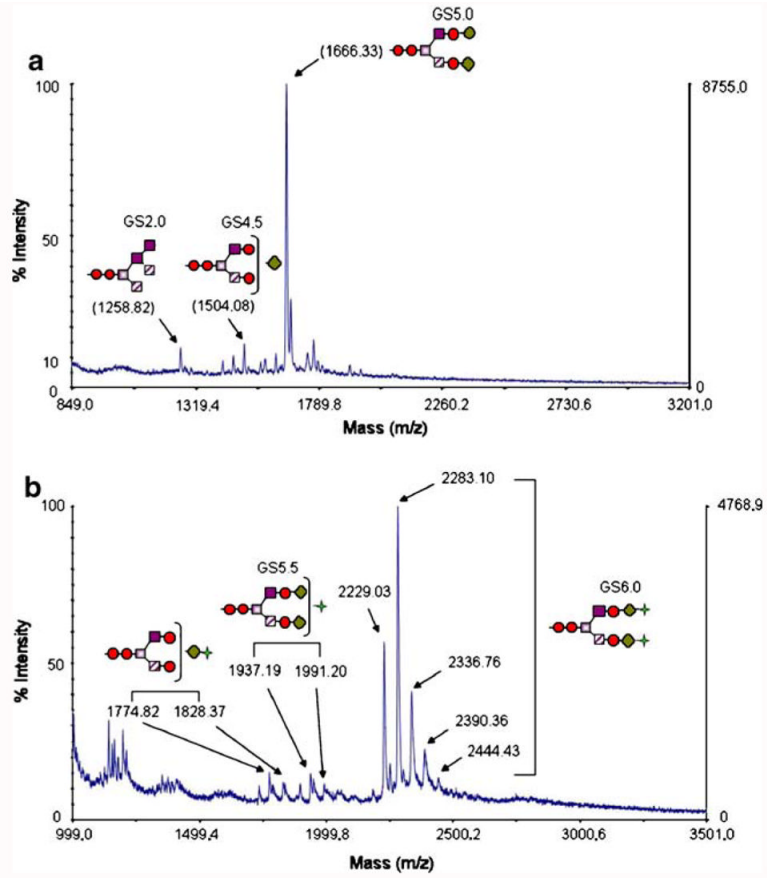

Fig. 4.

MALDI spectra of released glycan structures. Glycans were removed from rhLF with PNGase F and subjected to MALDI-TOF analysis in the positive ion mode for non-sialylated rhLF (**a**) and the negative ion mode for sialylated rhLF (**b**). The major peak observed in the non-sialylated rhLF N-glycan spectrum corresponds to $Gal_2GlcNAc_2Man_3GlcNAc_2$ (GS5.0), whereas the major peak in the sialylated rhLF N-glycan spectrum corresponds to $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$ (GS6.0). *Red circles* GlcNac, *grey squares* Man, *violet squares* Man, *diagonal-striped squares* Man, *green diamond* Gal, *green four-pointed star* Sia

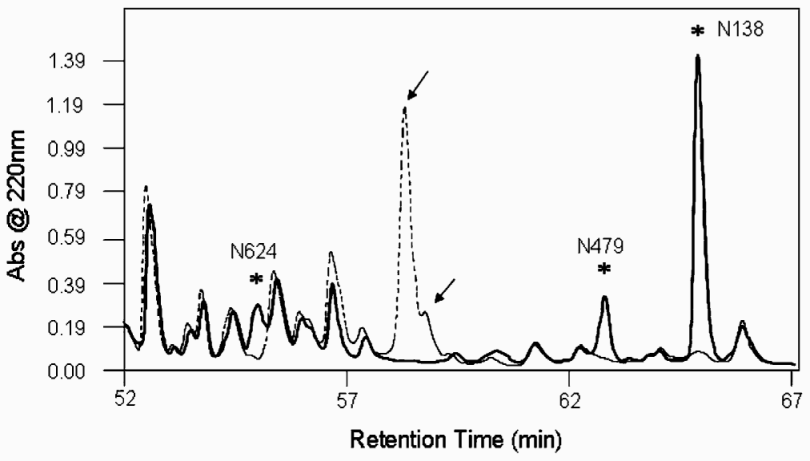

Fig. 5.

Peptide mapping of non-sialylated rhLF. The *dotted* chromatogram displays the chromatographic UV trace for tryptic peptides from glycosylated rhLF; the *solid line* corresponds to the trace for deglycosylated tryptic peptides. Fractions from the glycosylated sample were then treated with PNGase F and run on liquid chromatography-coupled mass spectrometer via nanospray to identify peptide sequences. Fragments corresponding to deglycosylated and non-glycosylated N-glycosylation sites (N138, N479, N624) were identified in both samples and peaks containing these fractions were indicated with asterisks. Glycopeptides (N138 and N479) were identified in peaks marked with *arrows*

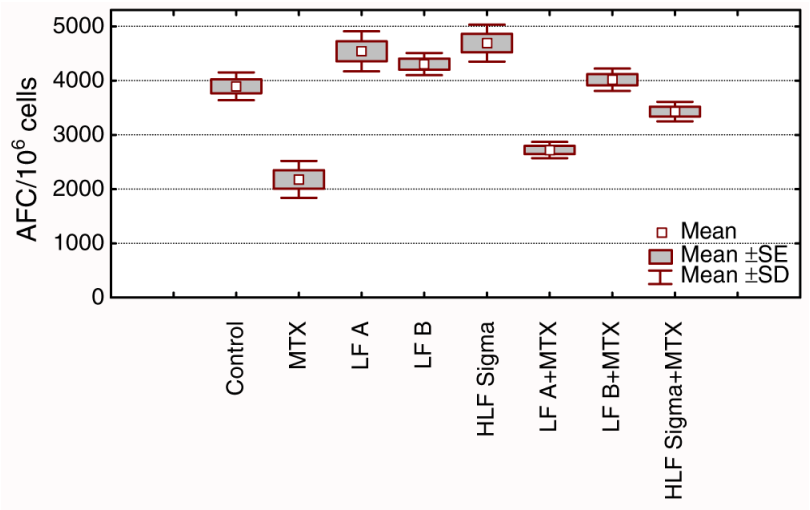

Fig. 6.

Reconstituting effect of lactoferrin on the secondary humoral immune response in mice suppressed by methotrexate. Splenocytes isolated from sheep red blood cell (*SRBC*) primed mice were incubated with SRBC, alone in the presence of methotrexate (*MTX*). Antibody forming cells (*AFC*) were evaluated after 4 days. Cells were cultured in the presence of non-sialylated LF (*LF A*), sialylated LF (*LF B*), or milk-derived LF (*HLF Sigma*). All LF concentrations were 1 μg/ml. The results are shown as mean values of AFC number from five wells±SE, calculated per 10^6 viable cells. Statistical analysis of groups: control *vs.* LF A NS; control *vs.* LF B NS; control *vs.* HLF Sigma p=0.0068; control *vs.* MTX p=0.0001; control *vs.* LF A+MTX p=0.0001; control *vs.* LF B+MTX NS; control *vs.* HLF Sigma+MTX NS; MTX *vs.* LF A+MTX NS; MTX *vs.* LF B+MTX p=0.0001; MTX *vs.* HLF Sigma+MTX p=0.0001 (ANOVA)

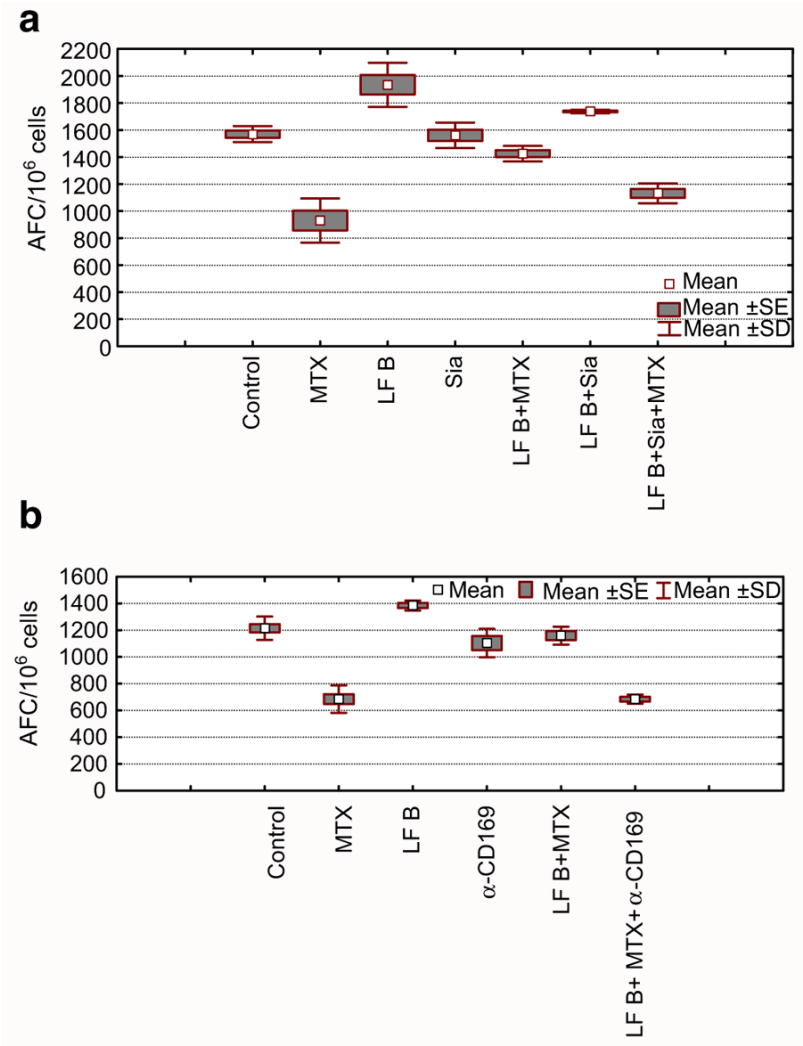

Fig. 7.

The activity of human recombinant lactoferrin in the secondary immune response *in vitro* is abolished by addition of sialic acid. Splenocytes isolated from sheep red blood cell (*SRBC*) primed mice were incubated with SRBC, alone in the presence of methotrexate (*MTX*). Antibody forming cells (*AFC*) were evaluated after 4 days. Cells were cultured in the presence of sialylated LF (*LF B*). Free sialic acid was added alone, or in combination with LF (**a**). Alternatively, anti-sialoadhesin monoclonal antibody CD169 (sialoadhesin) was added before LF (1:250) (**b**). **a** Control *vs.* LF B p=0.0001; control *vs* MTX p=0.0001; control *vs.* Sia NS; control *vs.* LF B+Sia NS; control *vs.* LF B+MTX NS; control *vs* LF B+Sia+MTX p=0.0001; MTX *vs.* LF B+MTX p=0.0001; MTX *vs* LF B+Sia+MTX NS; LF B+MTX *vs.* LF B+Sia +MTX p=0.0023 (ANOVA). **b** Control *vs.*LF B p=0.0291; control *vs.* MTX p=0.0001; control *vs.* Ab NS; control *vs.* LF B+Ab+MTX p=0.0001; LF B *vs.* LF B+Ab p=0.0001; MTX *vs.* LF B+MTX p=0.0001; MTX *vs.* LF B+Ab+MTX NS; LF B+MTX *vs.* LF B+Ab+MTX p=0.0001 (ANOVA)

Table 1

Strains and plasmids used in this study

Strain and plasmid	Description	Source
TOP10	*E. coli* (F-mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG)	Invitrogen (Carlsbad, CA)
yAS309	Glycoengineered *Pichia pastoris*	Li *et al.* [24]
BK422	pBK842 in yAS309	This study
BK427	pBK850 in yAS309	This study
pPICZA[a]	PpAOX1 promoter, Zeo^R	Invitrogen
pGAPZA[a]	PpGAPDH promoter, Zeo^R	Invitrogen
pBK833	*S. cerevisiae* α-mating factor pre+hLF in pPICZA	This study
pBK834	α-amylase signal sequence (ss)+hLF in pPICZA	This study
pBK835	Glucoamylase ss+hLF in pPICZA	This study
pBK836	Human serum albumin ss+hLF in pPICZA	This study
pBK837	Inulinase ss+hLF in pPICZA	This study
pBK838	Invertase ss+hLF in pPICZA	This study
pBK839	*P. pastoris* KAR2 ss+hLF in pPICZA	This study
pBK840	*S. cerevisiae* killer toxin 1 ss+hLF in pPICZA	This study
pBK841	*P. pastoris* phosphotase 1 ss+hLF in pPICZA	This study
pBK842	*S. cerevisiae* α-mating factor prepro+hLF in pPICZA	This study
pBK843	Chicken lysozyme ss+hLF in pPICZA	This study
pBK850	*S. cerevisiae* α-mating factor preproKR+hLF in pGAPZA	This study

[a]Identical signal sequence used with different promoter sequences.