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Novel recombinant human lactoferrin: Differential activation of oxidative stress related gene expression

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Abstract

Lactoferrin, an iron-binding protein found in high concentrations in mammalian exocrine secretions, is an important component of the host defense system. It is also a major protein of the secondary granules of neutrophils from which is released upon activation. Due to its potential clinical utility, recombinant human lactoferrin (rhLF) has been produced in various eukaryotic expression systems; however, none of these are fully compatible with humans. Most of the biopharmaceuticals approved by the FDA for use in humans are produced in mammalian expression systems. The Chinese hamster ovary cells (CHO) have become the system of choice for proteins that require post-translational modifications, such as glycoproteins.

The aim of this study was to scale-up expression and purification of rhLF in a CHO expression system, verify its glycan primary structure, and assess its biological properties in cell culture models.

A stable CHO cell line producing >200 mg/L of rhLF was developed and established. rhLF was purified by a single-step cation-exchange chromatography procedure. The highly homogenous rhLF has a molecular weight of approximately 80 kDa. MALDI-TOF mass spectrometric analysis revealed N-linked, partially sialylated glycans at two glycosylation sites, typical for human milk LF. This novel rhLF showed a protective effect against oxidative stress in a similar manner to its natural counterpart. In addition, rhLF revealed a modulatory effect on cellular redox *via* upregulation of key antioxidant enzymes. These data imply that the CHO-derived rhLF is fully compatible with the native molecule, thus it has promise for human therapeutic applications.

Keywords

Recombinant human lactoferrin; Gene expression; Antioxidant; Inflammation

1. Introduction

Lactoferrin (LF) synthesized by epithelial cells and granulocytes is a first-line defense protein involved in protection against microbial infections (Lönnerdal and Iyer, 1995; Sánchez et al., 1992). LF is critical in prevention of systemic inflammation (Baveye et al., 1999; Baynes and Bezwoda, 1994; Kruzel et al., 2002) and can be considered as a pleiotropic agent acting *in vivo* as an “immune sensor” to direct specific immune responses toward immune homeostasis (Kruzel et al., 2007). LF bridges innate and adaptive immune functions by regulating target cell responses, using mechanisms which are highly dependent on the type of carbohydrates attached to the protein backbone. LF has also been shown to maintain iron homeostasis, playing an important role in modulation of inflammatory responses (Baveye et al., 1999).

Numerous forms of recombinant human lactoferrin (rhLF) have been produced in multiple expression systems, including transgenic animals and plants (Conesa et al., 2010). However, none of those recombinant molecules have been approved for systemic administration in humans due to their structural incompatibility. While the primary and secondary structure of the majority of these recombinant LFs are identical with the wild type (non-polymorphic) human LF, the glycosylation process inherent within each expression system renders a final product that is not fully compatible due to significant alterations in the glycan structure. In particular, rhLFs derived from yeast and fungal expression systems display high levels of mannose Nlinked glycans which may be immunogenic and antigenic, and thus limit potential for human therapeutic use. Indeed, glycosylation is an important post-translational modification which directly affects both protein structure and biological functions (Shental-Bechor and Levy, 2009; Marth and Grewal, 2008; Ohtsubo and Marth, 2006). The oligosaccharide component of glycoprotein is often critical for determination of pharmacological properties including *in vivo* activity, pharmacokinetics, and immunogenicity. For example, the glycan portion of immunoglobulins from patients with rheumatoid arthritis is devoid of galactose and sialic acid leading to generation of autoantibodies known as rheumatoid factor (Matsumoto et al., 2000). Similarly, studies revealed the importance of glycosylation to pathogenic recognition, to the modulation of the innate immune system, and to the control of immune cell homeostasis and inflammation (van Kooyk and Rabinovich, 2008).

In our previous work, a methylotrophic yeast *Pichia pastoris* strain capable of producing LF with human-like N-linked glycans of high uniformity was developed (Choi et al., 2008). This rhLF proved to be practically identical to natural human LF. Further studies on the N-glycan structure with terminal galactose (Gal2GlcNAc2Man3GlcNAc2) revealed the importance of N-acetylneuraminic acid as a terminal sugar in the propagation of specific immune responses (Choi et al., 2008). However, the most acceptable expression system by leaders in the pharmaceutical industry is one of mammalian platforms based on human epithelial kidney cells (HEK) or Chinese hamster ovary cells (CHO) (Sinclair and Elliott,

2005; Li and d'Anjou, 2009). The glycosylation machinery of the CHO expression system largely resembles that in humans, although there is greater heterogeneity in glyco-forms between production runs. Fortunately, batch variability can be minimized by optimization of protocols or *via* use of genetically engineered mammalian expression hosts (Hossler et al., 2009; Yamane-Ohnuki et al., 2004; Davies et al., 2001). The goal of this study was to test the biological activity of rhLF derived from the CHO scale-up expression protocol, thus, allowing for generation of human compatible glycoforms which could be used in preclinical testing and animal safety studies. The importance of this report relates to potential use of rhLF in the development of new therapeutic approaches for the systemic treatment of infectious diseases.

2. Materials and methods

All reagents for molecular biology were provided by GenScript (Piscataway, NJ, USA). Freestyle™ CHO expression media was purchased from Invitrogen (Carlsbad, CA, USA). POROS® XS Cation Exchange Resin was purchased from Life Technologies (Carlsbad, CA, USA). HiPrep 26/10 desalting column was a product of Amersham Biosciences (Piscataway, NJ, USA). All other reagents, including human milk-derived LF (Cat. No. L0520), were purchased from Sigma Chemical (St. Louis, MO, USA).

2.1. Expression construct, generation of production strains

The DNA sequence of human LF (Choi et al., 2008) was sub-cloned into a pTT5 vector at the EcoR I and Hind III sites and used for transfection. The CHO-3E7 (NCR) cells were cultured using Freestyle™ CHO expression medium supplemented with 8 mM glutamine (Hyclone, Logan, UT, USA), in a humidified 37 °C incubator with 5% CO₂. Transfection with human LF containing vector was accomplished by using polyethylenimine (PEI) at a 3:1 ratio of PEI to DNA. Clones were selected by methotrexate (MTX) in 3 rounds (50/200/500 nM) as described (Transfection of DG44 Cells & Development of Stable Cell Lines in Defined Medium, Life Technologies, Carlsbad, CA, USA). Clones excreting >200 mg/L of rhLF were selected for consideration of LF production.

2.2. Protein purification

LF purification was accomplished in accordance with published protocols (Loignon et al., 2008). Briefly, cell culture supernatant was harvested by centrifugation for 40min at 4000RPM at 4°C with further clarification by 0.45 µm Corning microfiltration. The supernatant's pH was adjusted with 1 M acetic acid to a pH of 5.5, and loaded onto POROS® XS Ion Exchange Resin column equilibrated with 50 mM NaAc buffer pH 5.5. Elution was accomplished with a linear gradient of 50 mM NaAc containing 1 M NaCl pH 5.5 over 30 column volumes (CV). A buffer exchange on combined LF fractions was performed using HiPrep 26/10 desalting column and Dulbecco's phosphate-buffered saline (DPBS) pH 7.0. Endotoxin levels were assessed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit according to the manufacturer's instructions (GenScript, Piscataway, NJ, USA). Protein concentration was estimated using the method of Bradford as described (Bradford, 1978). The iron content was measured by inductively coupled plasma mass spectrometry (ICP-MS) and expressed per gram of protein.

2.3. Western blot

Proteins were separated by 12% SDS-PAGE and then electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked, and then probed with rabbit anti-human LF antibody (anti-hLF; 1:1000; Sigma Chemical, St. Louis, MO, USA) and subsequently detected by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:2000) (GenScript, Piscataway, NJ, USA). The results were visualized using an Enhanced ECL Substrate Kit (GenScript, Piscataway, NJ, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

A high-protein binding 96-well plate (Hycult, Plymouth Meeting, PA, USA) was coated with 100 µL/well of rabbit anti-human LF antibody (Sigma Chemical, St. Louis, MO, USA) diluted 1:5000 in PBS overnight at 4°C. The antibody was aspirated and the plate was blocked for 1 h at room temperature with 200 µL of 3% bovine serum albumin (BSA) in PBS. The blocking solution was replaced with 100 µL of serial dilutions of hLF standard and samples to be assayed. Standard hLF was diluted 2-fold serially in PBS from 100 to 0.1 ng/mL; LF samples were typically diluted 1:100 and then 2-fold serially to 1:100,000. The 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. The wash buffer was aspirated and then 100 µL/well of 1:5000 diluted streptavidin-peroxidase anti-hLF (Hycult, Plymouth Meeting, PA, USA) was added and incubated for 1 h at room temperature. Wells were washed, 100 µL/well of 3,3',5,5'-tetramethylbenzidine (Hycult, Plymouth Meeting, PA, USA) was added, and the final reaction terminated with stop solution (Hycult, Plymouth Meeting, PA, USA) after which absorbance at 450 nm was measured.

2.5. MALDI-TOF analysis of glycans

N-glycans were released and separated from rhLF by PNGase F as previously described (Morelle et al., 2009). Molecular weight was determined using a Voyager linear matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA) with delayed extraction. The structural information of N-glycans composition was deduced from the Consortium for Functional Glycomics database (<http://www.functionalglycomics.org/static/consortium/consortium.shtml>).

2.6. Analysis of glycopeptides by in-gel tryptic digests

A sample of LF (50 µg) was resolved by 10% SDS-PAGE minigel (10 µg/lane) and stained with Biosafe Coomassie. The main protein bands were excised, reduced/alkylated and digested with trypsin. Individual digests were pooled and lyophilized. Samples were reconstituted in 0.1% TFA in 100% acetonitrile. Glycopeptides were isolated from the digests using ion-pairing normal-phase liquid chromatography (IP-NPLC), a method that selectively binds hydrophilic glycopeptides over more hydrophobic peptides (Ding et al., 2009). The IP-NPLC fractions were then analyzed by nanoLC-MS (Hi/Lo) on the Q-TOF Ultima.

2.7. Intracellular reactive oxygen species (ROS) measurements

Changes in intracellular ROS levels were determined using the fluorogenic probe 2'-7'-dihydro-dichlorofluorescein diacetate (H2DCF-DA; Probes, Eugene, OR, USA) (Boldogh et al., 2005), or carboxymethyl form 2'-7'-dihydro-dichlorofluorescein-DA. Briefly, U937 cells were grown to 70% confluence and loaded with 50 μ M H2DCF-DA at 37 °C for 30 min. Cells were washed twice with PBS and exposed to nucleic acid bases, nucleosides, or solvent. Changes in DCF fluorescence were recorded in FLx800 (Bio-Tek Instruments, Winooski, VT, USA) microplate reader at 485 nm excitation and 528 nm emission. Alternatively, in parallel experiments, changes in cellular ROS levels were determined by flow cytometry (BD FACSCanto flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA). The median fluorescence for 12,000 cells from three or more independent experiments was analyzed after correction for DCF extrusion, and expressed as $\pm$ S.E. The fluorescence distribution is represented by the position of the "center" of the distribution, represented mathematically by the median channel number (Flow Cytometry data analysis software: WinMDI 2.9). A fluorescent hydrogen peroxide sensor HyPer (Evrogen, Axxora, Farmingdale, NY, USA) was used to confirm changes in ROS levels, as published previously (Malinouski et al., 2011). A pHyPer-Cyto vector without targeting signal was used, representing cytoplasmic expression. Cells were transfected by use of N-TER Nanoparticle Transfection System as described by manufacturer instructions (Sigma–Aldrich, St. Louis, MO, USA). Seventy-two hours after transfection, cells were incubated in serum-free medium for 4h in the presence of LF, challenged, and changes in fluorescence were recorded in FLx800 microplate reader.

2.8. Gene expression profiling in whole blood cell (WBC) culture

A sample of fresh whole blood (2 mL) was diluted 1:10 with serum free RPMI 1645, and cells were plated onto 12 well plates as previously described (Damsgaard et al., 2009). LF was added at 10 μ g/mL concentration and culture was incubated for 2 h at 37 °C. Cells were collected by centrifugation and RNA isolated using the QIAamp RNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). RNA integrity was determined using an Agilent Bioanalyzer. A 300 ng aliquot of total RNA from each sample was labeled and amplified using the Epicenter TargetAmp Nano-gT Biotin-aRNA Labeling Kit for the Illumina® System (Cat. No. TAN07924). Both before and after the amplifications the RNA/cRNA concentrations and quality were checked with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 750 ng of each cRNA was hybridized to each array on the BeadChip (Illumina Human HT-12 v4 Gene Expression BeadChip) at 58° C overnight (14–20 h) following the Illumina Whole-Genome Gene Expression Direct Hybridization Protocol (Illumina, San Diego, CA, USA). Hybridized biotinylated cRNA was detected with 1 mg/mL Cyanine3-streptavidin (GE Healthcare, Waukesha, WI, USA; Code: PA43001). BeadChips were scanned with the Illumina iScan Reader. Raw data was acquired in Genome Studio Software (Illumina, San Diego, CA, USA), and exported to Genespring (Agilent Technologies, Santa Clara, CA, USA). Data was analyzed using GeneSpring Software. T-testing with Benjamin and Hochberg error correction was performed using a data cutoff of 0.05 and a fold change of 2 or greater. A reverse transcription reaction using 250 ng of total RNA, was done using the QIAGEN RT² First Strand Kit (QIAGEN, Valencia, CA, USA). cDNA Samples were assayed using QIAGEN

RT² PCR Array, Human Oxidative Stress and Antioxidant Defense (QIAGEN, Valencia, CA, USA). PCR array was done according to manufacture instructions using QIAGEN RT² qPCR Mastermix (Cat. No. 330500). Real time PCR was done using an ABI 7900 SDS Real Time instrument (Life Technologies, Carlsbad, CA, USA). Raw data was exported from the real-time instrument software and fold regulation was calculated using the Delta Delta CT method by the RT² Profiler™ PCR Array Data Analysis tool (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

2.9. Isoelectric point (pI) determination

Isoelectric focusing (IEF) was performed using a Novex® pH 3–10 IEF gel. Samples were diluted to 5 mg/mL in Novex 3-10 IEF Sample Buffer before loading. Gels were focused at 100 constant volts for 1 h, 200 constant volts for 1 h, followed by 500 constant volts for 30 min. Gels were fixed in 12% TCA for 30 min; stained in Coomassie Brilliant Blue R-250, destained in 10% acetic acid, and dried between cellophane sheets. The stained gel was digitized using a calibrated GE Healthcare Image Scanner III. The pI range was calculated from the Serva pI standards using Phoretix 1D software (Nonlinear Dynamics, version 10.1) and a first order Lagrange interpolation pI curve.

2.10. Bactericidal activity against *Streptococcus pneumoniae*

Cultures were maintained and used as described (Mirza et al., 2011), using early log phase cultures. Briefly, 10⁶ *S. pneumoniae* D39 (serotype 2) or TIGR4 (serotype 4) were incubated in assay saline (150mM sodium chloride, 50 µM calcium chloride, 1 mM magnesium chloride and 1 mM K₂PO₄; pH 7.2) in the presence or absence of 40 µg/mL of hLF or 40 µg/mL of rhLF. Cultures were incubated at 37°C for 1 h, then serially diluted and plated on blood agar to measure viability. Colony forming units (CFU) were enumerated after overnight incubation at 37°C in aerobic conditions. Bactericidal activity was calculated by subtracting log CFU obtained from incubation with hLF or rhLF from that of log CFU obtained in the absence of LFs. Experiments were completed using 3 samples for controls 7 samples per test.

2.11. Statistics

The differences across groups were determined by analysis of variance after testing homogeneity of variance by Levene's test. Individual grades were compared using the Tukey's test for multiple comparisons. Differences were considered significant when $p < 0.05$. The statistical analysis was performed using STATISTICA 6.0 for Windows.

3. Results

3.1. Production of rhLF

The CHO-transfected with hLF expressing vector clone number CHO PRC 40-097 was selected by 3 rounds of MTX treatment with the expression level assessed by ELISA at approximately 200mg/L. Higher expressing clones were not considered for production of rhLF due to possible incomplete glycosylation. The cell culture supernatant was clarified by centrifugation and purified on POROS® XS Ion Exchange Resin. Fig. 1 shows that single step purification provided homogenous rhLF as evidenced by HPLC and SDS-PAGE/

Western blot analyses. Human milk-derived LF (hLF) was used as a control. The hLF and rhLF are similar in molecular weight (approximately 80 kDa) and anti-human LF antibody recognition as determined by SDS-PAGE and Western blot, respectively. The high molecular weight aggregates of hLF, seen on SDS-PAGE and Western blot, were not present in rhLF. The rhLF obtained according to this protocol was partially saturated with iron (0.57 mg of Fe per 1 g of LF) and with endotoxin level <1 UE/mg protein.

3.2. Isoelectric point (pI)

A Novex[®] (pH 3-10) IEF gel was used to determine pI of the rhLF in comparison with hLF. Although Novex[®] can readily detect minor changes in protein glycosylation, and can resolve different isoforms of proteins of similar size; the data generated showed broader bands for all LFs tested. All human LFs showed moderately cationic characteristic ranges: milk-derived LF 7.0–7.8, neutrophil-derived LF 7.5–7.9, and rhLF 7.5–8.3. These results indicate a slightly lower pI values than previously described (Moguilevsky et al., 1985) but relative values between hLFs are in agreement.

3.3. MALDI-TOF analysis of glycans

Glycans were removed from rhLF with PNGase F, permethylated, and subjected to MALDI-TOF analysis in the positive ion mode. Analysis of N-linked glycans showed that fucosylation was common to all peaks resolved under these conditions (Fig. 2). Three minor peaks of mass 2966.5, 2606.3 and 2401.2 were identified, indicative of biantennary complex glycans with N-acetylneuraminic acid as a terminal molecule. The other glycans are not capped with neuraminic acid, with other human-type glycans present comprised of Fuc1Hex5HexNAc4, Fuc1Hex4HexNAc4, or Fuc1Hex3HexNAc4.

3.4. Analysis of glycopeptides by in-gel tryptic digests

The sequence of hLF contains three putative N-linked glycosylation sites at positions Asn138, Asn479 and Asn624. To determine N-glycosylation occupancy of each site, a sample of rhLF was digested with trypsin and analyzed. Glycopeptides were isolated from the digests using ion-pairing normal-phase liquid chromatography (IP-NPLC), and assessed by nanoLC-MS (Hi/Lo) on the Q-TOF Ultima. The peptide sequences were identified as TAGWNVPIGTLRPFLNWTGPPEIEA A V A R for Asn138 and TAG-WNIPMGLLFNQTG S C K for Asn479, with identified glycosylation exhibiting similar glycan diversity when compare to milk-derived human LF (Fig. 3). The molecular ion profiles obtained for the Asn138 glycosylation site are characterized by a higher degree of sialylated structure for both hLF and rhLF, when compared with the Asn479 glycosylation site. The spectra analyses indicate that low abundance glycans are tri-antennary rather than biantennary for both LFs analyzed. The peptide sequence identified as NGSDCPDK for Asn624 showed no relative glycan intensity. These results are in agreement with previous reports on glycosylation of hLF (van Berkel et al., 1996).

3.5. LF-induced gene expression in WBC culture

To assess the effects of rhLF on whole genome expression, a total RNA from WBC short term cultures was examined. The WBC population approximates the state of circulating cells

in vivo, contains physiological concentrations of all factors that influence immune cellular function, and is useful in determining effects of stimuli on the development of pathological conditions. Whole genome expression profiling was performed using Illumina HumanHT-12 v4 BeadChip using biological triplicates. The results of the whole genome expression arrays were verified at greater sensitivity by RT-PCR, using the QIAGEN human oxidative stress and antioxidant defense RT² profiler PCR array (samples assessed in triplicate). This PCR array, which includes families of four enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPX), peroxiredoxins (PRDX), and prostaglandin-endoperoxide synthase (PTGS) also known as cyclooxygenase (COX), were differentially induced. Fig. 4 demonstrates fold changes at 60 min, listed as expression of individual genes from Illumina BeadChips (blue) and confirmed by real-time qPCR (red). The top panel profiles the expression of all 84 genes related to oxidative stress and the bottom highlights four key antioxidant enzymes from the same experiment. The constitutive isoform of PTGS is up regulated by rhLF whereas inducible one is inhibited. The isoform differential activation is also evident for SOD family genes where the cytoplasmic (copper-zinc SOD1) isoform is up regulated by rhLF and mitochondrial (manganese superoxide dismutase; SOD2) and extracellular SOD3 are not. Results obtained from longer time exposure did not reveal additional information (data not shown).

3.6. LF modulates intracellular ROS levels induced by exogenous oxidants

rhLF upregulates the expression of GPX, PRDX and SOD1 (Fig. 4), thus we examined whether it increases antioxidant status of cells. To do so, U937 cells were exposed to glucose oxidase (GOx), which primarily generates superoxide anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is converted into H_2O_2 by SOD, and H_2O_2 is detoxified by GPX and/or PRDX (D'Autraux and Toledano, 2007). Without LF pre-treatment, GOx (1 μ U) induced ~3-fold increase in DCF fluorescence (Fig. 5). Pre-treatment of cells with human milk-derived LF (hLF) or rhLF significantly decreased intracellular ROS levels (Fig. 5A). There were no differences observed between ROS levels of cells treated with natural hLF or treated with rhLF. Doses of rhLF that prevent an increase in ROS levels were also established. Data in Fig. 5B, show that 50 and 100 μ g/mL of rhLF significantly decreased ROS levels, while lower concentrations of LF (25, 12.5 μ g/mL) moderately affected GOx-induced ROS levels in our cell culture system. These results suggest that CHO-derived rhLF has a protective effect against ROS generated by GOx. In fact, decreases in ROS levels were similar to *N*-acetyl cysteine (NAC), a glutathione precursor and direct ROS scavenger. The used concentrations of rhLF and a dose of GOx (inducing 2–3-fold increase in ROS levels) were determined in preliminary studies (data not shown).

The redox chemistry of DCFH is highly complex and there are limitations and artifacts associated with the DCF assay, nevertheless DCFH is a convenient, easy-to-use probe (Kalyanaraman et al., 2012). To confirm antioxidant effects of rhLF and DCFH results, redox-sensitive fluorescence of biosensor (Belousov et al, 2006) expressed in the cytoplasmic (pHyPer-cyto) compartment were utilized as described previously (Hajas et al, 2013). Transfected cells were LF-treated than exposed to GOx. An increase in pHyPer-Cyto fluorescence appeared first in the cytoplasmic compartment upon GOx treatment as was previously documented (Hajas et al, 2013) showing an approximate 3-fold increase (Fig.

5C). rhLF and hLF pretreatment significantly decreased pHyPer-Cyto fluorescence (Fig. 5C). These results are in agreement with data generated by using DCF assays, and showing protective effect of rhLF against oxidative stress. Although further studies are required, these data are in line with iron binding property of LFs (Majka et al., 2013) and with those showing changes in expression of antioxidant genes (Fig. 4).

3.7. Bactericidal activity against *S. pneumoniae*

The biological activity of the rhLF was further investigated to confirm bactericidal activity, as classically reported for this molecule (Conesa et al., 2009; Mirza et al., 2011). Activity of the rhLF was compared to human milk-derived LF activity against two *S. pneumoniae* strains, representing the common serovars 2 and 4. Activity was measured using a dose of 40 µg/mL representing an LD₉₀. Strain D39 (serovar 2) treated with milk-derived human LF led to 88.7% growth reduction; D39 treated with the rhLF gave 91.9% growth reduction. Similarly, TIGR4 (serovar 4) treated with hLF led to 88.9% growth reduction; TIGR4 treated with rhLF gave 90.2% reduction. This indicates that the antibacterial activity of the rhLF against the two common *S. pneumoniae* strains is not significantly different from natural derived human LF.

4. Discussion

The choice of a suitable expression system depends largely on the biochemical and biological properties of the protein of interest. Eukaryotic expression systems are most commonly used for production of recombinant proteins as therapeutics. In particular, stably transfected adherent CHO cells or non-adherent lymphoid cell lines are preferred by the pharmaceutical industry because their protein post-translational modifications largely resemble naturally occurring patterns in humans. rhLF has been historically produced in various expression systems, including transgenic plants and animals, but none of the resultant products have been tested and approved for human use for intravenous (systemic) administration. Although there are two rhLFs available for larger studies, one from transgenic rice (Nandi et al., 2002) and the other from *Aspergillus awamori* (Ward et al., 1997), both present a health threat if administered intravenously due to the antigenic nature inherent within their non-homologous glycosylation patterns. Furthermore, clinical studies using orally administered *Aspergillus awamori*-derived rhLF (Talactoferrin Alfa) in sepsis or non-small cell lung cancer failed to show significant improvement over the placebo group. Specifically, Talactoferrin Alfa was shown not to be systemically bioavailable (Mojaverian et al., 2003; Hayes et al, 2006) consequently its effectiveness was limited by oral delivery. There is growing evidence however that oral administration of bovine lactoferrin (bLF) can reduce incidence of many life-threatening syndromes, including first episode of late-onset sepsis (Manzoni et al., 2009) and invasive fungal infections in very low birth weight (VLBW) neonates (Manzoni et al, 2012). Bovine LF given orally has shown clinical utility in treatment of enteric disorders of viral, bacterial or parasitic origin (Ochoa and Cleary, 2009). These observations are in agreement with the protective effect of breastfeeding against pathogens causing diarrhea or other disorders in infants and toddlers (Huffman and Combest, 1990). Yet, there remains no clinical data on effectiveness of systemically (parenterally) delivered LF in humans. The limiting factor for such studies is

related to lack of fully compatible, non-antigenic rhLFs. The major concern relates to incompatibility of the glycan structure. Indeed, glycosylation is a common but complex type of post-translational modification of proteins, directly affecting protein structure, trafficking, cell signaling and cell to cell recognition events (Shental-Bechor and Levy, 2009; Fukuda et al., 1989; Marth and Grewal, 2008; Ohtsubo and Marth, 2006). Moreover, changes in protein glycosylation have been related to the onset and/or progression of several diseases (Freeze and Aebi, 2005; Higai et al., 2003; Parekh et al., 1985; Saldova et al., 2007; Arnold et al., 2011; Blomme et al., 2009; An et al., 2009). Additionally, glycosylation and glycan diversity are directly related to modulating microbial adhesion and invasion during infection (Marth and Grewal, 2008).

In this study the production of rhLF in a mammalian cell expression system using stably transfected CHO was described. Purification of rhLF secreted to the cell culture media was accomplished with a single step on POROS® XS Ion Exchange Resin at low pH (5.5). Biochemical and physiochemical properties of rhLF were subsequently compared to those of human milk-derived LF. The CHO-derived rhLF proved to be biologically active and equivalent to that of the natural product (milk-derived LF), with the added feature that it possesses human-like glycosylation pattern. The tryptic digestion of rhLF and nanoLC–MS/MS analysis confirmed the previous report by van Berkel et al. (1996) that only two of the three putative N-linked glycosylation sites at position Asn138 and Asn479 are occupied. In contrast to *Pichia pastoris* expressed rhLF (Choi et al., 2008), the CHO-derived rhLF is not glycosylated at position Asn624. The fact of rhLF glycosylation at Asp138 and Asn479 but not at Asp624 makes this novel rhLF identical to human milk derived LF described earlier (Spik et al., 1988). Also it was demonstrated that rhLF N-glycans, like in the human milk-derived LF, are partially sialylated, and sialylation was considered critical in reconstruction of MTX-inhibited secondary humoral immune response (Choi et al., 2008). The ability of this novel rhLF to control microbial growth and limit severe manifestations of infectious pathologies was also similar to its natural counterpart as confirmed in multiple immunological assays including testing of adjuvant activity to boost efficacy of the *Bacillus Calmette-Guérin* (BCG) vaccine (Hwang et al., 2009). In that particular study, it was shown that the rhLF was superior to milk-derived LF when used as an adjuvant combined with the BCG vaccine; study outcomes yielded greater immune responses to antigens, as well as provided significant reduction in lung pathology resulting from subsequent challenge with the virulent organisms.

LF plays an important role in cellular redox modulation (Kruzel et al., 2010). It was found that rhLF effectively decreased the GOx-induced changes in intracellular ROS in a manner similar to that of milk-derived human LF. These findings are in concert with our previous study where it was demonstrated that LF decreased ROS levels *in vivo* induced by NADPH oxidase of ragweed pollen extract (Kruzelet al., 2006). LF also inhibited infiltration and accumulation of inflammatory cells into lung tissue (Hwang et al, 2005,2007). These data and those from gene array experiments suggested that the protective action of LF involves two mechanisms, one iron-dependent and the other manifested at a subsequent stage that regulates critical allergic responses; the latter being iron-independent. In this study it was hypothesized that LF may induce molecules that are involved in controlling intracellular

oxidative stress levels. Indeed, the whole blood cells exposed to rhLF confirmed the activation of gene expression for several antioxidant markers. The induction of antioxidant defense system is an appealing strategy for controlling inflammatory responses in which oxidative stress plays an important role. There are several components of the antioxidant defense system and their expression may vary depending upon the cell type and the nature of the oxidative stress. Here we demonstrate differential effect of rhLF on the families of four antioxidant enzymes: SOD, GPX, PRDX, and PTGS, also known as COX. In case of PTGS the induction of constitutive form - PTGS1 demonstrates the anti-inflammatory effect of rhLF in WBC. In contrast, LPS and other pro-inflammatory stimuli, typically up regulate the inducible form of PTGS 2 (Font-Nieves et al., 2012). Also, rhLF showed the ability to induce SOD1 (copper-zinc SOD), which is present in the cytoplasm and nucleus, but not mitochondrial SOD2 or extracellular SOD3. This suggests that rhLF-induced overexpression of SOD1 is directed to more efficient dismutation of intracellular superoxide radicals. LF acts as an iron scavenger (Majka et al., 2013) and lowers cellular oxidative stress by decreasing ROS levels. Because the LF used in this study was only partially iron saturated (0.57 mg of Fe/g of LF), the antioxidant activity reported here may also be due to sequestration of iron from the media of cultured cells. If this is the case the $O_2^{\bullet-}$ generated by GOx was not directly converted in H_2O_2 , then to hydroxyl radical by which is measured by the redox-sensitive probe. These observations also suggest that in rhLF treated cells the oxidase-generated $O_2^{\bullet-}$ was processed by the increased levels of SOD, and the H_2O_2 was detoxified in the GPX and/or PRDX pathways. A question that arises, however, is why exogenous LF would have an effect on WBC, since there is endogenous LF always present in circulation? Indeed, under normal physiologic conditions the serum LF level is very low (300 ng/mL). Yet, it should be noted that upon microbial infection the level of LF can increase up to 100-fold. LF is an acute phase protein for which plasma concentrations rise significantly upon infection or trauma. Thus the experimental design employed in this study, utilizing 10 μ g/mL rhLF added to cultures, mimics the acute inflammatory conditions *in vivo*, during which LF is released from neutrophils for modulation of immune responses in a defined localized response directed against infectious assault. In such a situation, high local concentrations of LF may drive both innate and secondary defense mechanisms, leading to development of specific adaptive immunity. Given that experiments on gene expression are performed only at the 2h time point, it is not surprising that exogenous LF, by mimicking acute inflammation, induced expression of the antioxidant defense system mediators to prevent adverse effects of inflammation driven ROS.

It appears likely, that if LF is given parenterally in humans could also affect the function of other major circulatory iron-carrier protein, transferrin (TF). Although human serum TF and LF are quite similar in sequence and structure, and coordinate iron in the same manner, they differ in their affinities for iron as well as their receptor binding properties (Wally and Buchanan, 2007). It is plausible that internalization and endocytic trafficking of TF, which is highly dependent on a wide concentration range of hydrogen peroxide (Cheng and Vieira, 2006), could be significantly impaired in a presence of exogenous LF. Indeed, the oxidative stress-dependent changes in endocytic trafficking may be primed by multiple and varied mechanisms contributing to pathogenesis and disease (Cheng and Vieira, 2006). However, extrapolation to disease contribution relies on data obtained from different experimental

models; data in patients are scarce and sometimes contradictory. Because of the potential therapeutic utility of rhLF further studies are needed to identify and define approaches aimed at the interception of metal-carrier proteins and their antioxidant and antimicrobial activities.

Finally, it is of interest that in addition to activation of host innate immune function, the CHO-derived rhLF also maintained antibacterial function. The limited anti-bacterial screening of rhLF against the two common serovars of *S. pneumoniae* indicated there was no significant difference when compared to milk-derived LF. This is similar in nature to results reported for rhLF from transgenic rice (Conesa et al., 2009). While full minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) profiles are warranted, it is comforting to note that rhLF activity at LD₉₀ was practically identical to the milk-derived LF in these two strains.

5. Conclusions

These studies were undertaken to produce rhLF using the CHO expression platform, a system of choice for glycoprotein therapeutics. We developed a protocol for scale-up expression and purification of rhLF, verified its glycan primary structure, and assessed its biological properties in comparison with the milk-derived LF. The rhLF demonstrated high biological potency and was compatible with its natural counterpart. In addition, rhLF showed a modulatory effect on cellular redox *via* upregulation of key antioxidant enzymes. Overall, these studies indicate that this novel rhLF holds strong promise for use as a therapeutic, and may overcome existing barriers for systemic administration in humans.

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Abbreviations

hLF human milk lactoferrin

rhLF	recombinant human lactoferrin
TF	transferrin
LPS	lipopolysaccharide
HEK	human epithelial kidney cells
CHO	Chinese hamster ovary cells
ELISA	enzyme-linked immunosorbent assay
CFU	colony forming units
MTX	methotrexate
BCG	<i>Bacillus Calmette-Guérin</i> vaccine
ROS	reactive oxygen species
SOD	superoxide dismutase
GPX	glutathione peroxidase
PRDX	peroxiredoxins
PTGS	prostaglandin-endoperoxide synthase
COX	cyclooxygenase
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
ICP-MS	inductively coupled plasma mass spectrometry

Fig. 1. HPLC chromatogram of rhLF. The rhLF derived from a single step purification of supernatants from transfected CHO cells was subjected to molecular analysis. Insert: SDS-PAGE (left panel) and Western blot (right panel) show a direct comparison to milk-derived human LF. Border left lanes represent high range molecular weight standards.

Fig. 2. MALDI-TOF analysis of permethylated N-glycans from rhLF. The MALDI-TOF spectra of released glycan structure are depicted. The predicted structures of the rhLF glycans deduced from composition, MS/MS analysis, endoglycosidase digestion, and information of biosynthetic pathways are shown schematically.

Fig. 3. Mass spectra analysis of the tryptic glycopeptide ions. Comparison of mass spectra profiles of hLF and rhLF tryptic digest peptides identified by AA sequence as Asn138 (TAGWNVPIGTLPFLNWTGPPEIEAAVAR) and Asn479 (TAGWNIPMGLLFNQTGSCK) glycosylation sites. No relative glycan intensity was detected for Asn624 (NGSDCPDK). Carbohydrate legend identical to that for Fig. 2.

Fig. 4.

Validation of whole genome gene expression microarray by RT PCR. Comparison of relative fold changes at 2 h in expression of individual genes was accomplished using the Illumina HumanHT-12 v4 Gene Expression BeadChip (blue) and Human Oxidative Stress and Antioxidant Defense RT² Profiler PCR Array gene panel (red). The top panel profiles the expression of all 84 genes related to oxidative stress. The bottom panel highlights four key antioxidant enzymes from the same experiment. The relative fold change in expression of four enzyme family isoforms is shown for superoxide dismutase (SOD), glutathione peroxidase (GPX), peroxiredoxins (PRDX), and prostaglandin-endoperoxide synthase (PTGS); also known as cyclooxygenase (COX). Differential activation is revealed within family isoforms. All these enzymes are critical for the metabolic neutralization of ROS. Differential expression shown was calculated using normalized and averaged biological triplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5. Comparison of natural and recombinant human LF to protect human macrophage cells from oxidative stress. (A) rhLF decreases ROS levels over time as determined by DCF assay. (B) Dose-dependent protection of cells by rhLF from GOx generated ROS at 30 min post exposure. (C) Decreased levels of ROS by rhLF in GOx-exposed cells as measured by the redox biosensor HyPerCoty. Comparisons were made to natural human lactoferrin (hLF). HyPerCoty fluorescence was determined at 30 min after GOx addition. The data are expressed as the mean $\pm$ SD. Results were analyzed for significant differences using ANOVA procedures and Student's t-tests (Sigma Plot 11.0). $\blacklozenge$, glucose oxidase (GOx 1 μ U); $\blacksquare$, mock-treated; $\circ$, NAC (10mM for 3 h + mock-treated); $\bullet$, NAC (10mM for 3 h + GOx); $\square$, hLF (100 μ g for 1 h + GOx); $\lozenge$, rhLF (100 μ g for 1 h + GOx). Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).