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A multifunctional bioactive protein: Lactoferrin

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Abstract
Lactoferrin which is also known by the name of lactotransferrin (LTF), is a multifunctional iron-binding glycoprotein of the transferrin family and is found in almost all human mucosal secretions as well as in the specific granules of polymorphonuclear leukocytes in blood. A variety of functions have been found to associated with this protein along with its contribution to antimicrobial host defense mechanism. Moreover, it has been shown to have direct effects on some of the pathogenic microorganisms through bacteriostatic and microbial iron uptake induction. Several studies have shown that the protein synergistically interacts with immunoglobins, complement, and neutrophil cationic proteins which act against Gram-negative bacteria. Further, both the whole protein and a cationic N-terminus peptide fragment directly damage the outer membrane of Gram-negative bacteria suggesting a mechanism for its supplemental antimicrobial effects. It has appeared logical that antimicrobial activity of the protein arises from sequestration of environmental iron thereby causing nutritional deprivation of iron in susceptible organisms. Lactoferrin has diverse role where it can be used as an immunotherapeutic and can also play a role in immunodiagnostics. Still its overall physiologic role remains yet to be defined clearly inside the living system.

Keywords: lactoferrin, antimicrobial, immunomodulator, antioxidant

1. Introduction
Milk is the primary source of nutrients for young mammalians. It is recognized as being nutritionally balanced and has therefore attracted a lot of scientific interest over the years. Various properties of intact milk proteins have been reported including satiating, antimicrobial, mineral binding, antilipidemic and anticancer properties (Paesano, 2014; Kanwar *et al*., 2014; Zhang *et al*., 2014; Nakamura *et al*., 2013) [38, 22, 33]. Identification of large number of peptides in milk protein hydrolysate make the milk proteins as one of the most important source of bioactive peptide. Several authors have suggested that milk protein-derived bioactive proteins may be used as prophylactic agents to alleviate symptoms of various diseases in humans. Side-effects of various drugs used to cure/slow down the progress of specific diseases in humans may sometimes outweigh their benefits (Onishi, 2011; Agrawal *et al*., 2009) [37]. Further increasing awareness regarding potential benefits of milk protein derived bioactive peptide among the people laid path of growing milk nutraceutical market (Nagpal *et al*., 2011) [32].
Lactoferrin (Lf) is a non-heme iron binding glycoprotein with molecular weight of 78 kDa that contains around 690 amino acid residues. It belongs to the transferrin (Tf) family. This protein is almost found in all exocrine secretions including saliva, tears, semen, vaginal fluids, gastrointestinal fluids, nasal mucosa and bronchial mucosa of human being (Iigo *et al*., 2009; Birgens *et al*., 1985) [20, 3]. Lactoferrin is also found in milk of bovine, caprine, camel and human (Saltanat, 2009) [45]. Lf is also known for its anti-bacterial, antifungal, antiviral, antimicrobial, anti-oxidant, anti-inflammatory, anti-parasitic, anti-allergic and most importantly anti-cancerous properties (Iigo *et al*., 2009; Parhi *et al*., 2012) [56, 40]. Lf is the second most abundant milk protein after casein and its highest concentration is found in human colostrum and then human milk followed by cow milk (Sanchez *et al*., 1992) [46]. Development of drug-resistant cancers imposed question mark on the use of chemotherapeutic agents. This limitation raises the need of a natural substitute that has generalized acceptance and can possibly completely eradicate the primary tumor, thus eliminating the risk of recurrence. In this context Lf has got the potential to be used as anticancer bio-molecule.

Lactoferrin: Structure and Functions
The structure of Lf consists of a single polypeptide chain which is folded into two lobes

(N and C lobes) with 33%–41% homology (González-Chávez *et al*., 2008). Two lobes are linked through an α-helical residue, making it a flexible molecule. The two lobes of Lf are made of α-helix and β-sheet, and each lobe can bind either Fe^{+2} or Fe^{+3} ions in synergy with the carbonate ion (CO_3^{2-}) (Iafisco *et al*., 2011) [19]. Amongst transferrin family the latoferrin has highest iron binding affinity. As lactoferrin have various physiological functions such as antimicrobial/antiviral activities, immunomodulatory activity, and antioxidant activity (Wakabayashi *et al*., 2006; Burrow *et al*., 2011) [55, 6]. So, it is considered as crucial components of host defense system.

Lactoferrin is the major iron transporter protein in blood plasma. In its natural form lactoferrin, is partially saturated with iron and hence can be fully saturated with iron from the external environment (Tsuda *et al*., 2004) [51]. Lactoferrin acts as a signaling molecule in various pathways and to exert their cytotoxic effects Human Lf (hLf) and bovine Lf (bLf) cause cell cycle arrest the cell cycle and leads to apoptosis (programmed cell death) in cancer cells while bovine lactoferricin B inhibits cell growth by triggering mitochondrial related apoptosis (intrinsic apoptotic pathway) and disrupting the cell membrane.

Sources of lactoferrin

Lf is an important part of the innate immune system (Wakabayashi *et al*., 2006) [55]. Lf is continuously synthesized in body and is released into the exocrine fluids like saliva (Reitamo *et al*., 1980) [42], tears (McClellan, 1997) [30] and vaginal fluids (Valore *et al*., 2002) [52], or only at well-defined stages of cell differentiation such as, the granules of neutrophils (Breton-Gorius *et al*., 1980) [4]. Glandular epithelial cells secrete Lf in milk source. Various concentrations of Lf is found in the milk obtained from different sources (Masson *et al*., 1969) [29]. During an infection or an inflammatory condition, the levels of Lf are raised in the body (Caccavo *et al*., 2003) [7] making Lf a biomarker for inflammatory conditions.

Antimicrobial activity of lactoferrin

Risk of development of resistance to antibiotics raise the needs for alternatives antimicrobials and lacteferrin is one of the promising antimicrobial molecule that have potential to fills the gape (Li *et al*., 1995) [27]. The antibacterial activity of lactoferrin is mediated through its iron sequestering ability by virtue of which makes iron inaccessible to bacteria and hampered their growth and division (Bullen *et al*., 1972) [5]. Lf and Lf derived peptide has bacteriostatic activity against both Gram-positive and Gram-negative bacteria (Ellison *et al*., 1988) [9]. *Staphylococcus epidermidis* is one of the most predominant infectious agents in individual implemented with intraocular lenses leading to a characteristic biofilm formation on the soft contact lenses. It is observed that Lf induces the binding of this bacterium with the anionic cell wall preferentially to vancomycin thereby allowing its entry into the bacteria (Leitch and Willcox, 1999) [26]. Lf also facilitate the penetration of lysozyme which binds to teichoic acid and compensate the charges on cell wall (Leitch and Willcox, 1999) [6]. Lf causes depolarization of the bacterial membrane making it permeable and eventually metabolic injury. Lf is also used to treat periodontal diseases by acting against plaque forming oral microorganisms like *Streptococcus mitis, Streptococcus gordoni, Streptococcus salivarius* and *Streptococcus mutans*.

Lactoferrin also prevent the colonization of *Giardia lambia*, a most common protozoal infection of human intestine by acting on Giardia trophozoites plasmalemma, endomembrane and cytoskeleton (Ochoa *et al*., 2008) [35].

In another study, antibacterial activity of bLf hydrolysate was assessed by using different enzyme including, rennet and pepsin against *Escherichia coli* and *Bacillus subtilis* which revealed that Lf-cin B was the most potent antibacterial peptide and was isolated from both rennet and pepsin LFH (Sekine *et al*., 2015). It was demonstrated that pepsin hydrolysate derivatives of bLf had stronger bifidogenic activity than natural against *Bifidobacterium breve* and *Bifidobacterium longum* species (Oda *et al*., 2013) [36]. Several modifications have been attempted in bLf in order to use it as a food preservative. It was found that Glycosylated lactoferrin (gLf) showed substantial Fe-binding capacity and excellent emulsifying properties and also revealed its ability to inhibit the growth of *E. coli* at 50 °C completely (Haversen *et al*, 2002) [17]. Hence, all these studies offer new possibilities for Lf as a food preservative. In another study it was observed that nanoformulated Fe-bLf was more effective in the treatment of Salmonella-infected mice than the standard therapy using ciprofloxacin (Gupta *et al*., 2014) [13]. Lactoferrin has ability to damage fungal cell membrane that alter its permeability and also its iron chelating properties attributed to antifungal activity (Wakabayashi *et al*., 2000) [54]. Lf also exhibits antiprotozoal activity and the mechanism by which this is done varies from its antibacterial and antifungal aspects. Although Lf had no role in inhibiting the entry of these protozoa into the animal system but it did not allow the growth of these protozoans in the host (Roseanu *et al*., 2000) [43].

Use of lactoferrin as antiviral compound is one of the most recent properties. Although the research regarding antiviral activity of Lf is in early phase however, there are only a very few cases in which Lf failed to benefit as an antiviral activity. Lf exhibited antiviral activity against a number of viruses including herpes simplex virus, cytomegalovirus, hepatitis B and C virus (HBV and HCV) and human immunodeficiency virus (HIV) (Hara *et al*., 2002; Ikeda *et al*., 1998; Roy *et al*., 2012) [15, 21, 44]. A new perspective in the studies of antimicrobial activity of Lf is due to its potent prophylactic and therapeutic ability in a broad spectrum. Unlike to all these antimicrobial effects, in some protozoans like Trichomonas, Lf helps in effective binding, and successful internalization in these parasites (Tachezy *et al*., 1998) [49].

Lactoferrin and Immunity

Beside diverse function of Lf in various body fluids, iron free form of Lf is the integral component of cytoplasmic secondary granules of neutrophils thus have role in first line defense. During inflammation, Lf is released and the concentration of Lf at the site of inflammation is increased from 0.4–2.0 μg/mL to 200 μg/mL playing a major role in the feedback mechanism of inflammatory response (Hiss *et al*., 2008). In the kidney Lf is synthesized locally where, it sequestrating free iron from urine and makes it available for metabolic functions (Abrin *et al*., 2000) [1].

Lf acts as immune modulator by interacting with specific cell receptors of epithelial and immune cells and as a lipopolysaccharide to pro-inflammatory bacterial elements (Elass-Rochard *et al*., 1995; Legrand *et al*., 2008) [8, 25]. At cellular level Lf significantly affects the differentiation, maturation, activation, migration, proliferation and functions

of immune cells by using, nuclear factor-kappa B (NF-κB) and MAP kinase signaling pathway (Gahr *et al*., 1991) [11]. Lf from bovine milk showed proteinase inhibitory activity against *Porphyromonas gingivalis*, a bacterial pathogen, by inhibiting Arg- and Lys-specific proteolytic activities (Manzoni *et al*., 2012) [28]. The bovine Lf at molecular level influence maturation of lymphocyte and release of cytokines in bone marrow microenvironment (Touyz, 2000) [50]. Anti-inflammatory action of Lf alleviate stress by preventing the excess inflammatory response (Ye *et al*., 2014) [57]. It was demonstrated that Lf knockout mice shown high susceptibility to inflammation-induced colorectal dysplasia, mainly due to NF-κB and AKT/mTOR signaling, regulation of cell apoptosis and proliferation. On the basis of above study, it can be inferred that anti-carcinogenic property of Lf is attribution of its anti-inflammatory function (Gutteridge *et al*., 1979) [14].

Free form of iron plays a pivotal role in generation of reactive oxygen species (ROS) and leads to lipid peroxidation of cell membranes using iron-dependent Haber-Weiss reaction. Inefficiency of certain vital enzyme like, catalase, glutathione peroxidase and superoxide dismutase lead to over production of hydroxyl radicals further increases the oxidative stress (Nozaki *et al*., 2002) [34]. It is hypothesized that Iron sequestration by Lf from the microenvironment limits the oxidative damage to bio-membranes by hampering lipid peroxidation. Lf also regulate the systemic inflammatory response in controlled manner so that there is minimum damage to surrounding tissues (Gahr *et al*., 1991; Pajkrt *et al*., 1996) [11, 39]. Antioxidant mechanism is one of the attribute by virtue of which oral administration of Lf shown to support improved immune response (Mulder *et al*., 2008) [31].

Lf is considered as important component in first line host defense, as it plays vital role in innate as well as adaptive immune response (Legrand *et al*., 2008, Kruzel *et al*., 2002) [25, 24]. It was revealed that Lf potentiate the phagocytic activity neutrophils (Wakabayashi *et al*., 2003) [36], increased activity of NK cells (Shau *et al*., 1992) and also involved in macrophages activation by increased production of cytokines and nitric oxide (NO) that, reduces the proliferation of intracellular pathogens (Kawai *et al*., 2007) [23]. Production of pro inflammatory cytokines such as, TNF-α, IL-6 and IL-1β by Lf according to the requirement helps to confer its immune modulatory activity. Lf regulate the production of antigen presenting cells (APCs) like, macrophages, dendritic cells and B cells which presents the processed antigen to CD4+ T cells via major histocompatibility complex II (MHC II) (Puddu *et al*., 2009) [41] there by it play active role in specific immune response against pathogens. Lf found to reduces the production of cytokines, TNF-α, IL-6 and IL-1β that were induced by Bacille Calmette-Guerin strain of *Mycobacterium bovis.*

It is reported that all T cell subsets including δγ T cells have been express Lf receptors (Fischer *et al*., 2006) [10]. Lf shown to down regulate the leukocyte function associated antigen (LFA-1), an adhesion molecule present on CD4+ and CD8+ T cells, in human peripheral blood mononuclear cells when cultured in presence of human Lf. Expression of human T cell ζ–chain, T cell receptor complex involved in receptor signaling were enhanced by hLf (Nichols *et al.,* 2015) observed that when concanavalin A (ConA) activated murine splenocytes were cultured in the presence of bovine or human Lf resulted in reduced production of IFN-γ and IL-2. There are various studies that have proven the immune modulatory function of Lf such as oral delivery of Lf to the mice bearing tumor cells showed an increase in lymphoid and intestinal CD4+ and CD8+ T cells (Wang *et al*., 2000); increased population of circulating leukocytes CD3+CD4+, CD3+ TCRγδ+, and granulocyte were seen in mice with orally administered Lf (Wakabayashi *et al*., 2006) [55]. Recently presence of lactoferrin in feces has been introduced as a biomarker for the diagnosis and monitoring of inflammatory bowel disease (IBD). It could also be used as tool to investigate and quantify the effect of granulocyte and monocyte adsorptive apheresis (GMA) in ulcerative colitis (UC) (Hashiguchi *et al*., 2015) [16]. Hence, lactoferrin has diverse role, it can be an immunotherapeutic and can also play a role in immunodiagnostics.

Conclusions

The advantages of this natural molecule prove its potential as a natural therapeutic agent that can be used in various fields of research including cancer. The role of Lf as anti-bacterial and anti-fungal agent had been beneficial in its use as a bactericidal and fungicidal agent in lotions and creams. Its use can be extended to topical applications as well. An interesting aspect of using Lf as an anti-cancer agent by delivering it to the body in the form of ice-creams, tablets and oral supplements in the form of NPs have been researched upon. With its role in being able to combat deadly viruses like HCV and HBV also poses a need for its use as an anti-viral agent for human immunodeficiency virus (HIV) and other potent viruses that cause health risks. The role of this natural molecule as anti-inflammatory agent needs further research. It stands as a biomarker for inflammatory conditions and its potential role as a therapeutic molecule needs to be taken forward.

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