Review

Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application

Domenico Caccavo[1], Nelly M. Pellegrino[1], Maria Altamura[1], Amelia Rigon[2], Luigi Amati[3], Antonio Amoroso[4], Emilio Jirillo[1]

[1]*Department of 'Clinica Medica, Immunologia e Malattie Infettive', University of Bari, Bari, Italy*
[2]*University 'Campus Bio-Medico', Rome, Italy*
[3]*IRCCS for Gastroenterological Diseases, Castellana Grotte, Bari, Italy*
[4]*Department of 'Medicina Clinica', University of 'La Sapienza', Rome, Italy*

Lactoferrin is an iron-binding glycoprotein present in various secretions (*e.g.* milk, tears, saliva, pancreatic juice, *etc.*). It is also stored in specific granules of polymorphonuclear granulocytes from which it is released following activation. Lactoferrin exerts a bactericidal activity by damaging the outer membrane of Gram-negative bacteria, as well as immunoregulatory functions by decreasing the release of interleukin-1 (IL-1), IL-2 and tumor necrosis factor-α (TNF-α) and enhancing monocyte and natural killer cell cytotoxicity. Lactoferrin binds with high affinity to lipid A, the toxic moiety of the lipopolysaccharide, or endotoxin from Gram-negative bacteria. Lipopolysaccharide interaction with monocytes/macrophages results in the production and release of TNF-α, that plays an important role in inducing septic shock. In this respect, it has recently been demonstrated that lactoferrin inhibits the lipopolysaccharide interaction with CD14 on monocytes/macrophages by competition with the lipopolysaccharide binding protein. Therefore, besides its bactericidal activity, lactoferrin may also act by neutralizing the toxic effects of lipopolysaccharide and this protective role against endotoxin lethal shock has been demonstrated in animal models. Moreover, *in vitro* and *in vivo* neutralization of endotoxin by a human lactoferrin-derived peptide was also reported and lactoferrin or lactoferrin-derived peptides could represent useful tools for the treatment of endotoxin-induced septic shock. The recent production and characterization of monoclonal antibodies against different epitopes of human lactoferrin, including monoclonal antibodies selectively neutralizing lactoferrin binding to lipid A, may allow a better elucidation of the consequence of lactoferrin-lipopolysaccharide interaction.

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein that shows manifold functions in mammalian species, even though contrasting results have frequently been reported about its effects. In spite of the name and the capacity of LF to bind and transport iron, this is not the prominent activity exerted by this molecule. Many LF effects are likely related to its cationic nature which allows LF binding to several molecules, either soluble or anchored on cell surfaces.

The biological role of LF is not completely elucidated and, in several aspects, it still remains an enigma. Nevertheless, the bulk of data available from the current literature indicates that this multifunctional molecule basically acts as an antimicrobial and anti-inflammatory agent, the antimicrobial effects being directed against bacteria, fungi and viruses. Recently, other *in vivo* and *in vitro* LF-mediated activities have been reported, including immunoregulatory functions, as well as tumor growth inhibition. Therefore, knowledge of physiological role and potential therapeutic applications of LF are rapidly expanding. Encouraging results have been reported in animal models mostly regarding LF treatment in the course of bacterial sepsis.

Received 19 November 2001
Revised 16 July 2002, 24 October 2002
Accepted 29 October 2002

Correspondence to: Domenico Caccavo MD PhD, Immunologia, Policlinico, Piazza G. Cesare 4, 70124 Bari, Italy
Tel: +39 080 5478492; Fax: +39 080 5478537
E-mail: caccavo@midim.uniba.it

DOI 10.1179/096805102125001000

In this review, the most recent knowledge about LF functions and its potential therapeutic applications will be discussed.

SALIENT FEATURES OF LACTOFERRIN

LF is a ~80 kDa glycoprotein, found in milk, tears, saliva and other secretions, such as bile, pancreatic juice, small intestinal secretions, bronchial mucus, vaginal mucus, and seminal plasma.[1,2] It is also stored in the secondary granules of polymorphonuclear leukocytes (PMNs).[3] The molecule consists of two globular lobes, the N lobe (residues 1–333), and the C lobe (residues 346–692), that are connected by a three-turn helix (residues 333–343).[4] Each lobe contains an iron site and is subdivided into two domains, so that the molecule is represented by four domains, *i.e.* N_I, N_{II}, C_I, and C_{II}.[4]

Although LF is structurally related to serum transferrin (TF), its affinity for iron is about 300 times higher than that of TF, which allows LF to retain iron also at low pH. In addition, the LF isoelectric point (pI) is 8.7, which contributes to its cationic nature.[5]

No differences have been detected between milk and PMN LF regarding M_r, pI, and iron–protein complex stability.[6] Nevertheless, milk and PMN LF may differ in their carbohydrate composition, the major difference being the absence of α1,3- and α-1,6-linked fucose residues in PMN LF.[7]

In humans, the highest LF concentrations are found in colostrum, mature breast milk, tear fluid and seminal plasma,[8–12] and human milk LF levels are significantly higher than those detected in bovine milk.[8] LF is detected in human plasma or serum at low concentrations and is predominantly derived from PMNs,[13] even though contrasting results have been reported about the correlation between PMN count and LF plasma levels.[13,14] Several factors may influence the results of LF measurement in plasma and serum, including the anticoagulant used, differences in LF iron saturation, the interval between venipuncture and analysis, and the LF polymerization.[15,16] Higher LF concentrations are present in males than in females[17–20] and, in females, augmented levels are detectable either during pregnancy[20] or after menopause,[17] thus indicating that LF concentrations may be influenced by endocrine activity. In this respect, the quantification of LF levels in vaginal mucus of women throughout the menstrual cycle demonstrated highest LF concentration in mucus just after menses (62.9–218 μg/mg of total mucus proteins) and lowest ones before the onset of menses (3.87–11.4 μg/mg proteins).[21] In the same group of subjects, the overall concentration of vaginal mucus proteins showed no significant differences during menstrual cycle. Plasma LF concentration was highest just before or during menses; however, the marked variation demonstrated in vaginal mucus was not seen. Interestingly, in women using oral contraceptives, the mean LF concentration in vaginal mucus was markedly lower than in the control group (never exceeding 19.8 μg/mg of mucus proteins) and LF levels did not show monthly variation.[21]

An estrogen-dependent synthesis of LF was first demonstrated in the uteri of mice receiving diethylstilbestrol (DES) on the previous 3 days.[22] In fact, by Northern blot analysis of RNA from uteri, it was found that an increase in the level of LF mRNA of at least 300-fold could be induced in mice after 3 days of hormone exposure, whereas a weak signal was detectable in material from control animals. It has also been reported that LF mRNA is expressed in various tissues during development; while LF mRNA expression is not increased by the synthetic estrogen DES in non-reproductive tissues, such as liver, spleen, and lung, it is markedly enhanced in neonatal reproductive tract tissues.[23]

The human LF gene is differentially regulated in different tissues and in the 5′-flanking region of the gene are present several regulatory elements that can contribute to the specific expression of the gene in different cell types.[24] The increase of LF mRNA after estrogen treatment depends on a complex interaction among transcription factors acting on the estrogen response element (ERE) of the LF gene.[24] Studies performed utilizing the human endometrium carcinoma cell line RL95-2 or the human endometrium carcinoma cell line HEC-1B have shown that: (i) the estrogen-stimulated transcription from the human LF gene is mediated through an imperfect estrogen response element (ERE);[25] (ii) an estrogen-related receptor (*i.e.* hERR1) modulates the estrogen receptor-mediated response of human LF gene promoter;[25] and (iii) the estrogen-related receptor α1 (ERRα1) actively modulates the estrogen response of LF gene, as well as other estrogen-responsive genes.[26]

The responsiveness of LF gene expression to estrogen was studied *in vivo* in primate endometrium and results obtained by Western blotting and immunohistochemistry have documented that LF was not detectable in the untreated ovariectomized monkey endometrium, was instead elevated by estrogen treatment, and was suppressed by sequential, combined administration of estrogen plus progesterone.[27]

With respect to LF clearance, experiments conducted in rats have demonstrated that LF removal from circulation occurs by uptake of the molecule by the liver Kupffer cells, liver endothelial cells, and hepatocytes,[28] but also monocytes/macrophages and other cells of the reticular endothelial system (RES) can remove LF. In fact, prolongation of the half-life of injected hololactoferrin was obtained by blocking RES.[29] In humans, [^{125}I]-labelled LF is rapidly cleared from plasma[30] and organ radioactivity counting shows that LF is taken up by the

liver and spleen, and 99% of administered LF is recovered in the urine within the first 24 h.[30]

CELL RECEPTORS FOR LACTOFERRIN

Cell receptors for lactoferrin (LFRs) have been demonstrated on different cell types, including T- and B-lymphocytes, monocytes/macrophages, platelets, liver cells, and gastrointestinal tract cells. LFRs on lymphocytes were described in 1976 by Van Snik and Masson,[31] who demonstrated the binding of lactoferrin to the cell membrane of mouse peritoneal lymphocytes and macrophages. Later, Birgens *et al.*[32] analyzed the presence of LFRs in normal and leukaemic human blood cells and observed that LF binding to monocytes was about 10 times higher than binding to PMNs and lymphocytes. LF bound with low affinity also to lymphocytes obtained from patients with chronic lymphocytic leukaemia, whereas a high affinity binding could be demonstrated to lymphoblasts as well as to myeloblasts.

A lymphocyte LFR was found on human lymphocytes following cell stimulation with phytohemagglutinin (PHA).[33] The appearance of receptors was time dependent reaching the maximal expression after 2 days of mitogen stimulation, which suggested an induction of biosynthesis of the receptors. Western blot analysis utilizing Triton X-100 extract of PHA-stimulated lymphocytes and [^{125}I]-labelled LF showed that receptors were represented by two protein bands of 100 kDa and 110 kDa.[33] Receptors were then purified by affinity chromatography and their interaction with LF was reversible and dependent on concentration and pH.[34] The interaction between human LF (hLF) and lymphocyte receptors has also been investigated using hLF fragments obtained by mild tryptic hydrolysis and evidence has been provided that the binding site of hLF for lymphocyte receptors is located in the N-terminal domain I (residues 4–90 and/or 258–281).[34]

Monoclonal antibodies (mAbs) against LF receptors present on the cell membrane of a human T-lymphoblastic cell line (Jurkat cells) have been produced,[35] and one recognized both soluble LF-binding protein and membrane lymphocyte LFR. A 95 kDa LF binding protein was isolated from the cell culture medium which corresponded to the soluble form of the 105 kDa lymphocyte receptor. In the same study using fluorescence and electron microscopy, it was documented that LFRs were localized both inside and at the cell surface of the Jurkat cells. Additional experiments showed that Jurkat cells have about 300,000 LF binding sites.[36] Following LF-receptor interaction, Jurkat cells internalized LF, which was found in endosome vesicles; then, the cells released into the culture medium both degraded and intact LF, a 30–40% fraction of the ligand being degraded at each round of endocytosis.[36]

The interaction between LF and B-lymphocytes was investigated by Kawasaki *et al.*,[37] who reported that the N-terminal basic region containing N-terminal Gly was involved in the LF–B-lymphocyte interaction and acylation of the amino groups of LF significantly reduced its binding to B-lymphocytes.

Studies on LF–monocyte interactions have shown that LF bound to human monocytes by specific binding indicating the presence of LFRs.[38] About $1.6 \cdot 10^6$ receptors/monocyte are present. Competitive binding experiments have demonstrated that neither human transferrin nor human IgG inhibited the binding of [^{125}I]-LF to the cells, thus suggesting the presence of specific receptors. Interestingly, the binding of LF to adherent monocytes had about 100-fold lower affinity than to cells in suspension.[38]

The ability of LF and transferrin to interact with monocytes was compared utilizing the monocytic cell line U937.[39] It was found that total binding of LF to the cells was about 10 times greater when compared with the binding of transferrin, but most of the LF binding was non-specific and uptake of iron was more rapid from transferrin than from LF. The binding of [^{125}I]-LF was not inhibited by 5-fold molar excess of unlabelled transferrin or *vice versa*, which confirmed the presence of different receptors for LF and transferrin on human monocytes. Treatment of the cells with heparinase, that cleaves acid sugar residues, did not inhibit the binding, suggesting that electrostatic interactions between LF and acidic cell membrane components are not important.

Experiments carried out on hLF binding to the cells of the human monocytic leukaemia cell line THP-1 documented that binding markedly increased as the cells matured into macrophages, following stimulation with phorbol-12-myristate 13-acetate.[40] Scatchard analysis of the [^{125}I]-LF binding to THP-1 macrophages indicated the presence of high- and low-affinity receptor sites. The number of these high- and low-affinity receptors was estimated to be $2.4 \cdot 10^6$ and $2.5 \cdot 10^6$ per cell, respectively. The binding was not affected by the iron saturation of hLF, but was markedly reduced following acetylation, suggesting that amino residues of the polypeptide portion of the molecule play a crucial role in the binding. In the same study, it was reported that a short oligosaccharide structure, Gal-β-1-αaGlcNAc-β1-3-Gal is recognized by THP-1-derived macrophages, and this recognition partly contributes to the binding of LF to cells. Further characterization of LF-binding proteins of human macrophages revealed the presence on THP-1 cells of 35, 50 and/or 80, and 35–37 kDa proteins, that represent LF-binding proteins with polylactosamine-binding ability. Recently, the N-terminal amino acid sequence of the 50-kDa protein was determined[41] and such a sequence, *i.e.* Lys-Gln-Lys-Val-Ala-Gly-Lys-Gln-Pro-Val, has not been found in the N-terminal regions of known proteins. An antibody

which recognized this N-terminal amino acid sequence reacted with 50-, 60-, and 80-kDa proteins as assessed by immunoblotting and immunoprecipitation, thus indicating the presence on THP-1 cells of 50-, 60-, and 80-kDa lectin-like proteins with the same N-terminal amino acid sequence.

Human platelets bear LFRs and platelet LFRs share immunological and chemical properties with lymphocyte LFRs.[42] LF, as well as the N-terminal fragment of LF (residues 3–281) and a synthetic octodecapeptide (residues 20–37) corresponding to one of the two external loops, inhibit ADP-induced platelet aggregation. The inhibition requires the binding of LF to its receptors and not to platelet glycoprotein IIb-IIIa. The presence of LFRs on megakaryocytes has been evaluated utilizing the megakaryocytic Dami cell line[43] and LFRs were only demonstrated on the subpopulation of the largest cells. Of note, the mouse monoclonal antibody raised against LFR of Jurkat cells allowed the characterization of LFRs on megakaryocytes as a 105-kDa protein on Western blots. Therefore, it is conceivable that the platelet/megakaryocyte LFRs are identical to lymphocyte LFRs.

LF can interact with different receptors on liver cells before internalization, such as asialoglycoprotein receptors (ASGP) and low-density lipoprotein receptor-related protein (LRP). Rat hepatocytes bind LF by a 45-kDa membrane protein identical to rat hepatic lectin 1 (RHL-1), the major subunit of the asialoglycoprotein receptor,[44] by a galactose-independent mechanism. Deacylation of purified asialoglycoprotein receptor *in vitro* abolished receptor lectin activity with the complete loss of specific [^{125}I]-LF binding.[45] Other investigations have provided evidence that isolated rat parenchymal liver cells bind specifically LF, which, in turn, inhibits both binding and uptake of apolipoprotein E (apoE)-bearing lipoproteins by parenchymal liver cells *in vivo* and *in vitro*.[46,47] Removal by aminopeptidase M of the 14 N-terminal amino acids of LF, including 4 arginine residues at position 2–5, enhances its affinity for parenchymal liver cells and significantly inhibits the β-very low density lipoprotein binding.[48] Studies on the involvement of lipoprotein receptor-related protein (LRP)/α_2-macroglobulin receptor (α_2-MR) and its associated protein in the endocytosis of LF into rat liver cells have demonstrated that endosomal LF binding site is LRP/α_2-MR.[49] The complex LF-LRP/α_2-MR co-migrates into endosomes where it is detectable by immunoblotting at a position around 600 kDa. The LF-LRP/α_2-MR interaction is optimal at pH 5.8, which explains the finding of intact receptor-LF complexes in the acidic endosomal compartment.

Intestinal LFRs have been described in several species.[50–53] A human LFR was isolated from brush-border membranes (BBM) from human small intestine and the molecular weight of the receptor was represented by 110 kDa and 37 kDa subunits under non-reducing and reducing conditions, respectively.[54] Following deglycosylation of the protein, the molecular weight of the subunits decreases to 34 kDa, which indicates that the subunits are glycosylated. Competitive binding studies with bovine LF (bLF) and TF showed that the binding was specific. Scatchard plot analysis indicated the presence of $4.3 \cdot 10^{14}$ binding sites/mg membrane protein with an affinity constant of $0.3 \cdot 10^{6}$ M^{-1}. The binding was pH dependent with an optimum between pH 6.5 and 7.5.

The interaction between hLF, bLF and mouse LF with a subclone derived from a human carcinoma cell line HT29 (*i.e.* HT29-18-C1) was investigated.[55] Both hLF and bLF bound to two types of binding sites with different affinity. LFRs on the surface of the cells were identified as heparan sulphate and chondroitin sulphate glycosaminoglycans and the hLF binding was mediated by basic residues located in the N-terminal lobe, as demonstrated utilizing N-terminal deleted hLF variants.

Recently, the entire coding region of an intestinal hLFR cDNA was cloned by PCR based on amino acid sequences of purified native LFR.[56] Then, the recombinant LFR was expressed in a baculovirus-insect cell system and purified by immobilized hLF affinity chromatography. The molecular weight was 136 kDa under non-reducing conditions and 34 kDa under reducing conditions. The biochemical properties of recombinant hLFR were similar to those of native LFR. Reverse transcriptase-PCR documented that the gene was expressed at high levels in fetal small intestine and in adult heart and at low levels in Caco-2 cells. Phosphatidylinositol-specific phospholipase C treatment of Caco-2 cells demonstrated that this hLFR is glycosylphosphatidylinositolanchored.

The putative role of intestinal LFRs in intestinal iron absorption was investigated by Mikogami *et al.*,[57] who analyzed the effect of intracellular iron depletion on the cell surface binding of hLF to human enterocytes and its intracellular uptake using HT29-18-C1 cells. The addition of picolinic acid, an iron chelator, to the culture medium resulted in an enhancement of LF binding that was correlated with a decrease in intracellular iron content. Scatchard analysis indicated that the increased LF binding resulted from an increase of LFRs rather than an enhancement of the binding affinity for LF. These results suggest that biosynthesis of LFRs in intestinal cells can be regulated in response to the levels of intracellular chelatable iron.

It is worth mentioning that labelling of LF with fluorochromes or radioactive compounds may affect its binding to and/or its uptake from cells. For instance, labelling of hLF with fluorescein 5′-isothiocyanate (FITC) inhibits LF binding to PHA-stimulated human lymphocytes.[58] In fact, most of the FITC binds to three close lysine residues (Lys 263, Lys 280, and Lys 282) located in the N-terminal domain. Therefore, the use of FITC-labelled LF for analyzing the

LF-activated lymphocyte interaction, may lead to erroneous interpretations of experimental results.

With respect to LF radio-iodination, Peen *et al.*[59] have recently studied in Wistar rats the uptake of [^{125}I]-LF labelled by two different methods, *i.e.* LF was radio-iodinated either with a solid-phase lactoperoxidase/glucose oxidase iodination ([^{125}I]-LF) or coupled to tyramine-cellobiose ([^{125}I]-TC-LF). They found that liver uptake of [^{125}I]-LF was almost exclusively carried out by Kupffer cells (94% *versus* 5% of hepatocytes and 0.4% of liver endothelial cells), whereas the uptake of [^{125}I]-TC-LF was carried out predominantly by hepatocytes (63%), in comparison with liver endothelial cells (22%), and Kupffer cells (15%). The authors concluded that, since [^{125}I]-LF formed large macro-aggregates as a result of oxidative radio-iodination, the almost exclusive Kupffer cell localization was not surprising, whereas [^{125}I]-TC-LF consisted of monomers (50%) and dimers/oligomers/micro-aggregates and was taken up preferentially by hepatocytes.

ANTIMICROBIAL ACTIVITY

In the 1960s, the ability of LF to inhibit bacterial growth was reported[60–62] and subsequently many studies have been performed to elucidate the mechanism(s) underlying LF-mediated antimicrobial activities.

LF exerts bacteriostatic and bactericidal effects against a variety of micro-organisms. Actually, the bacteriostatic activity of LF has been mainly investigated against several strains of *Escherichia coli*.[63–67] Nevertheless, the growth of some strains of *Staphylococcus aureus* as well as *Staphylococcus epidermidis* is also inhibited by LF, especially if associated with lysozyme.[68,69]

LF binds reversibly to two atoms of Fe^{3+} with high affinity ($K_a = 10^{20}$ l/mol) in the presence of bicarbonate.[70] Therefore, the iron-free form of LF, apolactoferrin (apoLF), may deprive bacteria of iron, thus inhibiting their metabolic activities *in vivo*. This hypothesis is supported by the following findings: (i) Fe-saturated LF is not bacteriostatic for some bacteria; and (ii) addition of Fe in the medium reverses such bacteriostasis.[71,72] On the other hand, bacterial interaction with LF through LF binding protein expressed on the bacterial membrane is advantageous for some micro-organisms, including *Neisseria* spp. as they are capable of utilizing LF-bound Fe by expressing specific receptors for LF on their outer membranes that take up and internalize iron-saturated protein.[73,74] LF binding proteins have been also demonstrated on the membrane of *Haemophilus influenzae*[75] (two proteins of ~105 kDa that are distinct from the transferring-binding protein) and *Moraxella catarrhalis*.[76] Interestingly, most pathogens that possess the ability to acquire Fe reside at mucosal surfaces where LF is the most important Fe-binding protein. Besides bacteria, even some protozoan parasites show the ability to bind to, and to acquire, iron from LF. For instance, *Trichomonas vaginalis* possesses two LF binding proteins and, following exposure of the parasite to LF, energy metabolism markedly increases, suggesting that the thricomonad receptors for LF may contribute to the virulence of this parasite.[77] Also, the protozoan *Leishmania chagasi* is able to take up iron for growth from LF and TF; it has been suggested that this may contribute to the ability of *Leishmania* to survive and grow in diverse environments.[78]

However, it should be pointed out that many *in vitro* studies on microbial iron acquisition from LF are conducted by growing micro-organisms under strict, iron-free conditions that are unlikely to occur *in vivo* and this must be taken into account when interpreting the experimental results.

Besides bacteriostasis, LF exerts a direct bactericidal activity unrelated to the nutritional deprivation of iron. Arnold *et al.*[79] demonstrated, for the first time in 1977, that apoLF obtained from human colostrum showed bactericidal activity against *Streptococcus mutans* and *Vibrio cholerae*. Bacterial growth was completely inhibited, even when subcultured in iron-rich medium after prior incubation with LF. In addition, the concentration of apoLF necessary for the inhibition was markedly less than the minimum required to bind all available iron in the medium, thus clearly indicating that LF was bactericidal rather than bacteriostatic for these micro-organisms.[79]

Physical conditions as well as the metabolic state of the bacteria may influence LF-mediated killing. Optimal bactericidal activity is obtained *in vitro* at pH 5 with minimal reduction at pH 6 and no antimicrobial activity is observed at pH 8. Also, temperature affects the killing rate, as complete inhibition of bacterial growth occurs at 37°C and no loss of bacterial viability is detected at 2°C. Finally, micro-organisms harvested in the exponential phase are more sensitive to LF, whereas those harvested in the stationary phase are more resistant.[72] In this respect, it is possible that LF acts by depleting essential reserves during exponential growth and, therefore, the bactericidal effect is mainly observed in this phase. In addition, the finding that LF-mediated killing occurs at 37°C suggests an energy dependence for LF bactericidal effect, *i.e.* a requirement for active bacterial metabolism during the killing or, alternatively, this may simply reflect a temperature dependence for activity of LF itself.

Although the mechanisms involved in bactericidal activity are not definitively elucidated, evidence has been provided that LF causes a significant and dose-dependent release of lipopolysaccharide (LPS), damaging the outer membrane of Gram-negative bacteria.[80] LF also enhances the antibacterial activity of lysozyme.[81] Transmission electron microscopy documented that *E. coli* cells, cultured with LF and lysozyme, become

enlarged and hypodense, suggesting that killing is caused by osmotic damage. Moreover, dialysis chamber studies have demonstrated that bacterial killing requires a direct contact between LF and bacteria, this interaction being related to direct LF–LPS binding.[81]

Appelmelk *et al.*[82] reported that LF binds directly to isolated lipid A and/or intact LPS from several Gram-negative pathogens, including *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *H. influenzae*, *M. catarrhalis*, *Shigella flexneri*, and *Helicobacter pylori*. In addition, polymyxin B inhibits the LF–LPS interaction in a dose-dependent fashion, indicating that LF and polymyxin B may recognize the same binding site or sites in close proximity. Thus, it has been suggested that LF and polymyxin B could share some effects on LPS bio-activity, such as the inhibition of LPS-mediated release of inflammatory cytokines.[82] LF binding to lipid A/LPS was also evaluated by Caccavo *et al.*[83] who confirmed that lipid A, in the whole LPS molecular structure, likely represented the main determinant recognized by LF. Moreover, utilizing a panel of smooth (S) LPSs or rough (R) LPSs from *Salmonella minnesota* with different core length (*i.e.* Ra, Rb, Rc, Rd1, Rd2, and Re), the same authors demonstrated that the binding of LF to S-form LPS is weaker than that to lipid A, and the rate of binding to R-form LPSs is inversely related to core length.[83] Taken together, these findings indicate that the polysaccharide *O*-chain, as well as oligosaccharide core structures, could interfere with the LF–lipid A interaction. The binding of LF to whole cells of *Salmonella typhimurium* (S form) and to their isogenic R mutants, as well as the antibacterial effects of LF against such bacteria have also been evaluated.[84] The rate of LF binding to bacterial cells was inversely related to the oligosaccharide core length and, interestingly, bacteria with the shortest core showed maximal susceptibility to the LF-mediated antimicrobial activity. These findings strengthen the hypothesis that LPSs and oligosaccharides could shield the outer membrane of Gram-negative bacteria from the LF attack. Further studies on the LF–LPS interaction have shown that, in the LF molecule, there are two LPS binding sites located in the N-terminal domain I and in the C-terminal lobe, respectively. The former involves residues 28–34 of the loop region and represents the high-affinity binding site with a K_d of 3.6 ± 1 nM, the latter represents the low-affinity binding site with a K_d of 390 ± 20 nM.[85]

Glycosylation of hLF does not affect its capacity to bind iron or the affinity towards LPS and human lysozyme.[86] However, unglycosylated hLF is much more susceptible to tryptic proteolysis than glycosylated hLF, as the former is almost completely digested following a 4 h incubation with trypsin.[86] Therefore, the prominent function of glycosylation is probably to protect hLF against proteolysis.

The LF binding to LPS, heparin, human lysozyme, and DNA has been evaluated using N-terminal deleted hLF variants.[87] No binding occurred with a hLF mutant lacking the first five residues (*i.e.* Gly^1-Arg^2-Arg^3-Arg^4-Arg^5), thus indicating that this N-terminal stretch of hLF was essential for binding. In addition, an anti-hLF monoclonal antibody recognizing an N-lobe epitope including Arg^5 completely inhibited hLF interaction with ligands, thus clearly indicating that the same N-terminal stretch was involved in the hLF binding to all these molecules. The hLF interactions with LPS and human lyzozyme was also investigated at different NaCl concentrations. Decreasing the salt concentration increased hLF binding to LPS and human lyzozyme up to 75% and 55%, respectively, whereas increasing salt concentration above 0.4 M abolished the binding. Taken together, these results indicate that the cationic N-terminal is essential for the hLF electrostatic interaction with the ligands.

The interaction of LPS Re and lipid A with LF was recently investigated using physical techniques, such as Fourier-transform infrared spectroscopy and it was found that LF binds to the phosphate group within the lipid A part inducing a rigidification of the acyl chains of LPS. The secondary structure of the protein, however, was not changed.[88]

Bellamy *et al.*[89] reported that proteolytic digestion of LF with gastric pepsin yields several peptides. A peptide from the N-terminal region, consisting of a loop of 18 amino acid residues formed by a disulphide bond between cysteine residues 20 and 37, is called human lactoferricin (hLFcin) and shows a marked bactericidal activity. The homologous peptide derived from bovine LF (bLFcin) is composed of residues 19 to 36 and also exhibits strong antibacterial activity. The minimal inhibitory molar concentration of bLFcin has been determined for different bacterial species, including *E. coli, K. pneumoniae, Ps. aeruginosa, Staphylococcus aureus*, and *Listeria monocytogenes*, and it was at least 10-fold lower in comparison with that of whole LF. hLFcin includes residues 28–34, that represent the LF high-affinity binding site for LPS. In addition, synthetic peptides, *i.e.* HLT1, corresponding to the loop region of hLFcin (residues 20–35), and HLT2, similar to the positively charged portion of the loop region (residues 24–35) have a significant antibacterial activity and specifically bind to LPS.[90]

bLFcin depolarizes the cytoplasmic membrane of *E. coli* and induces fusion of negatively charged liposomes.[91] When exposed to 30 mg/ml bLFcin, bacterial cells were intact and the content of the cells was still inside. However, even though cells did not appear swollen, a separation of the cell envelope was demonstrable by electron microscopy. In addition, on the bacterial surface were demonstrable blebs containing bilayer structures, extruding from the cytoplasmic membrane, and released to the surroundings of the bacteria. This may represent an additional mechanism by which LF and LF-derived peptides exerts their antibacterial activity.

A synthetic peptide corresponding to the first 33 residues at the N-terminal region of hLF (LF-33) shows neutralizing activity of endotoxin.[92] Anti-endotoxin activity was evaluated by determining the 50% endotoxin-neutralizing concentration (ENC_{50}) in a *Limulus* ELISA, and by evaluating the endotoxin-induced TNF-α secretion by RAW 264.7 cells. The evaluation of ENC_{50} clearly demonstrated the anti-endotoxin activity of LF-33, to a different extent, against lipid A and four different LPS preparations purified from *E. coli*, *Salmonella abortus equi*, *Ps. aeruginosa*, and *N. meningitidis*, respectively. This effect was higher in comparison with that exerted by polymyxin B. A synthetic peptide lacking the first six residues (LF-27) showed 10-fold less potent anti-endotoxin effect or no detectable activity, indicating the importance of these residues in neutralizing endotoxin. LF-33 also inhibited in a dose-dependent fashion the endotoxin-induced TNF-α secretion by RAW 264.7, whereas LF-27 had no effect, confirming the results obtained in the *Limulus* ELISA.[92]

Although the bulk of data on its antimicrobial activity clearly indicates that LF acts as a bacteriostatic/bactericidal agent, the question arises why among the same bacterial species some strains are LF-sensitive whereas others are not. With respect to Gram-negative bacteria, one possible hypothesis, as discussed above, is that the polysaccharide *O*-chain of the LPS somehow protects bacterial cells from LF attack. However, further studies could indicate other putative mechanism(s) by which bacteria may escape LF-mediated killing.

LF also exerts antimicrobial activity against fungi and, in 1971, the inhibition of *Candida albicans* growth by iron-unsaturated LF was demonstrated *in vitro*.[93] In the same work, no differences were found in the distribution of LF in leukocytes from patients with chronic candidiasis or controls; in addition, the mean concentrations of LF in parotid fluids from the two groups were not significantly different.

On the cell-surface, *C. albicans* expresses mannoprotein constituents, consisting of > 90% mannose, 3–5% protein and small amounts of phosphorus, that represent the major fungal antigens and seem to mediate the interaction between the fungus and lymphocytes.[94,95] A mannoprotein fraction, namely MP-F2, which induces lymphoproliferation and production of interleukin (IL)-2 and interferon (IFN)-γ,[95] strongly stimulates LF release from PMNs and enhances PMN ability to inhibit the candidal growth.[96] On the other hand, LF alone, even in the absence of PMNs, inhibits in a dose-dependent fashion candidal growth, and neutralizing anti-LF antibodies reverse such an inhibition, suggesting that LF plays a crucial role in the PMN-mediated anti-*Candida* activity.[96] An N-terminal LF fragment (*i.e.* LFcin) exerts killing activity against *C. albicans* similar to the LF-mediated bactericidal effect.[61] Experiments performed with [^{14}C]-bLFcin have demonstrated a relationship between bLFcin binding rate to *C. albicans* and the extent of killing, clearly indicating that fungicidal effects are related to LF interaction with the cell surface.[97]

Recently, fungicidal activity of LF in combination with antifungal drugs (*e.g.* fluconazole, amphotericin B, and 5-fluorocytosine) has been evaluated *in vitro* against clinical isolates of *Candida.* Synergistic effects against the growth of *Candida* were observed, the most successful combination being LF-fluconazole.[98] Based on these preliminary results, clinical studies are in progress to elucidate better the *in vivo* effect of this combination therapy.

In the last few years, the antiviral effects of LF have also been evaluated and results indicate that several viruses (*e.g.* human immunodeficiency virus [HIV]-1 and HIV-2, herpes simplex virus type 1, influenza virus, respiratory syncitial virus, rotavirus, hepatitis C virus [HCV], and cytomegalovirus) are inhibited *in vitro* by hLF and/or bLF.[99–108] The ability of LF to interact with virus particles seems to be crucial for its antiviral activity. LF strongly binds to the V3 loop region of HIV-1 and HIV-2 gp120 envelope proteins, and shielding of this domain results in the inhibition of virus–cell fusion and entry into the cells.[104] Interestingly, plasma levels of LF are significantly decreased in HIV-1 infected patients compared to controls and such a reduction may play a role in the aetiopathogenesis of AIDS.[109] LF prevents infection of HCV in cultured human hepatocytes (PH5CH8), a cell line susceptible to infection of HCV, by inhibiting viral entry to the cells.[106] Pre-incubation of HCV with LF is required to prevent HCV infection of PH5CH8 cells, whereas pre-incubation of LF with the cells displays no inhibitory effect, thus indicating that anti-HCV activity is strongly related to the HCV–LF interaction.[106] In this respect, LF binds to two HCV envelope proteins, namely E1 and E2, but the LF N-terminal loop region, important for the antibacterial activity, has a little role in the HCV E2-LF interaction.[103] Based on these *in vitro* results, LF has been proposed as a potential candidate for the treatment of patients with chronic hepatitis.

IMMUNOREGULATORY FUNCTIONS AND EFFECTS ON INFLAMMATION

Several effects of LF on immune functions and inflammation have been described, although contrasting results were frequently reported.

In mice, primary antibody response, to either sheep erythrocytes or trinitrophenolate *Brucella abortus*, evaluated utilizing the plaque-forming cells (PFC) assay, was reduced *in vitro* by LF.[110] However, in another report, the humoral immune response to sheep red blood cells, expressed as number of PFC, was increased up to 5 times *in vivo*, when human milk LF was injected in mice at concentrations of 1–10 μg/ml before immunization.[111]

A trivial explanation for this discrepancy could be that results of the former study were obtained *in vitro* and those of the latter *in vivo*. Moreover, in the *in vivo* study, LF was injected into mice 3 h before immunization and it is known that LF is rapidly cleared at the hepatic level. Finally, the inhibition achieved *in vitro* did not exceed 50–60% even in the presence of high LF concentrations and this finding was not sufficiently analyzed by the authors. Therefore, the results reported on the effects of LF on humoral immune response are quite inconclusive and further investigations are needed.

LF also stimulates thymocyte maturation and promotes T-cell differentiation. In fact, following overnight incubation with hLF, $CD4^-CD8^-$ thymocytes acquire the $CD4^+$ phenotype, a phenotype characteristic of T-helper lymphocytes.[111]

LF up-regulates the expression of CD4 antigen in the Jurkat-lymphoblastic T-cells through the activation of a transduction pathway inducing a cascade of phosphorylation of several proteins on their tyrosine residues.[112] In these cells, Lck and Erk2 isoforms of mitogen-activated protein kinase (MAP kinase) are implicated in the up-regulation of CD4 induced by LF.

As far as cytotoxic activities are concerned, contrasting effects of human LF on lymphocyte- and monocyte-mediated cytotoxic activity and antibody-dependent cell-mediated cytotoxicity have been reported.[113,114] Nishiya and Horwitz[113] reported that hLF increased cytotoxic activity against the K562 cell line mediated by monocytes, but it had no effect on lymphocyte-mediated activity. The authors drew such a conclusion from the finding that LF markedly enhanced adherent cell cytotoxic activity, whereas no effect was seen on non-adherent cells at all LF concentrations tested. Later, however, the same authors found that adherent cell preparations were contaminated with up to 6% NK cells[115] and, therefore, they suggested that caution must be paid in attributing all cytotoxic activities in adherent cell preparations to monocytes. On the other hand, more recently, Shau *et al.*[116] documented that NK activity against K562 cells mediated by the lymphocyte fraction of peripheral blood mononuclear cell is significantly enhanced by LF. In the same work, it was also demonstrated that lymphokine-activated killer (LAK) cell activity is strongly increased by LF. Since NK and LAK cells are known to lyse virus-infected cells, antiviral effects of LF could be indirectly mediated by its ability to enhance cytotoxic functions. On the other hand, LF binds to specific DNA sequences and this interaction leads to transcriptional activation.[117] Therefore, some LF-mediated effects at the cellular level may depend, at least in part, on its involvement in communication, signalling, and regulation of gene expression.

It is well known that CpG motifs in bacterial DNA, as well as oligodeoxynucleotides containing these motifs, are potent, direct B-cell activators.[118] Recently, using a human Burkitt lymphoma B-cell line (Ramos cells) it has been demonstrated that both LF and LFcin bound to oligodeoxynucleotides and inhibited their immunostimulatory effects on human B-cells.[119] In particular, LF significantly inhibited both binding and internalization of oligodeoxynucleotides from Ramos cells, whereas TF did not, indicating that this effect was specific. The process was inhibited by high salt concentrations suggesting that LF binding to oligodeoxynucleotides occurs via charge–charge interaction.

LF enhances phagocytic cell function and, in fact, when macrophages or human blood monocytes are co-cultured with intracellular amastigote forms of *Trypanosoma cruzi* in the presence of hLF, both the percentage of phagocytes associating with such intracellular amastigote forms and the number of organisms per 100 host cells significantly increase.[120] Iron does not enhance the uptake of microorganisms by monocytes/macrophages, but it is required to stimulate intracellular killing, as the number of intracellular amastigote forms for 100 cells is not modified by apoLF, whereas it is significantly decreased by 20% or 100% iron saturation of the molecule.[121] LF effects on phagocytic activities are not shared by other iron-binding proteins and, for instance, TF does not increase the capacity of monocytes/macrophages to either take up or kill micro-organisms. This suggests that the mere ability to bind and transport iron is not sufficient for increasing phagocytosis.

LF inhibits mitogen- and allo-antigen-induced lymphocyte proliferation,[122] the IL-1, IL-2, and TNF-α production in mixed lymphocyte reaction and also the release of IL-1 and TNF-α from PBMCs following LPS stimulation.[123] hLF and bLF, as well as bLFcin suppress an *in vitro* IL-6 response in a monocytic cell line (THP-1) when stimulated by LPS.[124] In addition, *in vivo* experiments conducted in a murine model, showed that pretreatment of LPS-injected mice with bLF causes a significant reduction in IL-6 and TNF-α levels.[125]

CD14 is a ~55 kDa glycoprotein present either anchored on the surface membrane (mCD14) of monocytes/ macrophages or as a soluble protein (sCD14) with serum concentrations of 2–6 μg/ml.[126–128] CD14 represents the receptor for LPS molecules, and LPS interactions with mCD14 and Toll-like receptors lead to the release of pro-inflammatory mediators, mainly TNF-α and IL-1β from monocytes/ macrophages.[129–131] In addition, LPS binds to a serum acute phase protein, namely the LPS-binding protein (LBP) and the complex LPS–LBP enhances cell sensitivity to LPS. LBP, especially at low endotoxin concentrations, plays an important role in mediating the biological effects of LPS.[132,133]

On endothelial cells, LPS induces the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM 1) and endothelial-leukocyte adhesion molecule 1 (E-selectin).[134,135] The LPS-mediated activation of human endothelial and epithelial cells is mediated by LBP and sCD14.[134,135]

LF binding to LPS inhibits LPS interaction with mCD14 by competition with LBP, since either LF or LBP interact with the lipid A moiety of LPS.[136] Besides amino acid residues 28–34, another basic cluster (*i.e.* residues 1–5) of the N-terminal region of LF competes with LBP for the binding to LPS.[136]

hLF binds specifically with a high affinity also to sCD14 and this interaction leads to the inhibition of ICAM-1 and E-selectin expression on endothelial cells.[137] The possibility that, besides LF–LPS interaction, LF binding to CD14 may be responsible for the inhibition of pro-inflammatory cytokines cannot be ruled out.

It is well known that, following phagocytic cell activation, hydroxyl radical formation from superoxide and hydrogen peroxide plays a crucial role in the tissue injury associated with inflammation.[138] LF decreases the generation of extracellular hydroxyl radicals by activated human PMNs, since it rapidly chelates iron in a non-catalytic form.[139] On the other hand, the uptake of LF by mononuclear phagocytes inhibits their ability to form hydroxyl radicals and protects them from membrane autoperoxidation.[140] On these grounds, a protective function of LF at the site of inflammation could be hypothesized. However, the cleavage of iron-loaded LF by proteases (enzymes present at high concentrations at inflammatory sites) yields pro-oxidant compounds that increase the damage resulting from O_2^- and H_2O_2 exposure.[141] These data suggest that interaction between LF and phagocytes may have contrasting effects, *i.e.* both anti- and pro-oxidant consequences. One can hypothesize that biological conditions may differently direct LF functions. In this respect, although in free radical biology LF acts as an antioxidant agent, under conditions of high protease activity LF could contribute to oxidant-mediated tissue injury.

LF has no effects on the activation of the very early complement components (C1 and C4) but inhibits C3 deposition on immune complexes, important for the solubilization of immune precipitates.[142,143] Thus, it is possible that LF exerts its anti-inflammatory function also by modulating the complement cascade activation.

Another putative mechanism by which LF may exert an anti-inflammatory function is via its effect on IL-8 production.[144] IL-8 is a chemokine secreted from many cells, including endothelial cells, in response to various stimuli, such as LPS and pro-inflammatory cytokines.[145,146] IL-8 binds to cell surface proteoglycans present on the endothelium and this binding increases the local concentration of IL-8, which, in turn, initiates both activation and recruitment of leukocytes at sites of inflammation.[147] hLF decreases both IL-8 mRNA and protein expression induced by LPS/sCD14 in human umbilical vein endothelial cells (HUVECs).[144] Experiments performed with mutated hLF have documented that hLF variants lacking residues 1–5 or changed at residues 28–34 are not effective in the inhibition of IL-8 production by HUVECs, thus strengthening the importance of these residues in LF biological functions.[144] These results provide evidence that anti-inflammatory activity of LF is, at least in part, mediated by its ability to inhibit IL-8 expression on endothelial cells, thus reducing the recruitment of leukocytes at sites of inflammation.

POTENTIAL THERAPEUTIC APPROACH WITH LACTOFERRIN

As a result of the different physiological roles exerted by LF, it was conceivable to test its potential therapeutic applications in different diseases. The very short half-life of LF, which is cleared from circulation within a few minutes (in rats more than 50% is cleared after 10 min),[59] may represent a limitation to its therapeutic use. Nevertheless, several reports, almost exclusively conducted in animal models, suggest a therapeutic potential for this molecule.

First experiments *in vivo* were performed in 1972 by Bullen *et al.*,[148] who evaluated, in an animal model, the capacity of milk to induce resistance to bacterial infection in infants. To this end, newly born guinea-pigs were dosed orally with 10^6 *E. coli* O111 cells. Then, suckled animals were kept with their mothers, whereas artificially fed animals were separated immediately from their mothers. The animals were followed-up for 6 days and it was clearly demonstrated that in artificially fed guinea-pigs the numbers of *E. coli* O111 cells in both the small and the large intestine were significantly greater than those in suckled animals. In the same study, evidence was provided that milk also exerted *in vitro* a powerful bacteriostatic effect on the same bacteria. Although there was no conclusive evidence for a direct role of LF, the authors suggested that it was involved in bacteriostasis, presumably acting in concert with specific *E. coli* antibodies.[148] In a similar way, results of a study conducted in humans have documented that breast feeding of infants exerts a protective effect against infection[149] and babies who were breast fed for 13 weeks or more had significantly less gastrointestinal illness than those who were bottle fed. However, the LF-mediated protective effect seemed to be restricted to gastrointestinal tract infections, since no protection of breast feeding was observed against ear, mouth, or skin infections.

In a murine model, mice were administered intravenously with bLF 24 h before a challenge with a lethal dose of *E. coli.* About 70% of animals treated with bLF survived challenge, whereas in the control groups (*i.e.* mice treated with *E. coli* alone or mice treated with bovine serum albumin) the survival rates were 4% and 8%, respectively.[150]

LF exerts its antibacterial activity *in vivo* against different bacteria, including *Clostridia*, as demonstrated in

another murine model. In mice orally inoculated with *Clostridium* spp. (*Cl. ramosum*, *Cl. paraputrificum*, and *Cl. perfringens*), the administration of bLF suppressed bacterial proliferation.[151] The bacteriostatic rate was dependent on the concentration of bLF, as well as the duration of feeding. In addition, a hydrolysate, obtained by digestion of bLF with porcine pepsin, showed the same inhibitory effect on *Clostridium* proliferation as observed with undigested bLF.[151] Of interest, supplementation of diet with bLF and pepsin-generated hydrolysate of bLF results in significant suppression of bacterial translocation from intestine to the mesenteric lymph nodes.[152]

The effects of LF on gastritis induced by *Helicobacter* spp. has been evaluated in mice infected with *Helicobacter felis*, which causes a hypertrophic gastropathy with inflammatory cell infiltration. In these animals, the stomach is significantly enlarged after infection and the enlargement is positively correlated to the degree of inflammation. *H. felis*-infected mice were treated by daily oral administration of either control proteins or recombinant hLF, metronidazole, tetracycline, or combinations of LF plus metronidazole or tetracycline. Only the combination of LF and tetracycline reduced significantly the infected stomach size, indicating that combination of LF with tetracycline enhances the therapeutic effectiveness of the antibiotics.[153] In humans, only one study was reported on the treatment of healthy *H. pylori*-infected volunteers with recombinant hLF. Results are quite disappointing since no adverse events occurred, which indicates the safety of LF administration, but the treatment was ineffective.[154]

The antibacterial and anti-inflammatory properties of LF were investigated with an animal model of experimental urinary tract infection.[155] At 30 min after instillation of 10^8 *E. coli* bacteria (strain O6:K5:H) into the urinary bladder of C3H/Tif and C3H/HeN mice, bLF, hLF, or synthetic peptide sequences from the antibacterial region of hLF were given orally. One day after inoculation with bacteria, the numbers of micro-organisms present in the bladder and kidneys of the hLF- and bLF-treated mice were significantly lower when compared to those in control groups and a notable percentage of hLF-treated mice had no detectable *E. coli* in the kidneys. In addition, the levels of PMNs and IL-6 in the urine, used as parameters of the inflammatory response, were significantly reduced in LF-treated mice. The results of this work not only pointed out that hLF was more effective as an antibacterial and anti-inflammatory agent than bLF, but also demonstrated that LF exerts its therapeutic effects even at mucosal sites remote to the intestinal tract.

It is well known that septic shock is associated with the release of endotoxin from Gram-negative bacteria into the circulation.[156] On this basis, patients with Gram-negative bacteraemia and septic shock have been treated with encouraging results with a human mAb against endotoxin in a randomized, double blind trial.[157]

Therefore, LPS could be a target for treating septic shock, which represents an important cause of morbidity and mortality among hospitalized patients.

As discussed above, LF is able to bind to the lipid A moiety of the LPS molecule, thus reducing the noxious biological effects of endotoxins. The intravenous administrations of bLF to mice 24 h before a challenge with a lethal dose of *E. coli* protects animals as indicated by the finding that ~70% of mice pretreated with bLF survived after challenge, whereas the survival rates in control mice treated with *E. coli* alone and pretreated with BSA were 4% and 8%, respectively. hLF showed the same protective effect as bLF.[125]

In another animal model of septic shock, the galactosamine-sensitized mouse model, the effect of the synthetic LF-derived peptide LF-33 was investigated.[92] The injection of small amounts of LF-33 (2.5 mg/animal) simultaneously with 125 ng of *E. coli* LPS reduced the lethality from 93% to 6%. LF-33 also significantly reduced the lethality when injected i.v. 10 min subsequent to the i.p. injection of endotoxin. This protective effect was correlated with the reduction of the TNF-α serum levels. These results indicate that, in addition to the *in vitro* anti-endotoxin activity, LF-33 also acts *in vivo* neutralizing the detrimental biological activity of LPS.

The effects of LF feeding against septic shock induced by intravenously administered endotoxins were evaluated in germ-free piglets.[158] Results have documented that LF prefeeding was associated with a significant decrease in mortality in comparison with the control group, represented by animals fed BSA. These results are the first evidence that oral administration of LF may protect from septic shock.

More recently, the endotoxin inactivation by orally administered LF-enriched colostrums or LF alone was demonstrated in rats in which endotoxaemia was induced by enteral application of *E. coli*.[159] In control animals, as well as in animals treated with casein-enriched colostrum, endotoxin values markedly increased after 5 h, whereas the administration of LF-enriched colostrum and, mostly, LF alone decreased endotoxin values more than 50%.

Finally, the capacity of LF to modify articular inflammation was recently studied in murine models of autoimmune and septic arthritis.[160] To this end, collagen arthritis and *Staph. aureus* septic arthritis was induced in DBA/1 mice and Swiss mice, respectively. Then, inflamed joints were injected peri-articularly with hLF and arthritis was monitored for 3 days. In DBA/1 mice, the results were compared with those obtained in mice treated with corticosteroids or in the control group injected with saline. DBA/1 mice injected with hLF showed significant reduction of local inflammation for up to 3 days, achieving 71% of the effect of corticosteroid. Peri-articular injection of [^{125}I]-LF documented that 25% of LF was retained in paws after 6 h. Since

serum levels of IL-6 were not significantly affected, a predominant local anti-inflammatory effect was suggested. Peri-articular administration of LF also suppressed significantly paw inflammation in *Staph. aureus*-infected Swiss mice and did not enhance bacterial survival. Thus, since LF is a protein without reported toxic effects, its therapeutic activity in human arthritis could be evaluated.

Acknowledgements

The authors' work reported in this paper was supported in part by grants from Ministero della Salute, MIUR, and AIRC, Italy.

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