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Immunoregulatory role of lactoferrin-lipopolysaccharide interactions

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Abstract Lactoferrin (Lf) is a mammalian exclusive protein widely distributed in milk and exocrine secretions exhibiting multifunctional properties. Many of the proven or proposed functions of Lf, apart from its iron binding activity, depend on its capacity to bind to other macromolecules. Lf can bind and sequester lipopolysaccharide (LPS), thus preventing pro-inflammatory pathway activation, sepsis and tissue damage. However, the interplay between Lf and LPS is complex, and may result in different outcomes, including both suppression of the inflammatory response and immune activation. These findings are critically relevant in the development of Lf-based therapeutic interventions in humans. Understanding the molecular basis and functional consequences of Lf-LPS interaction will provide insights for determining its role in health and disease.

Keywords Lactoferrin · Lipopolysaccharide · Inflammation · Immune response

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Introduction

Lactoferrin (Lf), a member of the transferrin family, is an 80 kDa iron-binding glycoprotein abundantly found in exocrine secretions of mammals, in particular in milk and fluids of the digestive tract, constitutively released by mucosal epithelia and by neutrophils upon inflammation (Legrand et al. 2008). Lf is a key element in the host defence system (Legrand et al. 2005; Valenti and Antonini 2005; Ward et al. 2002). It is now well recognized that this molecule plays a direct antimicrobial role in secretions and at the surface of epithelia, by limiting the proliferation and adhesion of microbes and/or by killing them (Valenti and Antonini 2005). These properties are mainly related to its ability to sequester iron in biological fluids or to destabilize the membranes of microorganisms. However, iron-independent microbicidal activities, requiring direct interaction between Lf and microbial surface components, have been subsequently demonstrated (Legrand et al. 2008). In addition to the antimicrobial properties, Lf ability to modulate the overall immune response and to protect against viral infections and septic shock have been largely described (Legrand et al. 2005; Valenti and Antonini 2005; Ward et al. 2002). In this respect, it is noteworthy that Lf concentrations are elevated locally in inflammatory disorders including neurodegenerative diseases (Kawamata et al. 1993), inflammatory bowel disease (Uchida et al. 1994), arthritis (Decoteau et al. 1972),

and allergic inflammation (van de Graaf et al. 1991). Although the cellular and molecular mechanisms accounting for the immunomodulatory effects of Lf are far from being fully elucidated, both in vitro and in vivo studies suggest the existence of multiple mechanisms that include modulation of cytokine/chemokine production, regulation of reactive oxygen species production, and of immune cell recruitment. It is now clear that at least some of the Lf biological activities do not merely depend on its iron-binding capacity, but may arise from its interaction with a variety of molecules. In this respect, the capacity of Lf to influence either negatively or positively cytokine production relies, at least in part, on its ability to bind and sequester both lipopolysaccharide (LPS) and its receptor CD14, as well as CpG bacterial DNA, thus preventing the downstream activation of pro-inflammatory pathways, septic shock and tissue damage (Appelmelk et al. 1994; Baveye et al. 2000a; Baveye et al. 2000b; Britigan et al. 2001). The outcome of Gram-negative infections is dependent not only by an individual's ability to recognize endotoxin and respond to its presence but also by numerous phenomena that inactivate endotoxin and/or prevent harmful reactions to it (Munford 2005). Until now, many detoxification mechanisms have been described acting in different body compartments, including proteins that facilitate LPS sequestration or prevent endotoxin interaction with its receptors. In this respect, Lf represents one of the most efficacious mechanisms of LPS neutralization, both in tissues and secretions, activated by the innate response in peripheral tissues during the inflammatory processes.

Lf interaction with LPS: molecular basis

Lf is a monomeric highly cationic (pI 8.4–9.0) glycoprotein with a single polypeptide chain of about 690 amino acid residues. Crystallographic analysis of Lf from different species revealed a highly conserved three-dimensional structure, but with differences in detail between species (Baker and Baker 2005).

Recently, Lf has been described as a molecule with a double face, composed by an internal portion, highly conserved between species and endowed with iron binding capacity, and an external surface strongly cationic and prone to interact with a number of negatively charged macromolecules. Although belonging to the transferrin family of proteins, some key properties differentiate Lf from other transferrins. The first is the biological location. Transferrin, as an iron delivery protein, is mainly located in the bloodstream while Lf is found mostly in exocrine secretions. Differently from transferrin, Lf retains iron binding capacity at low pH ($\sim$4), typically observed at infection and inflammation sites (Valenti and Antonini 2005). Moreover, while in intestinal epithelial cells the transferrin localization is restricted to the cytoplasm upon internalization, Lf can be also found into the nucleus (Ashida et al. 2004). Lastly, the highly cationic nature represents a defining feature for Lf. The positive surface charge is mainly concentrated in three distinct regions: at the N-terminus (1–7 amino acid), in the first helix (13–30 amino acid) and in the region that connects the two lobes. This property is crucial for its bactericidal ability, mediated through its lactoferricin (Lfcin) domain (Bellamy et al. 1992), and it is likely critical also for many of its demonstrated binding properties to cell-surface molecules such as glycosaminoglycans (Mann et al. 1994; Wu et al. 1995), to bacterial surface molecules (Senkovich et al. 2007; Valenti and Antonini 2005) and LPS (Appelmelk et al. 1994; Elass-Rochard et al. 1995; van Berkel et al. 1997).

LPS, a major constituent of the Gram-negative bacteria outer membrane, is one of the most potent inducer of the innate immune response. Recognition of various form of LPS from different strains of Gram-negative bacteria triggers a signaling cascade that results in the release of pro-inflammatory mediators, such as cytokines and chemokines, as well as small molecules, such as lipid mediators and reactive oxygen species (Beutler and Rietschel 2003). LPS is known to initiate the morbidity and mortality associated with Gram-negative sepsis, as well as to modulate a myriad other host innate inflammatory responses. Specifically, LPS has been characterized as the 'prototypical stimuli' for host activation through myeloid cells (neutrophils, monocytes, macrophages, dendritic cells) and non-myeloid cells (fibroblasts, platelets), as well as other innate host defense mechanisms, such as serum complement, and specific components within the intrinsic coagulation pathway (Dixon and Darveau 2005).

LPS is ubiquitous within our environment, in vivo and in vitro, and can express potent bioactivity in extremely small amounts (Westphal et al. 1981). This

bacterial component is a complex molecule consisting of three parts: a core oligosaccharide, a distal hydrophilic O side chain, and a highly conserved lipid A portion (Raetz and Whitfield 2002). The lipid A moiety is the main pathogen-associated molecular pattern of LPS, and is responsible for its toxic pro-inflammatory properties.

Structurally, Lf contains a highly basic arginin-rich region close to the N-terminus which binds to a variety of anionic biological molecules (Appelmelk et al. 1994; Britigan et al. 2001; Mann et al. 1994; Wu et al. 1995). Studies carried out with natural human Lf (hLf) and N-terminally deleted hLf variants provided evidence for the essential role of the N-terminal penultimate stretch of four arginine residues (Arg^2-Arg^3-Arg^4-Arg^5) in hLf interaction with physiologically relevant ligands, including polyanions such as heparin, lipid A and DNA, and human lisozyme (van Berkel et al. 1997). Iron-saturated and native hLf bound with identical affinities to these molecules (van Berkel et al. 1997). Conversely, by side-directed mutagenesis of the second basic cluster (Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}), Ellas-Rochard and co-workers suggested that hLf binding to *Escherichia Coli* LPS 055B5 is mediated by this region (Elass-Rochard et al. 1995). In addition to a high affinity (K_d 3.6 ± 1 nM) site located at the N-terminus, these authors also found a low-affinity (K_d 390 ± 20 nM) LPS-binding site located on the C-lobe of hLf, which is exposed at high protein concentrations (Elass-Rochard et al. 1995).

The formation of Lf-LPS complexes occurs through electrostatic interactions. It was shown that Lf binds to the phosphate group within the lipid A moiety inducing rigidification of the acyl chain of LPS. The secondary structure of Lf was, however, not changed (Brandenburg et al. 2001). Binding saturation was found to lie at a [Lf]:[Lipid A] ratio of 1:3–1:5 M and promotes the conversion of the molecular shape of lipid A from a conical form (active) into a cylindrical form (inactive), in keeping with previous studies suggesting that the conical shape of lipid A is a prerequisite for its endotoxic activity (Brandenburg et al. 1997; Schromm et al. 2000). Lf was shown to intercalate into phospholipid liposomes and to block the LBP-mediated intercalation of LPS, suggesting that conversion from an active to inactive form occurs at the plasma membrane level (Brandenburg et al. 2001).

LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and Toll-like receptor 4 (TLR4) (Lu et al. 2008). LBP is a 60 kDa acute-phase serum protein which directly binds to LPS and facilitates the association between LPS and CD14. Since the discovery of the LBP/CD14 host activation pathway (Schumann et al. 1990; Wright et al. 1990) it has become increasingly clear that the release of numerous inflammatory mediators and the expression of cell adhesion molecules necessary in sustaining inflammation occur in response to LBP and/or CD14 complexed with microbial components (Pugin et al. 1993; Wright 1995). Of note, it has been reported that hLf binds specifically and with a high affinity to sCD14. Affinity chromatography studies showed that hLf interacts not only with free sCD14 but also, though with different binding properties, with sCD14 complexed to LPS (Baveye et al. 2000a). Lastly, evidence has been provided that hLf also prevents the LBP-mediated binding of LPS to the CD14 receptor. Maximal inhibition of LPS interaction with the cell occurred when hLf and LBP were simultaneously added or pre-incubated together prior to their addition to the cultures, but not when hLf was added after LBP interaction with LPS has occurred. These results suggest that hLf competes with LBP for LPS binding, and this competition negatively affects the subsequent interaction of LPS with CD14 (Elass-Rochard et al. 1998).

Lf-derived peptides

The overall three-dimensional structure of Lf is very similar to that of the other member of the transferrin family except for a unique, highly positively charged N-terminal region. The N-terminus of Lf from human and other mammals contains cationic bioactive peptides, collectively known as lactoferricins (Lfcins). These Lf-derived peptides are released in the stomach and mucosal secretions by gastric pepsin action (Kuwata et al. 1998), as well as at the site of infection by either bacterial or mammalian proteases (Bellamy et al. 1992). The composition of Lfcins is characterized by a relatively large proportion of basic amino acids and a number of hydrophobic residues, especially tryptophan, that render them uniquely

suitable for interacting with LPS and disrupting the membrane structure. Lactoferricin peptides, LfcinB and LfcinH, derived from bovine lactoferrin (bLf) and hLf, respectively, not only retain many of the biological activities of the intact protein but in some cases, they can exert even more potent effects than the native molecule (Gifford et al. 2005). Although the exact mechanisms of this action remain elusive, it is thought that an electrostatic attraction first binds the cationic peptide to the outside of the bacterial cell. Support to these proposed initial binding sites has been provided by the fact that both Lfcins have the ability to bind and release LPS from the outer membrane of Gram-negative bacteria (Chapple et al. 1998, Yamauchi et al. 1993), and LfcinB can bind teichoic acid from Gram-positive bacteria (Vorland et al. 1999). Following its binding to the outer lipid layer of bacteria, Lfcin then crosses this barrier to interact with intracellular targets. Both Lfcins induce a compromise in bacterial cytoplasmic membrane permeability, allowing the passage of small ions, and resulting in the loss of both the transmembrane electrochemical and pH gradients (Gifford et al. 2005). Studies also indicate that LfcinB is capable of inhibiting macromolecular synthesis in both Gram-negative and -positive bacteria (Ulvatne et al. 2004). Recent results show that the Lfcin bactericidal domain, exposed at the Lf surface, can also serve as a macromolecular binding surface. By crystallographic analysis it has been shown that Lfcin domain of hLf specifically binds the pneumococcal surface protein A (Senkovich et al. 2007). Since this protein is a major virulence factor, its interaction with Lfcin represents a clear-cut example of the importance of this region in the antibacterial activity of Lf.

Role of glycosylation in the regulation of LPS-induced immune responses

Growing evidence indicates that many pathogens depend on binding to glycans for pathogenesis. Thus, glycosylation patterns of Lf may likely represent an important determinant to understanding the molecular basis of Lf various activities. Lf, as many eukaryotic proteins, is modified by N-linked glycosylation by enzymatically catalysed processes. Some differences among species are observed, having hLf three and bLf five potential N-glycosylation sites (Haridas et al. 1995; Moore et al. 1997). Studies characterizing the Lf structure have demonstrated that only a part of these potential sites are usually glycosylated, two in hLf and four in bLf (Spik et al. 1988; van Berkel et al. 1996; Wei et al. 2001). In particular, it has been reported that differential utilization of these sites may result in distinct glycosylation variants leading to heterogeneity in linked glycan chains (Derisbourg et al. 1990; Samyn-Petit et al. 2003; Spik et al. 1988; Spik et al. 1982; van Berkel et al. 1996). Indeed, N-glycan chains of milk-derived hLf contain a variable number of sialic acid residues, and the external N-acetylglucosamine can be fucosylated. Unlike the milk-derived Lf, hLf isolated from polymorphonuclear leucocytes lacks fucose residues and this seemingly predisposes this form to transduce certain signals that do not require fucose-specific receptors such as mannose receptor (MR) and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), exhibiting equal affinity to fucose and mannose (Derisbourg et al. 1990; Legrand et al. 1995; Weis et al. 1998). Conversely, bLf protein is linked with complex and high-mannose-type glycans. These glycans are structurally heterogeneous and present at four N-linked glycosylation sites, which are linked with high-mannose-type glycans only or with both complex and high-mannose types (Coddeville et al. 1992; Wei et al. 2001). On the basis of the glycosylation pattern, two different bLf isoforms, referred to as bLf-a and bLf-b, have been isolated from normal milk (Wei et al. 2001). Interestingly, the concentration of bLf-a, which has a higher molecular mass than bLf-b, in colostrum is greater than in normal milk. As bLf-a was found to exhibit a stronger bactericidal activity against *Escherichia coli* than bLf-b, it was suggested that bLf-a may play an important healthy role in colostrum drinking calves (Yoshida et al. 2000). Lastly, the presence of mannose-type glycans in bLf molecule has been described to be responsible for its capacity to induce mannose receptor-dependent delayed type hypersensitivity (DTH) response to ovalbumin (OVA) in mice (Kocieba et al. 2002; Zimecki et al. 2002).

Although glycosylation has no influence on folding in the majority of the Lf species (Baker and Baker 2009), most of the glycosylation sites are exposed on the external surface of the molecule and have been supposed to play a role in Lf interaction with viruses

(Valenti and Antonini 2005), toxins (Chung et al. 2007), sialic acid-binding immunoglobulin superfamily lectins (Choi et al. 2008) and C-type lectin receptors on immune cells (Groot et al. 2005; Zimecki et al. 2002). However, no evidence for the direct involvement of glysosylation in Lf binding to LPS has been provided yet. In this respect, studies with enzymatically deglycosylated hLf did not reveal any role of glycosylation in Lf binding to iron (van Berkel et al. 1995), human intestinal receptors (Kawakami and Lonnerdal 1991), bacterial receptors (Alcantara et al. 1992), and LPS (van Berkel et al. 1995). However, unglycosylated recombinant hLF was found to be much more susceptible to tryptic proteolysis (van Berkel et al. 1995).

Although glycosylation does not apparently play any role in Lf binding to LPS, it cannot be excluded that it may influence some endotoxin-induced immune responses. Some authors reported that the capability of Lf in reducing the effector phase of DTH to sheep red blood cells (SRBC) and in stimulating DTH to OVA in mice is dependent on MR as these effects are potently inhibited by methyl-α-D-mannopyranoside (MMan) but not by galactose. In this respect, it is of interest that bLf and hLf, which differ in terms of sugar composition relevant in recognition by MR, exhibit different activities in the selected tests (Zimecki et al. 2002), with bLf showing a higher activity than hLf. Similarly, bLf stably complexed with monophosphoryl lipid A (MLP) derived from the Gram-negative bacteria *Hafnia alvei*, effectively provides adjuvant activity to the immune response to SRBC and OVA in treated mice (Chodaczek et al. 2006). Interestingly, the adjuvant action of bLF was inhibited by co-administration of mannose-bovine serum albumin (BSA) or mannose, but not by galactose-BSA or galactose (Chodaczek et al. 2006). Although the direct Lf binding to C-type lectin receptors has been demonstrated for DC-SIGN (Groot et al. 2005) but not for MR, we cannot exclude the possibility that some Lf inhibitory effects could be due to its activation of this class of receptors. Interestingly, Nigou and colleagues demonstrated that purified mannose-capped lipoarabinomannans (MannLAMs) derived from *Mycobacterium bovis* bacillus Calmette-Guérin and *Mycobacterium tuberculosis* are able to inhibit IL-12 production by human dendritic cells (DCs) stimulated with LPS, and that the anti-inflammatory effects of MannLAM are mediated by their binding to the MR (Nigou et al. 2001). Moreover, Pathak and co-workers have reported that LPS-induced IL-12 p40 expression can be inhibited by MannLAM in a murine macrophage cell line by a mechanism IL-10-independent that involves the expression of IRAK-M, a negative regulator of TLR signalling (Pathak et al. 2005).

BLf inhibits HIV entry in human DCs by binding to the C-type lectin receptor DC-SIGN. Indeed, bLf is a markedly stronger inhibitor of virus transmission than hLf (Groot et al. 2005). Interestingly, MannLAM binding to DC-SIGN has been implicated in mycobacteria infection and suppression of immune functions in DCs (Geijtenbeek et al. 2003). In particular, MannLAM binding to DC-SIGN prevents mycobacteria- or LPS-induced DC maturation, thus suggesting that DC-SIGN, upon binding of MannLAM, interferes with TLR-mediated signals (Geijtenbeek et al. 2003). Indeed, interaction of different pathogens with DC-SIGN activates the Raf-1-acetylation-dependent signalling pathway to modulate signals triggered by different TLRs (Gringhuis et al. 2007). These findings are not only consisting with Lf recognition of MR and DC-SIGN, but also suggest that binding of Lf to C-type lectin receptors may be necessary for at least some Lf-dependent immune functions.

All together these results suggest that Lf, particularly the bovine form, can contrast LPS-induced inflammation by acting through multiple mechanisms involving specific receptors on cell targets other than direct binding to LPS.

Lf-mediated anti-inflammatory mechanisms

Until now, many detoxification mechanisms have been described acting in different body compartments, including proteins that facilitate LPS sequestration or prevent endotoxin interaction with its receptors. In this respect, Lf represents one of the most efficacious mechanisms of LPS neutralization, both in tissues and secretions, activated by the innate response in peripheral tissues during the inflammatory processes.

Several in vitro and in vivo studies have demonstrated that Lf can inhibit, in a concentration- and time-dependent manner, a number of LPS-induced effects. In this respect, it has been reported that Lf counteracts the ability of LPS to prime human

neutrophils for enhanced superoxide formation (Cohen et al. 1992). Furthermore, hLf, at LPS serum concentrations observed in pathological conditions, blocks the LPS-induced production of oxygen free radicals by competing with L-selectin, a serum-independent LPS receptor in neutrophils, for LPS binding (Baveye et al. 2000b). In keeping with these results and with the anti-inflammatory activity of Lf, it has been shown that the LPS-triggered release of IL-1, IL-6 and TNF-α in monocytes is inhibited in the presence of bLf, hLf and LfcinB (Choe and Lee 1999; Crouch et al. 1992; Haversen et al. 2002; Mattsby-Baltzer et al. 1996). Furthermore, it has been reported that hLf down-modulates the LPS-induced expression of some adhesion molecules, i.e., ICAM-1 and E-selectin, in endothelial cells (Baveye et al. 2000a). This effect was shown to rely on the hLf capacity to bind specifically and with high affinity to sCD14 and to LPS-CD14 complexes. This observation suggested that Lf can modulate the recruitment of immune cells to inflammatory sites by down-regulating the adhesion of leukocytes to endothelial cells. In keeping with this hypothesis, it has been reported that hLf inhibits LPS-induced expression of IL-8, and competes with this chemokine for its binding to proteoglycans of endothelial cells (Elass et al. 2002).

The capacity of Lf to modulate the LPS-induced inflammatory process has been also well documented in vivo. Indeed, several studies (Kruzel et al. 2000; Lee et al. 1998; Talukder and Harada 2007; Yajima et al. 2005; Zagulski et al. 1989; Zhang et al. 1999) have demonstrated that hLf, bLf and Lfcin-derived peptides administration protects animals against sub-lethal doses of LPS. Interestingly, the optimal protection against induced septicaemia required a 12–24 h pre-injection of hLf and bLf, suggesting that this protein may act by other mechanisms than simple LPS scavenging (Zagulski et al. 1989). Growing evidence indicates that progression of systemic inflammatory response syndrome into sepsis is due to the cellular damage and death induced by acute inflammatory response. In this respect, Kruzel and colleagues have recently reported that hLf protects against oxidative stress-induced mitochondrial dysfunction and DNA damage, both in cell culture and within an animal model of endotoxemia (Kruzel et al. 2009). In keeping with the anti-inflammatory effects of Lf observed in in vitro studies, serum levels of LPS-induced pro-inflammatory factors such as IL-6, TNF-α and nitric oxide were found significantly reduced in animals treated with hLf and bLf, in comparison with untreated controls after LPS inoculation (Artym et al. 2004; Hayashida et al. 2004; Kruzel et al. 2002; Machnicki et al. 1993). The protective role of Lf in LPS-induced pathologies has been further evaluated in other disease models. Indeed, mice were protected by hLf from hepatitis (Yamaguchi et al. 2001), by bLf from arthritis (Hayashida et al. 2004) and diarrhoea (Talukder and Harada 2007), and by both Lfs against preterm delivery (Mitsuhashi et al. 2000; Otsuki et al. 2005; Sasaki et al. 2004) after LPS challenge. The exact mechanisms by which Lf exerts preventive and/or therapeutic potential are not yet known, however, some of the effects could be due to Lf intrinsic capacity to bind LPS or to subvert LPS triggered pathways.

Role of Lf-bound LPS in immune activation

Despite the well recognized activity of Lf as a powerful scavenger of endotoxins, some studies documented that Lf-bound LPS retains the capacity to stimulate mouse and human cells. Lf binds to the lipid A portion of LPS via charge-charge interaction. The portion of Lf that binds to anionic molecules, including lipid A, is limited to its N-terminus arginine rich domain (van Berkel et al. 1997). Thus, it is likely that bound LPS can still expose the unbound part of lipid A that is recognized by LPS receptors such as TLR4. Such a Lf-LPS complex recognition would then result in macrophage activation. In this regard, it has been reported that Lf-bound LPS retains clotting capacity in a conventional Limulus assay (LAL), the standard method for detection of endotoxin contamination (Brandenburg et al. 2001). Of note, the lipid A backbone is also the epitope being recognized in this assay, thus explaining why the Lf-LPS complex is found to be LAL positive (Brandenburg et al. 2001, Na et al. 2004). Collectively, these results suggest that lipid A can be recognized even after Lf-LPS complex has been formed, and that this complex retains the capacity to activate macrophages. In keeping with this assumption, it has been reported that Lf-LPS complexes can still prime human monocytes and stimulate B lymphocyte proliferation (Wang et al. 1995). Furthermore, Na and co-workers reported that when LPS and purified Lf were mixed, and formed a

complex, induction of pro-inflammatory mediators or tolerance, rather than inhibition of LPS challenge, were observed in RAW 264.7 cells and peritoneal macrophages harvested from C3H/HeN mice (Na et al. 2004). Comparative studies carried out with LPS responsive and LPS hypo-responsive mice demonstrated a strong dependency of the Lf-LPS complex triggered signals on TLR4, leading to the conclusion that the immunomodulatory properties of Lf could be due, at least in part, to LPS binding (Na et al. 2004).

Despite these observations, the intimate relationship between Lf and LPS does not completely account for the different biological activities ascribed to this molecule. In keeping with these results, we have reported that the capacity of Lf to induce a type I IFN mediated antiviral state, but not TNF-α production, relies on the function of TLR4 in responding cells (Puddu et al. 2007). Our results showing that TLR4 is not essential for Lf-induced production of TNF-α by murine peritoneal macrophages, strongly suggest that this molecule induces macrophage activation via TLR4-dependent and -independent mechanisms. Accordingly, it has been recently reported that Lf-induced IL-6 secretion and CD40 expression in murine peritoneal macrophages were achieved via TLR4-independent and -dependent mechanisms, respectively, thus indicating potentially separate pathways for Lf-mediated macrophage events in innate immunity (Curran et al. 2006). Likewise, a dichotomous nature of Lf binding to monocyte/macrophage-differentiated HL-60 cells, one being mediated by specific Lf receptors whereas the other occurring mainly via LPS receptors after formation of Lf-LPS complexes, was also reported (Miyazawa et al. 1991). In addition, as reported above, Lf-MLP complexes stimulated the humoral immune response to OVA and SRBC in mice, that resulted significantly weaker when both components were inoculated separately (Nigou et al. 2001).

Thus, Lf binding to LPS may represent an important aspect, but does not entirely account for all immunomodulatory effects of this molecule. This

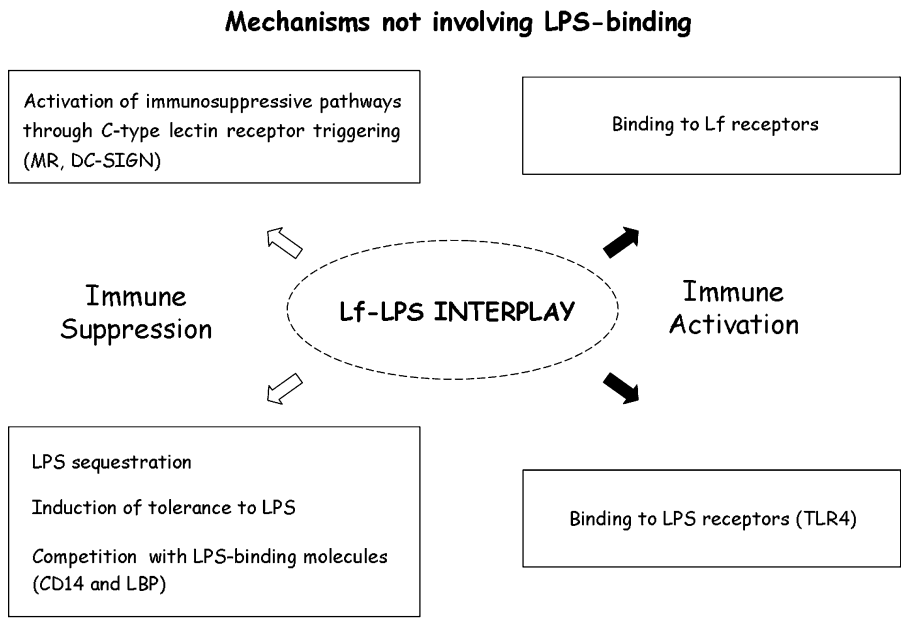

Fig. 1 Lf interplay with LPS: role in the regulation of the immune response. A schematic representation, highlighting the behaviour of Lf in both suppression and promotion of the immune response by means of different mechanisms involving or not LPS binding, is shown. Lf is able to mediate the immune suppression through different mechanisms involving sequestration of free endotoxin, induction of tolerance to LPS, and competition with other LPS binding molecules. Lf could play the same biological function also by directly binding C-type lectin receptors with a consequent triggering of the immunosuppressive signalling cascade. Furthermore the immune response could be activated through mechanisms based on the engagement of TLR4 by Lf-LPS complexes or the binding of Lf to its own receptors on cellular surface

aspect could be mostly relevant in those cell types, such as the macrophages, in which TLR4 function is of critical importance in the regulation of their activity.

Concluding remarks

Lf is a first-line defence protein involved in protection against a multitude of microbial infections, that dampens systemic inflammation. In this respect, it represents one of the most efficacious mechanism activated by the innate response to neutralize LPS. However, this molecule behaves as a factotum molecule by efficiently suppressing endotoxin-induced excessive immune reaction in sepsis or promoting, in particular conditions, a protective response that keeps the host alerted against pathogen insult. The pleiotropic action of this molecules is mostly due to its strongly cationic nature that allows its binding to a plethora of molecules ubiquitously expressed on different cell targets. As schematically represented in Fig. 1, Lf, exploiting its versatile nature, can suppress inflammation by different mechanisms involving or not direct LPS binding. The intrinsic properties of Lf to specifically bind LPS as well as to interact with host molecules involved in endotoxin sensing represent a mechanism to avoid excessive host's response to LPS challenge. In this respect, Lf can counteract LPS toxicity by acting through different strategies including scavenging of free endotoxin, Lf-LPS complex induced tolerance to endotoxin, other than competition with LPS binding molecules. In addition, a different scenario involving activation of immunosuppressive pathways through C-type lectin receptor engagement is starting to emerge as a critical event in the Lf-mediated suppression of LPS-induced inflammation that needs further investigation. On the other hand, the Lf-LPS interplay may lead to a completely different outcome involving or not the engagement of TLR4 by Lf-LPS complexes. In this scenario, immune activation can be mediated by Lf-bound lipid A in TLR4-dependent manner or/and by Lf itself through TLR4-independent pathways.

Lf is a versatile molecule shaped by nature to defend the mammals from external aggression. Its capacity to exert a dual role in the regulation of the immune response by either suppressing inflammation or inducing immune activation renders this molecule a promising candidate in the development of novel, side-effect free therapeutic strategies to fight immune-mediated disorders.

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