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A critical review of the roles of host lactoferrin in immunity

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Abstract Lactoferrin (Lf) is an essential element of innate immunity, which refers to antigen-nonspecific defense mechanisms that a host uses immediately or within hours after exposure to an antigen. Following infection, Lf is released from neutrophils (PMNs) in blood and inflamed tissues and, such as other soluble pattern-recognition receptors of the innate immunity, Lf recognizes unique microbial molecules called pathogen-associated molecular patterns (PAMPs): LPS from the gram-negative cell wall and bacterial unmethylated CpG DNA. However, unlike classical PAMPs receptors involved in the activation of immune cells, Lf may act either as a competitor for these receptors or as a partner molecule, depending on the physiological status of the organism. These immunomodulatory properties are explained by the ability of Lf to interact with proteoglycans and receptors on the surface of mammalian cells: cells of the innate (NK cells, neutrophils, macrophages, basophils, neutrophils and mast cells) and adaptive [lymphocytes and antigen-presenting cells (APCs)] immune systems, and also epithelial and endothelial cells. Through these interactions, Lf is able to modulate the migration, maturation and functions of immune cells, and thus to influence both adaptive and innate immunities. The understanding of the roles of the host-expressed Lf in immunity comes from in vivo and in vitro studies with exogenous Lf which, although informative, rarely reflect the pathological, or non-pathological, conditions in the organism. In this review, the data from the literature will be critically analyzed in order to present a real picture of the regulatory roles of host Lf in immunity.

Keywords Lactoferrin · Immunity · Immunomodulation · Immune cells · Lipopolysaccharides

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Introduction

Depending on the organism, immunity may involve innate immunity only, as for most invertebrates, or both innate and adaptive immunities, as for mammals. Whatever the systems brought into play, they are always complex and tightly regulated because they intend to be as harmful as possible against the infectious agents whereas they must be as harmless as possible for the host itself. Lactoferrin (Lf) is definitely one of the molecules that play key roles not only against microbes but also against excessive and harmful host responses in mammals: a sword and a shield at the same time. Those two aspects of Lf roles in host defense, together with its potency to modulate both innate and adaptive immunities, make

Lf an amazing multifunctional molecule in immunity. Until now, a plethora of studies aimed to demonstrate activities of Lf in immunity (reviewed in Legrand et al. 2004a, 2005, 2008); we will try to depict here the actual roles of host-expressed Lf in the immune responses.

Evidences for roles of Lf in immunity : in vivo and in vitro experiments

In vivo evidences

The multifunctionality of Lf makes the understanding of its activities very tricky (reviewed in Legrand et al. 2008). The literature is indeed crammed of evidences for roles of Lf in the host defense but, unfortunately, most of them are not necessarily informative on the actual mechanisms of action of the host molecule in immunity. Indeed, whereas many in vivo studies reported protective effects against infections or septic shock (reviewed in Legrand et al. 2005, 2006), it is not perfectly clear whether those effects are the result of a direct anti-microbial activity or of a boost of cell-mediated immunity modulation. However, recent studies undoubtly demonstrate that bovine Lf (bLf) supports immune and antioxidant status in healthy human volunteers (Mulder et al. 2008). In addition to the evidence that Lf levels markedly increased in biological fluids of patients suffering of inflammatory diseases (Bennett and Kokocinski 1978), the main in vivo indications of the role of Lf in the host defense came from observations on organisms suffering a lack of Lf synthesis. In particular, Breton-Gorius et al. (1980) showed a lack of specific granules in neutrophils from a patient with recurrent infections and, recently Ward et al. (2008), demonstrated a stimulus-dependent impairment of the neutrophil oxidative burst response in Lf-deficient mice. Conversely, Guillen et al. (2002) showed that transgenic mice expressing human Lf (hLf) exhibit enhanced Th1 response to *S. aureus*. However, although very demonstrative, this study and almost all others were performed with Lf from exogenous sources, usually coming from species different from that of the animal model used in the experiments, usually administered by oral route but also by intraveinous injection, and generally in large amounts. All these parameters are likely to generate effects that greatly differ from those of the host protein in physiological conditions, but which do reflect the immunomodulatory potency of Lf.

In vitro evidences

In vitro approaches are very suited to comprehend the actual roles of Lf in immunity, provided that the studies are conducted in homologous conditions (e.g., hLf with human cells) and with reagents of the highest purity and quality. As we will see thereafter, Lf binds the lipopolysaccharides (LPS) with high affinity (Appelmelk et al. 1994; Elass-Rochard et al. 1995) and these pro-inflammatory components are common contaminants in Lf preparations which can readily activate transduction pathways of the inflammatory response (usually MAP kinase and NFκB pathways; reviewed in Guha and Mackman (2001)). Furthermore, Lf protease degradation may generate peptides with different and/or higher activities than the whole protein [e.g., lactoferricin (Lfc), the N-terminal domain of Lf (Gifford et al. 2005)]. All the in vitro studies have demonstrated a role of Lf in the modulation of the activation of the cells involved either in innate immunity (most particularly macrophages, neutrophils, basophils, esosinophils, mastocytes and NK Cells) or in adaptive immunity (B and T-cells and dendritic cells), either as a pro-inflammatory or an anti-inflammatory agent (reviewed in Legrand et al. 2005, 2006). In most cases, the modulation of the activity of immune cells by Lf results in decreased or increased expressions of pro-inflammatory interleukins (IL-1, IL-6, IL-18, TNF-α) by immune cells or adhesion molecules by endothelial cells (E-selectin; Elass et al. 2002). Whereas most of the anti-inflammatory properties of Lf may be explained by a neutralizing effect of Lf against endotoxins, especially LPS, and their related receptors (see thereafter; reviewed in Legrand et al. 2005, 2006), the pro-inflammatory effects are much more controversial. Owing to the putative presence of LPS in Lf preparations used in some experiments, it is likely that the LPS itself may be the causative agent of pro-inflammatory effects. However, as discussed thereafter, it may be hypothesized that, in some instances, Lf may serve as a carrier for LPS. In addition to endotoxin binding, interactions of Lf with membrane components on cells, such as proteoglycans and cell receptors (reviewed in Suzuki et al. 2005; Legrand et al. 2008), were reported through in

vitro experiments which support a direct action of Lf on the activity of immune cells, most particularly on the proliferation, maturation and differentiation of lymphocytes (Mazurier et al. 1989; Bi et al. 1997).

Occurrence of host Lf in immunity: where, when and how?

The wide-spread distribution of the molecule in the organism testifies that Lf is in the front line of the host defense system. Lf is secreted in the apo-form from epithelial cells in most exocrine fluids such as saliva, bile, pancreatic and gastric fluids, tears and, more particularly milk (Montreuil et al. 1960). In biological fluids and at the surface of mucosa, Lf is believed to exert its antibacterial activities, either by direct interactions with microbes or by competition with bacterial siderophores (Jenssen and Hancock 2009). It represents, like other antibacterial proteins and peptides in fluids, an important key molecule of innate immunity.

When a microbe penetrates the tissues, itself and released endotoxins will activate the so-called "sentinel cells", such as macrophages, basophils, eosinophils, fibroblasts and dendritic cells, which will release very potent pro-inflammatory molecules (particularly IL-1, IL-6 and TNF-α). Both components and endotoxins will then activate the endothelial cells of which the permeability properties will be greatly modified. Endothelial cells will thus allow not only the passage of soluble molecules such as the complement and antibodies, but also the recruitment of blood-circulating neutrophils. This important step is made possible by the expression of specific adhesion receptors on activated endothelial cells (P- and E-selectins) and chemiokines (Il-8). Obviously, the recruitment of neutrophils by activated endothelial cells will initiate their activation and, as a result, a partial release of the content of their secondary granules in blood. Since Lf is a major component of secondary granules, its concentration in plasma may greatly increase from 0.4 to 2 mg l^{-1} to up 200 mg l^{-1} (Bennett and Kokocinski 1978). Plasma Lf is then rapidly cleared by the liver parenchymal cells (Debanne et al. 1985). In fact, plasma Lf only represents the tip of the iceberg since most of neutrophil Lf is delivered by neutrophils at very high concentration at the sites of inflammation where maximal activation of cells occurs. Degranulated Lf is in the form of the apo protein, so that its iron-chelating properties can be directed against microbes together with the direct microbicidal activity of the molecule. Besides its antimicrobial role, an important function of Lf is the modulation of the activity of the sentinel cells, either in pro- or anti-inflammatory ways (reviewed in Legrand et al. 2005, 2006).

These regulating activities take place thanks to the iron-binding properties of Lf and above all to its ability to interact with target molecules and cells. On one side, some in vitro experiments suggest that it may regulate proliferation, differentiation and activation of immune cells, thus strengthening, either directly or indirectly, the immune responses. On the other side, Lf plays anti-inflammatory activities able to lower harmfulness of the response. When tissues are infected, reactive oxygen species are abundantly produced, either generated by free iron released from necrosed tissues or overproduced by actived granulocytes. That oxydative burst, together with the excessive release of pro-inflammatory cytokines, mainly interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) highly contributes to the pathogenesis of the septic shock (Annane et al. 2005). The protective anti-inflammatory activity of Lf lies in its ability to bind free ferric ion but also exogenous pro-inflammatory bacterial components such as lipopolysaccharides (LPS) and their receptors. Whereas Lf iron binding has beneficial detoxication effects in infected or pathological tissues, binding to pro-inflammatory molecules has down-regulating effects on both the activation and recruitment of immune cells in inflamed tissues (reviewed in Legrand et al. 2005, 2006). Molecular and cellular basis supporting these assertions are developed in the following sections.

Lf modulates the activation of immune cells by microbial components

Lf binds to major pathogen-associated molecular patterns

Pathogens, especially bacteria, have common and relatively invariant molecular structures that are not shared with their host, known as Pathogen-Associated Molecular Patterns (PAMPs; reviewed in Akira and Hemmi 2003), which are powerful activation signals. The main PAMPs are the flagellin of bacterial

flagella, the peptidoglycan of Gram-positive bacteria, the lipopolysaccharide (LPS, also called endotoxin) of Gram-negative bacteria, double-stranded RNA (viruses) and unmethylated DNA. These molecules are recognized by the Pattern-Recognition Receptors (PPRs) which are the key elements of innate immunity, of which three main groups exist : the secreted PPRs (e.g., the C-reactive protein), the phagocytosis receptors (e.g., the mannose receptor), and the Toll-Like Receptors (TLRs).

Interestingly, it was reported that Lf binds to two major PAMPs: the LPS and unmethylated CpG DNA (Appelmelk et al. 1994; Elass-Rochard et al. 1995, 1998; Britigan et al. 2001). In the case of LPS, high-affinity interaction of Lf with the lipid A moiety of *E. coli* LPS was reported (Appelmelk et al. 1994). Using *E. coli* LPS, two binding sites with dissociation constants (Kds) of 3.6 ± 1 nM and 390 ± 20 nM were found on the N- and C-lobes of hLf (Elass-Rochard et al. 1995). It was shown that both sequences ^{1}GRRRR^{5} and ^{28}RKVRGPP^{34}, the same involved separately or in conjunction in the interactions with the 105 kDa Lf receptor, the LRP, glycosaminoglycans and DNA (Mann et al. 1994; Legrand et al. 1997; van Berkel et al. 1997) are required for the high-affinity binding of Lf to LPS (Elass-Rochard et al. 1995).

Although Lf could not be strictly considered as a PPR because it does not directly induce cell activation following binding to LPS or unmethylated CpG DNA, it may act as a competitor for the PPRs, or even, in some instances, as a transfer molecule.

Lf inhibits LPS signaling

A PPR such as the TLR4, the LPS signaling PPR, uses a serum transfer molecule called the LPS-binding protein (LBP), which transfers LPS to CD14. CD14 can either be soluble in serum or GPI-anchored to the cell membrane of neutrophils, monocytes/macrophages and lymphocytes (reviewed in Jerala (2007)). Soluble CD14 (sCD14) in complex with LPS binds to cells, such as endothelial cells, which do not express mCD14.

As shown through in vivo and in vitro experiments, hLf suppresses TNF-α, IL-1β, IL-6 productions in mononuclear cells in response to LPS activation (Miyazawa et al. 1991; Crouch et al. 1992; Mattsby-Baltzer et al. 1996; Haversen et al. 2002; Kruzel et al. 2002). In particular, orally-administered bLf was shown to have a beneficial effect on infections and protects animals against a lethal dose of LPS (Zagulski et al. 1989). It is now well admitted that the down-regulation of pro-inflammatory cytokines and protection against the septic shock is mainly related to the LPS-binding properties of Lf, through its lactoferricin (Lfc) domain (Elass-Rochard et al. 1995, 1998). Lf competes with serum LBP for LPS binding and therefore prevents the transfer of endotoxin to mCD14 presented at the surface of neutrophils (Elass-Rochard et al. 1998).This property accounts for the inhibition by Lf of LPS-induced activation and subsequent release of proinflammatory cytokines by immune cells. Another important finding was the evidence of high-affinity interactions ($Kd \approx 16 \pm 7$ nM) between Lf and sCD14 (Baveye et al. 2000a). It was showed that hLf interacts not only with free sCD14 but also, though with different binding properties, with sCD14 in complex with LPS or lipid A-2-keto-3-deoxyoctonic acid-heptose. As for LPS, the cationic N-terminal peptides of Lf are essential in the binding (Baveye et al. 2000a). Lf also suppresses the production of hydrogen peroxide mediated by the binding of LPS to L-selectin of neutrophils (Baveye et al. 2000b). Furthermore, the interaction between Lf and soluble CD14 (sCD14) inhibits the secretion of IL-8, a chemokine induced by the complex sCD14-LPS, by endothelial cells (Elass et al. 2002). The interaction of Lf with LPS and sCD14 interferes not only with the activation of immune cells but also with the expression of adhesion molecules on endothelial cells, necessary for the local recruitment of immune cells at inflammatory sites. In particular, Lf inhibits the (sCD14-LPS)-induced expression of E-selectin, ICAM-1 and IL-8 by human umbilical endothelial cells (Baveye et al. 2000a; Elass et al. 2002).

In conclusion, it is very likely that when Lf is released in high amounts at the inflammatory sites and in lesser extent in blood, it may prevent further binding of LPS to the receptors, thus preventing the activation of cells and playing a potent anti-inflammatory role in immunity.

Lf, a vector for LPS?

Several studies have reported that Lf may activate macrophages and induce IL-8, TNF-α and NO (Sorimachi et al. 1997). During infection, Lf secreted from neutrophil granules can bind to PMNs and

monocytes/macrophages (Gahr et al. 1991), and a connection was made between this binding and the promotion of the secretion of inflammatory molecules such as TNF-α (Sorimachi et al. 1997). Studies have shown that Lf may activate macrophages via TLR4-dependent and independent signalling pathways (Curran et al. 2006). This activation induces CD40 expression and IL-6 secretion. However, it is not clear whether Lf activates cells on its own, or if the observed effect is due to contaminating LPS in Lf preparations. Since (1) only traces of LPS are sufficient to trigger activation of cells, and (2) significant amounts of LPS were detected in commercial preparations of Lf, it is possible that many studies actually reported the effect of LPS or the Lf-LPS complex on immune cells.

Indeed, in some conditions, the Lf-LPS complex could be an inducer of inflammatory mediators in macrophages, through Toll-like receptor 4 (Na et al. 2004). Moreover, after a pre-treatment with the Lf-LPS complex, it was found that cells are rendering a tolerant state to LPS challenge (Na et al. 2004). It was also recently demonstrated that a complex of Lf with monophosphoryl lipid A is an efficient adjuvant of the immune responses (Chodaczek et al. 2006, 2008). Lastly, it was recently reported that the ability of Lf to form complexes with LPS would induce IFN-α/β expression and hence promote the antiviral activity of Lf (Puddu et al. 2007).

We thus hypothesize that depending on the Lf/LPS ratio at a particular site of inflammation or in a particular biological fluid, and depending on the nature and maturation of immune cells, Lf may act either as a LPS neutralizing molecule or as a LPS carrier, hence its immunomodulatory activity. In other words, when the Lf/LPS ratio is elevated, as when Lf is released from PMNs at the sites of inflammation, Lf could act as a LPS neutralizing and anti-inflammatory molecule. However, when Lf concentration is lower, Lf could serve as a carrier for LPS whose delivery would depend on the cell environment. It is indeed worth noting that most interactions of Lf with its receptors and ligands, including LPS, involve the basic Lfc domain of the molecule. Cell surface and extracellular matrix proteoglycans are precisely major targets of the Lfc domain (Mann et al. 1994; Ziere et al. 1996; Legrand et al. 1997). On most cells, in particular on T-lymphocytes, binding to proteoglycans accounts for more than 80% of total Lf binding on cell surface (Legrand et al. 1997). It is thus very likely that the Lf/LPS complex readily dissociates at the vicinity of the extracellular matrix (ECM) or in contact with the surface of cells. It is a fact that the affinity of Lf for LPS is about 500-fold lower than that for proteoglycans but the tremendous number of Lf-binding sites on cell proteoglycans (usually several millions sites per cell), and most particularly on ECM proteoglycans, largely compensates this difference. Interestingly, both nature and number of proteoglycans greatly depend on the nature of cells but also on their state of differentiation or maturation, but also of pathologies (Schwartz 2000). It is thus quite conceivable that dissociation of the Lf/LPS complex occurs more readily on certain cells than on others. After dissociation from proteoglycan-bound Lf, LPS would bind to any LPS receptor present on the cells, thus initiating the activation of cells.

Lf modulates the recruitment of immune cells

Cell migration is critical for a variety of biological processes. During influenza virus infection (pneumonia), bLf was shown to reduce the number of infiltrating leukocytes in bronchoalveolar lavage fluid, thus suppressing the hyper reaction of the host (Yamauchi et al. 2006). Lf also decreases the recruitment of eosinophils, reduces pollen antigen-induced allergic airway inflammation in a murine model of asthma (Kruzel et al. 2006), and reduces migration of Langherans cells in cutaneous inflammation (Griffiths et al. 2001). Such reduced migration of granulocytes, most particularly eosinophils, was recently reported (Bournazou et al. 2009). Furthermore, Lf can modulate fibroblast motility by regulating MMP-1 gene expression, a matrix metalloproteinase involved in the promotion of cell migration (Oh et al. 2001). Lastly, an in vivo study has shown that orally-administered recombinant hLf is able to prevent injury of non-steroidal anti-inflammatory drugs in the intestine of rats and mice and that this effect could be linked to attenuation of neutrophil migration to the intestine (Dial et al. 2005). Another aspect of Lf activity could be the inhibition of angiogenesis, probably by inducing IL-18 production in serum and blocking endothelial functions (Shimamura et al. 2004).

Although the exact mechanism of action of Lf in these processes was not clearly defined, it has been

demonstrated in vitro that Lf inhibits the (sCD14-LPS)-induced expression of E-selectin, ICAM-1 and IL-8 by human umbilical endothelial cells (Baveye et al. 2000a, b; Elass et al. 2002). These studies also pointed out the ability of Lf to compete with chemokines such as IL-8 for their binding to proteoglycans and their further presentation to leukocytes (discussed after). Other mechanisms of action of Lf in allergy were proposed. Indeed, Lf is over expressed in patients with allergies (Zweiman et al. 1990), a process which involves the activation of mast cells and basophils and IL-1β and TNF-α-triggered migration of APCs. In skin allergies, a mechanism by which Lf binds to keratinocytes and inhibits the release of TNF-α from these cells has been proposed (Cumberbatch et al. 2003). Another explanation has been found in the ability of Lf to displace tryptase (a potent pro-inflammatory protease released from mast cells) from heparin, and hence to destabilize the enzyme (Kimber et al. 2002). Lf apparently displaces tryptase from heparin which is known to maintain enzymatic activity. It was recently shown that inhibition occurs following Lf uptake by mast cells and interaction not only with tryptase but also with chymase and cathepsin G (He et al. 2003). These authors also showed an inhibition of anti-IgE induced histamine and tryptase releases from human colon mast cells by Lf (Elrod et al. 1997; He and Xie 2004).

The role of proteoglycans in the activity of Lf in immunity

Glycosaminoglycans and proteoglycans participate in a variety of biological processes including the inflammatory process (Taylor and Gallo 2006). Since Lf is a glycosaminoglycan-binding protein (Mann et al. 1994; Ziere et al. 1996; Legrand et al. 1997), it may be expected that massive Lf release from PMNs at the inflammatory sites and in blood interferes in proteoglycans functions.

Interleukin-8 (IL-8), a potent C-X-C chemokine activates LFA-1 integrins (LFA-1) on neutrophils but also binds, as a dimer and with a low affinity, to heparin and heparan sulfate molecules (reviewed in Mukaida 2000). Once firmly attached, the cells are directed by a chemo attractant gradient to transmigrate into tissue affected by injury or infection (Smith et al. 1991). The cell surface proteoglycans increase the local concentration of IL-8 that, in turn, regulates the activation of neutrophils through specific interactions with a G-protein coupled receptor. We showed in vitro that Lf inhibits the interaction of IL-8 to immobilized heparin, thus suggesting that the anti-inflammatory properties of Lf during septicemia is related, at least in part, to the ability of Lf to compete with chemokines for their binding to proteoglycans on cells and in the ECM (Elass et al. 2002).

Another role of Lf binding to glycosaminoglycans, still hypothetical though and above outlined, is the destabilization of the Lf-LPS complex, making LPS available to its signaling cell receptors.

Lf modulates the phagocyte capacity of cells

Promotion of lytic cell activity is an important aspect of Lf function. Lf is already expressed on resting PMNs where it could participate in the binding of micro-organisms (Deriy et al. 2000). It was shown in vitro that both release and cell binding promote the activation and phagocytosis of PMNs and monocytes/macrophages. Lf was reported as a promoter of motility, superoxide production and release of pro-inflammatory molecules such as NO, TNF-α and IL-8 (Gahr et al. 1991; Shinoda et al. 1996; Sorimachi et al. 1997) and a study indeed demonstrates enhanced phagocytosis against *S. aureus* (Kai et al. 2002). The molecular mechanisms explaining these activities are, however, controversial. Phagocytosis by PMNs is enhanced by the interaction of complement activation products, particularly complement factor C3. Nevertheless, it is unclear whether Lf activity is related to complement activation since Lf was shown either to inhibit (Kijlstra and Jeurissen 1982) or to activate (Rainard 1993) the classical and alternate pathways of complement. A more recent report shows that the Lfc domain of either hLf or bLf inhibits the classical complement pathway but not the alternative complement pathway (Samuelsen et al. 2004). Lastly, direct Lf binding to PMNs and opsonin-like activity could also be involved (Miyauchi et al. 1998).

Host Lf, a bridge between innate and adaptive immunities?

Whereas there is no doubt that Lf plays a key role in innate immunity, many studies have shown that Lf also intervenes in adaptive immunity. In particular,

oral administration of bLf seems to influence mucosal and systemic immune responses in mice (Sfeir et al. 2004). These effects may be explained by roles of Lf in the modulation of the maturation, differentiation and activity of lymphocytes, and in the promotion of antigen presentation to these cells.

Modulation of the maturation, differentiation and activity of lymphocytes by Lf

Many studies reported effects of Lf on the maturation and differentiation of T- and B-lymphocytes. Although most results were obtained from in vivo studies using oral administration of Lf, it may be assumed that increased concentrations of Lf in blood and biological fluids during inflammation (Bennett and Kokocinski 1978) would also influence the maturation and differentiation of lymphocytes. It was demonstrated that bLf administered orally to mice strongly elevated the pool of CD3 + T cells and CD4 + T cell content (Sfeir et al. 2004). Lf given orally to CP-immunosuppressed mice could reconstitute a T cell-mediated immune response by renewal of the T cell pool (Artym et al. 2003b). It was also recently demonstrated that a complex of Lf with monophoshoryl lipid A is an efficient adjuvant of the humoral and cellular immune responses (Chodaczek et al. 2006). Its stimulating effect on the immune system mainly concerns the maturation and differentiation of T-lymphocytes and the Th1/Th2 cytokine balance (Fischer et al. 2006). Under non-pathogenic conditions, Lf is able to stimulate the differentiation of T cells from their immature precursors through the induction of CD4 antigen (Zimecki et al. 1991; Dhennin-Duthille et al. 2000). A similar effect was described on isolated thymocytes incubated overnight with Lf. Furthermore, oral delivery of Lf significantly increased the number of CD4-positive cells in lymphoid tissus (Kuhara et al. 2000). Lf induces a Th1 polarization in diseases in which the ability to control infection or tumor relies on a strong response, but may also reduce Th1 cytokines to limit excessive inflammatory responses. Thus, Lf enhances both the ability of Th cells to assist the fungicidal actions of macrophages (Wakabayashi et al. 2003) and BCG vaccine effectiveness against challenge with *Mycobacterium tuberculosis* (Hwang et al. 2005, 2008). Similarly in Lf transgenic mice, an upregulation of the Th1 response was associated with *S. aureus* clearance (Guillen et al. 2002). Oral administration of Lf increased the splenocyte production of IFN-γ and Il-12 in response to Herpes simplex virus type 1 infection (Wakabayashi et al. 2004). An up-regulation of IFN-γ and TNF-α production by cervical lymph node cells stimulated by heat-killed *Candida albicans* was observed in Lf-treated mice compared with non-treated mice (Takakura et al. 2004). Lastly, the eradication of chronic hepatitis C virus by IFN therapy is favourized by administration of bLf that induces a Th-1 cytokine dominant environment in peripheral blood (Ishii et al. 2003).

Concerning the humoral response, Lf was shown, in vitro, to promote differentiation of splenic B cells (Zimecki et al. 1995). Lf also restored humoral immune response by peritoneal and alveolar cells in cyclophosphamide (CP)-immunocompromised mice (Artym et al. 2003a, 2004). Lastly, Lf binding to CpG-containing oligonucleotides was shown to inhibit their immunostimulatory effects on human B cells (Britigan et al. 2001).

Lf promotes the recruitment, maturation and activation of antigen-presenting cells

A connection between innate and adaptive immunities made by host-expressed Lf can be found at the very place where Lf is degranulated in large amounts from neutrophils, i.e., the inflammatory site. As described above, APCs, among which dendritic cells, the most important T helper cell activators, are present in all tissues. Recently, it has been shown that Lf acts as an alarmin to promote the recruitment and activation of APCs and antigen-specific immune responses (de la Rosa et al. 2008). It was also reported as a novel maturation factor for dendritic cells (Spadaro et al. 2008). Hence, Lf released at the inflammatory site would participate to adaptive immunity by promoting the maturation, activation and migration of APCs (reviewed in Puddu et al. 2009).

Putative mechanisms accounting for the effects of Lf in adaptive immunity

The exact mechanisms by which Lf modulates the maturation, differentiation, migration and activation of lymphocytes and APCs are not clearly known. As

mentioned before, most results are obtained from studies using oral-administered Lf whose action is mainly directed in the intestine. A proposed mechanism is that oral Lf induces IL-18 production in the small intestine, therefore leading to an increase in the level of Th1 cells (Ishii et al. 2003). Furthermore, it has been shown that bLf may promote systemic host immunity by activating the transcription in the small intestine of important genes such as IL-12p40, IFN-β and NOD2 (Yamauchi et al. 2006).

Two main hypothetical mechanisms may account for effects of Lf on lymphocytes and APCs. The first mechanism, formerly mentioned in this review, relies on the ability of Lf to complex PAMPs and to serve either as a PAMP-neutralizing molecule or as a PAMP vector. Indeed, LPS have profound effects on CD4 T-cell responses and are well-known for generating Th1 responses (McAleer and Vella 2008). They also upregulate costimulatory molecules on antigen-presenting cells (APCs). T cells that are primed in an LPS-stimulated environment are programmed for long-term survival following clonal expansion (McAleer and Vella 2008). Hence, it may be hypothesized that the LPS-binding ability of Lf plays a modulatory role in the physiology and functions of lymphocytes, in a similar way to cells of innate immunity. The second mechanism is based on interactions of Lf with receptors at the surface of cells which would be responsible for the activation of signalling pathways and/or the endocytosis and targeting of all or part of Lf in the nucleus of cells. Whereas three protein receptors have been identified on human cells, i.e., the lipoprotein-related receptor (LRP; Meilinger et al. 1995), the 105 kDa lymphocyte receptor identified as surface nucleolin (Legrand et al. 2004b), and a specific receptor evidenced on intestinal cells (reviewed in Suzuki et al. 2005), very little is known about the actual mechanisms of action of Lf on cells expressing these receptors. Interestingly, all three receptors permit signaling in cells and/or endocytosis of Lf. LRP, a Lf receptor at the surface of many cells has been recently shown to function as a mitogenic Lf receptor in osteoblastic cells, via p42/44 MAP kinase signaling (Grey et al. 2004). Such MAP kinase signaling was also observed in Jurkat lymphoblastic T cells owing to the 105 kDa Lf receptor (Dhennin-Duthille et al. 2000). It is also hypothesized that Lf may enter the cell and be targeted to the nucleus where it can act as a transcriptional activator (Oh et al. 2001). Recently, nucleolin ubiquitously expressed on dividing cells was pointed out as a possible Lf carrier between cell surface and nucleus (Legrand et al. 2004b). Interestingly, it was recently shown that Lf may down-regulate LPS-induced cytokines in THP1 through a mechanism involving Lf internalization, nuclear localization and interference with nuclear factor-κB (NF-κB; Haversen et al. 2002). The mechanisms of interference of Lf of NF-κB, a transcription factor playing a critical role in immune responses and inflammation, are not perfectly clear. However, Oh et al. (2004) showed that overexpressed Lf acts as a p53 gene transactivator through the stimulation of the IκB-kinase activity and NF-κB binding. These authors previously demonstrated a matrix metalloproteinase 1 gene transactivating activity by Lf through stress-activated MAPK signaling modules (Oh et al. 2001).

A role of host Lf in the maturation and differentiation of lymphocytes ?

As described above, most assertions of roles of Lf in adaptive immunity are based on either in vitro or in vivo experiments of which the physiological relevance may be questioned. Hence, the observations made in these experiments could poorly reflect the exact role of host Lf in adaptive immunity. For example, about vaccine efficiency, an adjuvant effect in the generation of delayed-type hypersensivity and in the bost of BCG efficacy to generate T helper response in mice was demonstrated (Zimecki et al. 2002). However, this adjuvant effect was attributed to bLf binding on the mannose receptor of immature skin APCs. Such binding has been recently confirmed in a study showing that bLf binding to DC-SIGN on dendritic cells blocks its interaction with HIV gp120 and subsequent virus transmission (Groot et al. 2005). Since glycans with polymannosidic structures are specific to bLf and not found on hLf, this example clearly illustrates a typical "non-specific" effect of exogenous Lf in in vivo experiments. Most importantly, since Lf is not synthesized/secreted in thymus or in any other primary or secondary lymphoid tissue, its presence in these tissues mostly originates from a transient release from degranulating neutrophils in blood. Therefore, unless in chronic and/or severe inflammatory diseases, a possible effect of host Lf on

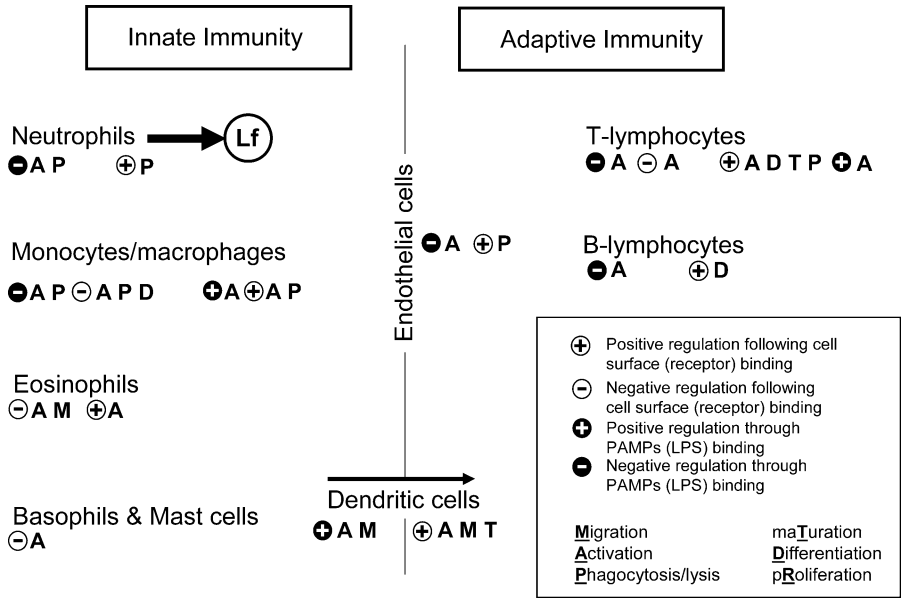

Fig. 1 Schematic representation of the influence of host Lf on immune cells. The scheme summarizes the known/admitted positive (*circled plus*) or negative (*circled minus*) effects of host Lf on cells of innate (*left*) and adaptive (*right*) immunities. It discriminates between effects connected with a direct binding of Lf to the surface of immune cells (*unfilled circle*), whatever the binding sites (proteoglycans, receptors, …) or the signalling mechanisms, and effects connected with the PAMPs (mainly LPS)-binding capability of Lf (*black filled circles*). The *letters* indicate the cell events modulated by Lf

maturation and differentiation of lymphocytes should be questioned.

Conclusion

Lf possesses pleiotropic roles which turn it into either a weapon or a shield in the host defense system. The beneficial effects of Lf administered in prevention or treatment of infectious pathologies have led to many applications for health. These applications have emphasized the importance of Lf in the regulation of immunity but most of them poorly account for the actual activities of host-expressed Lf in this control. It appears that Lf acts as an anti-microbial and anti-oxidant molecule, not only through direct interactions with microbes or through its iron-binding capability, but also by stimulating the migration and functions of cells of the innate and adaptive immunities (illustrated in Fig. 1). The immuno-modulatory properties of Lf are mainly related to its PAMPs (mostly LPS)-binding ability which generally turns Lf into an anti-inflammatory molecule able to protect the host from harmful immune responses. Conversely, it may be hypothesized that Lf also acts as a vector of PAMPs for immune cell activation. Other putative mechanisms, which still need further investigations, would require signalling or nuclear targeting following interactions with multifunctional or specific cell membrane receptors.

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