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Summary of the Ph.D. Thesis

THE ROLE OF LACTOFERRIN IN IMMUNE RESPONSE IN INFECTION AND INFLAMMATION

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Introduction

Lactoferrin (Lf) is a multifunctional iron-binding glycoprotein belonging to the transferrin family present in high concentration in colostrum, milk and in many exocrine secretions, such as tears, saliva or fluids of the digestive tract (Ward *et al.* 2002; Legrand & Mazurier 2010).

Human Lf (HLf) consists of a single polypeptide chain of 691 amino acid residues. Interestingly, Lf is a highly basic protein, with positive charges being distributed at the N-t (1–7 amino acid residues), along the outside of the first helix (13–30 amino acid residues), as well as in the interlobe region (Baker & Baker 2005). Many active clusters, for which the biological importance has been described, are located in this basic domain: N-t peptide (1–5 amino acid residues) is the sequence for nuclear targeting (Penco *et al.* 2001), lactoferricin (Lfcin), a pepsin-cleaved fragment (1–47 amino acid residues in HLf), for antimicrobial and antitumor activities and binding to proteoglycans (Bellamy *et al.* 1992), Lf11 (19–29 amino acid residues) (Japelj *et al.* 2005), and a sequence comprising seven amino acids (28–34 amino acid residues) (Elass-Rochard *et al.* 1995) for binding the bacterial endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria.

LF belongs to the Tf family, but its biological functions are not limited to iron metabolism regulation (Ando *et al.* 2010). Its beneficial effect ranges from the well-established antimicrobial activity against various microorganisms, including bacteria, viruses, fungi, and parasites, to immunomodulatory and antitumoral activities (Legrand *et al.* 2008).

The LPS-binding activity of Lf may account, at least in part, for the role of this protein in the modulation of the immune response and the inflammatory process. Macrophages pretreated with a premix of Lf and LPS were rendered tolerant to LPS stimulation (Na *et al.* 2004). Moreover, serum LPS binding protein (LBP) may participate in the role played by Lf in the modulation of the inflammatory response. It was demonstrated that Lf interacts with soluble CD14 (sCD14), resulting in the inhibition of signal transduction mediated by the CD14–LPS complex (Baveye *et al.* 2000). Lf inhibits proinflammatory responses not only through its ability to bind to key molecule on the surface of cell membrane, but also through its direct activity on immune cells and molecules (Legrand *et al.* 2005). Thus, Lf can inhibit production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Crouch *et al.* 1992; Haversen *et al.* 2002), but can also promote secretion of anti-inflammatory mediators including IL-10, IL-4, and tumor growth factor-β1 (TGF-β1) (Togawa *et al.* 2002; Zimecki *et al.* 2005). Alternatively, Lf may stimulate immune responses by direct actions on immune cells, such as maturation and differentiation of T-lymphocytes (Dhennin-Duthille *et al.* 2000), influence on Th1/Th2 cytokine balance (Ishii *et al.* 2003; Wakabayashi *et al.* 2003), and phagocytes activation through release of TNF-α (Sorimachi

et al. 1997), Toll-like receptor (TLR) -dependent and independent expression of CD40 and IL-6 secretion (Curran *et al.* 2006).

The presence of Lf specific receptors on immune cells suggests that the modulation of inflammation by Lf may be strongly connected to a direct effect via receptor-mediated signaling pathways. Thus, it was shown that at the cell surface, 80% of Lf binding is mediated through low affinity cell receptors such as sulphated chains of proteoglycans (Legrand *et al.* 1997). Other more specific receptors, that act alone or together with proteoglycans in binding and subsequent internalization of Lf in immune cells, have been described. A specific 105-kDa receptor was identified on lymphocytes, platelets and mammary gland cells (Mazurier *et al.* 1989; Leveugle *et al.* 1993; Damiens *et al.* 1998). Moreover, it was proposed that nucleolin could actually be this specific receptor (Legrand *et al.* 2004). Low-density lipoprotein receptor-related protein (LRP), a protein widely expressed on several cell types, was reported to account for receptor-mediated internalization of Lf and to act as a mitogenic receptor in osteoblastic cells (Grey *et al.* 2004). Recently, a small 34-37 kDa specific receptor was characterized at the surface of enterocytes, assisting Lf endocytosis via clathrin process, and a role in signal transduction that could be leading to IL-18 synthesis was hypothesized (Jiang *et al.* 2011; Jiang & Lonnerdal 2012). In macrophages, the multifunctional glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evidenced as a novel Lf receptor (Rawat *et al.* 2012). In monocytes, Lf downregulates LPS-induced proinflammatory cytokines secretion through a mechanism involving Lf translocation to the nucleus and inhibition of NF-κB activation (Haversen *et al.* 2002).

Lf and Lf-derived peptides were also demonstrated to possess anti-viral activity, inhibiting the growth of a variety of viruses, such as human immunodeficiency virus (HIV), Japanese encephalitis virus (JEV), rotaviruses, influenza viruses, hepatitis B and C virus (HBV and HCV), poliovirus (Berlutti *et al.* 2011). Possible mechanisms that could explain the protection of Lf to the host against viral infections involves either direct interaction with the viral particle, iron sequestration, or competitive binding to host cells, since Lf is present in many biological fluids and binds to most cells, thus preventing viruses to enter. Finally, an intracellular activity of Lf, involving apoptosis or inflammatory pathways could account for antiviral effects.

In our previous experiments we have demonstrated that Lf exhibits an anti-inflammatory activity regardless of the time of addition of Lf to the cells with respect to LPS challenge (Mattsby-Baltzer *et al.* 1996). On the basis of these results, the objectives of this thesis were to decipher the endocytosis mechanism through which Lf is internalized and exert its beneficial immunomodulatory effect and to investigate whether it involves transactivation of inflammatory genes.

The ability of Lf to inhibit HBV infection of target cell by preventing virus particles adsorption on cellular membrane was described (Hara *et al.* 2002), but the mechanism of anti-HBV action of Lf could not

clearly define whether or not this property depends on its structural integrity or is restricted to certain distinct regions of the protein. In this study, the capacity of seven Lf-derived peptides to prevent HBV infection and replication was investigated.

The anti-inflammatory activity of Lf

Lf exhibit immunological properties that bridges innate and adaptive immune functions in mammals (Legrand *et al.* 2006) (Fig. 1). It was reported that people with congenital or acquired Lf deficiency have recurring infections, thus demonstrating the relationship of Lf with the immune system (Breton-Gorius *et al.* 1980).

Lf is in the frontline of host defense regulating innate immune responses and may be considered a marker for inflammation due to the fact that its level is increasing during inflammation and in some pathologies (Roseanu & Brock 2006; Legrand *et al.* 2008). Lf was reported to modulate the inflammatory response by its capacity to bind bacterial LPS and LBP (Elass-Rochard *et al.* 1995), leading to down-regulating effects on both the activation and recruitment of immune cells in inflamed tissues. Lf inhibits LPS-induced cytokines production in THP-1 promonocytic cell line through different mechanisms suggesting Lf internalization, nuclear localization and interference with the nuclear factor NF-κB and/or MAPK pathway activation (Haversen *et al.* 2002).

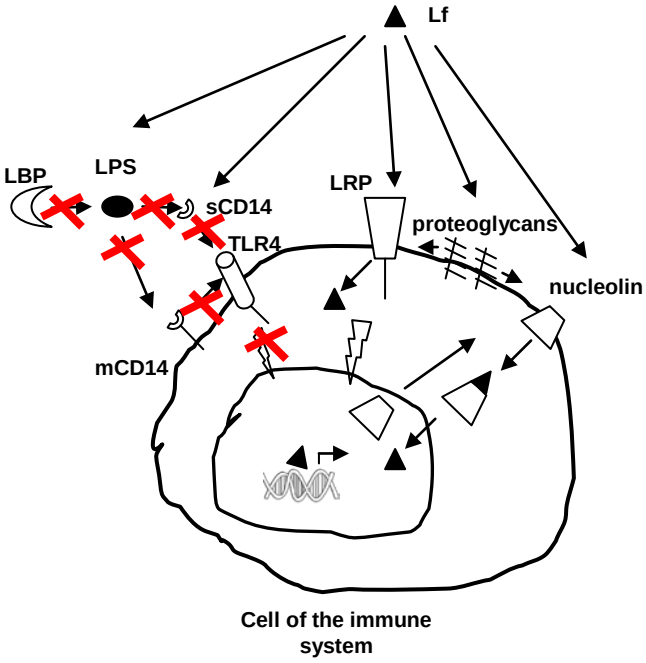

Figure 1. ***Schematic representation of the molecular mechanisms involved in the immunomodulatory activity of Lf.*** The interactions of Lf with lipopolysaccharide (LPS) and soluble serum CD14 (sCD14) results in impaired interactions of LPS with serum LPS binding protein (LBP) and subsequent impaired transfer of LPS to membranar CD14 (mCD14). In this way, the signaling pathway of Toll-like receptor 4 (TLR4) is blocked. On the other hand, the interactions of Lf with lipoprotein receptor-related protein (LRP), proteoglycans from the cell surface or nucleolin could lead to endocytosis and activation of signaling pathways or nuclear targeting. (Adapted from (Legrand *et al.* 2006; Ha *et al.* 2011).

The immunomodulatory properties of Lf can also be explained by its ability to interact with mammalian cells and modulate the immune response. Lf was shown to bind many cell types, hepatocytes,

intestinal cells, immune cells, melanoma cells, breast cancer cells (Legrand & Mazurier 2010; Roseanu *et al.* 2010).

The complex mechanism of action of Lf in inflammation is far from being completely elucidated. Thus, in our previous studies, we found that Lf exhibits an anti-inflammatory activity regardless of the time of addition, with respect to LPS stimulation, suggesting a complex mechanism of action (Mattsby-Baltzer *et al.* 1996).

In order to decipher the early steps of anti-inflammatory activity of Lf in immune cells, the endocytic pathways of Lf in THP-1 cells were investigated. Using specific inhibitors, clathrin- and caveolea-mediated endocytosis were studied.

Results and Discussions

Endocytosis and traffic of Lf in macrophage-like THP-1 cells

The endocytosis of Lf was described only in a few number of cell lines (Garre *et al.* 1992; Willnow *et al.* 1992; Ziere *et al.* 1992). Specific Lf receptors have also been described (Legrand *et al.* 2004; Suzuki *et al.* 2005; Lopez *et al.* 2008), but most binding sites on cell surface were reported to be of proteoglycan nature. Generally, these receptors are involved in signal transduction which may imply internalization of Lf.

Binding and internalization of Lf in THP-1 cells

After incubation for 1 h at 4 °C, HLf was shown to bind specifically to the plasma membrane of THP-1 macrophage-like cells (Fig. 2a, c).

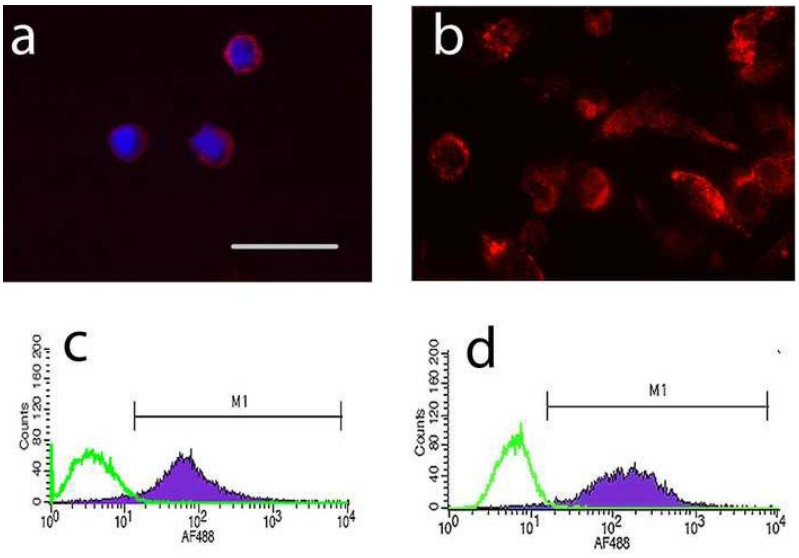

Figure 2. ***Binding and internalization of HLf in PMA-treated THP-1 cells.*** Cells were incubated for 1h / 4 °C (a) or 30 min/ 37 °C (b) with 0,1 mg/ml HLf-Texas Red (red) and visualized by fluorescence microscopy. Nuclei were visualized with DAPI (blue). The images are representative of at least two independent experiments. Scale bar 50 μm; THP-1 cells were incubated for 1h / 4 °C (c) or 30 min/ 37 °C (d) with 0.5 mg/ml HLf, and fluorescence staining was achieved by using goat anti-rabbit Alexa Fluor 488- conjugated IgG. Typical flow cytometry profile is shown after the cells were subjected to FACS binding or internalization protocols as described in Materials and Methods. Negative control, cells incubated with secondary Alexa Fluor 488-conjugated antibody only-green line; Sample-magenta filled histogram. M1>90 % specific signal (Florian *et al.* 2012).

An intense punctuated pattern of fluorescence, which was distributed in a random fashion throughout the cytoplasm was detected (Fig. 2b), suggesting a receptor-mediated endocytic process of HLf uptake by the THP-1 cells. We have reported in our previous study the presence of two types of binding sites for Lf on the surface of THP-1 cells: a very specific, high affinity type of binding site, which could allow the endocytosis of Lf in THP-1 cells, and a less specific, low affinity one, which could be involved only in the binding step of Lf on cell membrane (Roseanu *et al.* 2000).

Lf internalization in THP-1 cells is mainly clathrin-mediated

For the identification of the mechanism responsible for HLf uptake, THP-1 macrophage-like cells were treated with the inhibitors of clathrin-mediated coated vesicle formation, chlorpromazine (CP) and dansylcadaverine (DC) (Wu *et al.* 2003).

Lf internalization in THP-1 cells is partially mediated by acidic endosome-like organelles

By using specific inhibitors of vesicles acidification, the Lf internalization in macrophage-like THP-1 cells was studied. Thus, to check a potential role of pH in HLf endocytosis, inhibitors of endosomal acidification were employed: bafilomycin A1 (BAF A1) which specifically blocks the endosomal ATPase pumps (Popescu *et al.* 2005) and NH4Cl, which rises the endosomal pH (Eash *et al.* 2004).

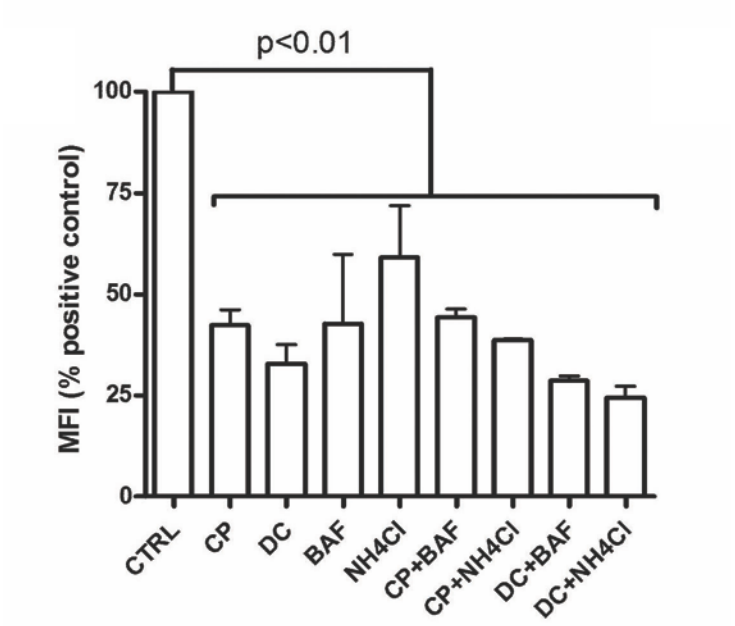

Figure 3. ***Requirement for an acidic compartment of human Lf (HLf).*** Flow cytometry analysis of HLf internalization in the presence of clathrin-dependent endocytosis inhibitors. Cells were incubated with endocytosis inhibitors for 30 min at 37 °C and then with 0.5 mg/mL HLf for 30 min at 37 °C. The results are represented as an average of the median fluorescence intensity (MFI) of 3 independent experiments using duplicate samples and positive control cells = 100%. One-way ANOVA showed a p-value = 0.0001 and a Dunnett's test proved the significant difference between each treated cell population and the control ($p < 0.01$) (Florian *et al.* 2012).

As shown in Fig. 3, flow cytometry experiments revealed that THP-1 cells treated with CP or DC for 30 min before HLf addition, led to a 50 % inhibition of the protein internalization compared with untreated cells, indicating that Lf endocytosis is a clathrin-mediated process. When cells were treated with endosomal acidification inhibitors, a 40 (for NH4Cl) to 60% (for BAF A1) inhibition was recorded, suggesting that the internalization of Lf may involve acidic endosome-like organelles (Fig. 3). Interestingly, when the same

endocytic pathway was inhibited, meaning that the cells were incubated with combinations of these inhibitors, no synergistic/additive effect could be detected (Florian *et al.* 2012).

Lf internalization in THP-1 cells is partially cholesterol-dependent

Different types of endocytosis can take place simultaneously, at least a part being affected by cholesterol sequestration (Rodal *et al.* 1999). It was of interest to evaluate whether the internalization of HLf into THP-1 cells is process which dependents on cholesterol. Methyl-beta-cyclodextrin (MβCD) was used as a cholesterol depletion agent and nystatin (NYS) for the efficient sequestration of plasma membrane cholesterol in order to block the cholesterol-dependent internalization routes (Subtil *et al.* 1999; Sharma *et al.* 2004).

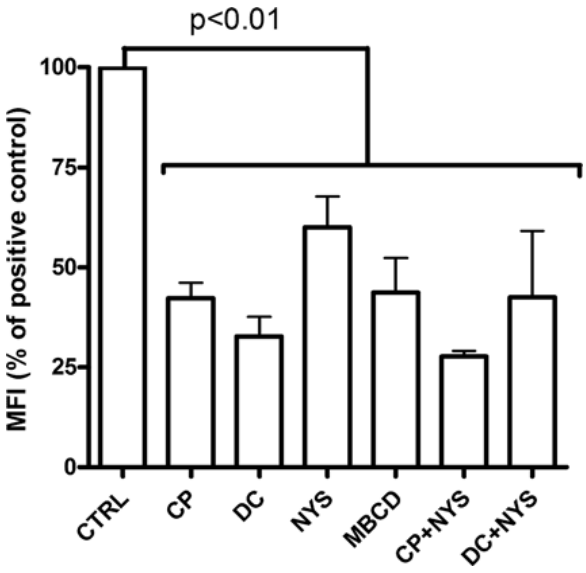

Figure 4. ***Effect of cholesterol inhibitors on HLf internalization.*** Cells were incubated with cholesterol inhibitors, methyl-beta-cyclodextrin (MβCD) and nystatin (NYS), for 30 min/ 37 °C and then with 0.5 mg/ml HLf for 30 min/ 37 °C. The results are represented as average of the median fluorescence intensity (MFI) of three independent experiments using duplicate samples and positive control cells=100 (Florian *et al.* 2012).

Flow cytometry analysis revealed as well a decrease of 40% of HLf internalization in the presence of NYS (Fig. 4). Surprisingly, when inhibitors of both, clathrin and cholesterol-mediated endocytosis were used, an additive effect was recorded (Florian *et al.* 2012). It can be concluded that HLf endocytosis is sensitive to the imbalances in the organization of the clathrin –coated pits.

All these data revealed that HLf uses mainly a clathrin-mediated pathway, a route which is also cholesterol-dependent and sensitive to endosomal acidification. It was reported that in *Entamoeba histolytica* the mechanism responsible for Lf endocytosis is not clathrin-mediated and involves caveolae-like microdomains (Leon-Sicairos *et al.* 2005). Caveola-mediated endocytosis seems not to be implicated in the internalization of HLf in THP-1 cells since only reduced or absent expression of Cav-1, a key protein in caveolae structure and functions, was previously detected in THP-1 cells (Llaverias *et al.* 2004). Caveola-dependent endocytosis is a very efficient but also a very slow process, bypassing the acidic endosomal compartments which are specific for the clathrin-mediated pathway.

Traffic and subcellular localization of Lf in THP-1 cells

To monitor the subcellular localization of HLf after internalization in macrophage-like cells, immunofluorescence techniques using various markers for different organelles were employed.

First, experiments were performed to investigate whether Lf resides within endosomal vesicles. As indicated in Figure 5, HLf partially overlapped with EEA-1, a marker specific for early endosomes. Interestingly, in electron microscopy experiments, Bi (Bi *et al.* 1996) also detected the presence of Lf in endosomal compartment, in Jurkat human lymphoblastic T-cell line. The clathrin-dependent endocytosis and recycling endosomes marker Tf, which was demonstrated to be mainly confined in the early endosomes, was shown to partially overlap with Lf in a region situated in the proximity of the cell membrane.

These results indicate that internalized HLf is partially recycled and partially degraded. Our results are in agreement with previous studies performed in Jurkat cells suggesting an uptake regulated by a receptor-mediated process, similar to Tf, but the pathway utilized by Lf after internalization diverged from the route utilized by Tf. The authors demonstrated that, after endocytosis, Jurkat cells released intact as well as degraded Lf into the culture medium, indicating that 30-40% of Lf is degraded at each round of endocytosis (Bi *et al.* 1996).

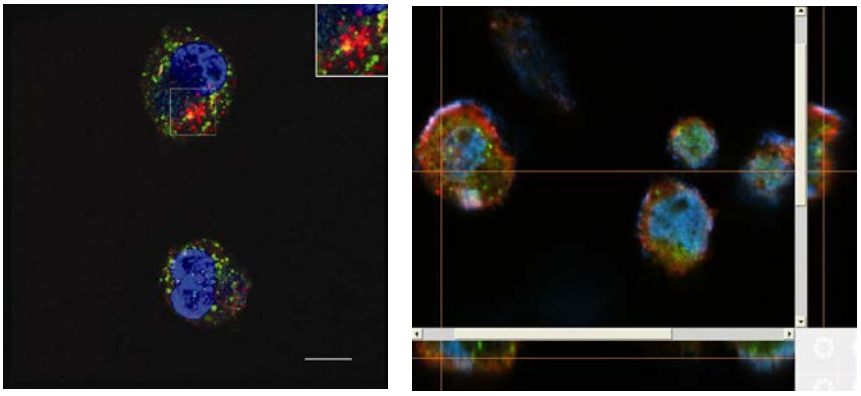

Figure 5. ***Subcellular localization of HLf in THP-1 cells differentiated to macrophages.*** Confocal imaging of cells treated with 0.1 mg/ml HLf-Texas Red (red) for 30 min/ 37 °C, fixed and then immunolabeled for early endosomes (green) by incubation with mouse anti-EEA1 and then goat anti-mouse Alexa Fluor 488-conjugated IgG. Nuclei were visualized with DAPI (blue). Cells were analyzed with a ZEISS LSM 710 confocal laser scanning microscope. Sequential scanning of 0.16-µm sections was used for co-localization studies. Optical sectioning analysis was performed using Axio Vision Rel. 4.8 software. Scale bar 10 µm.

Co-localization of Lf with endoplasmic reticulum marker

HLf intracellular localization was investigated in other subcellular compartments since the presence of HLf in early endosomes was not exclusive. Fluorescence microscopy experiments revealed that Lf co-localizes with the ER compartment. HLf accumulated in a peri-nuclear region in THP-1 macrophage-like cell after a 3.5h of chase at 37 °C, showing a partial overlapping with ER-Tracker, an ER specific cell-permeant

marker (Fig. 6 a-c). **To our knowledge, this is the first evidence that HLf reaches the ER compartment in macrophage-like cells.**

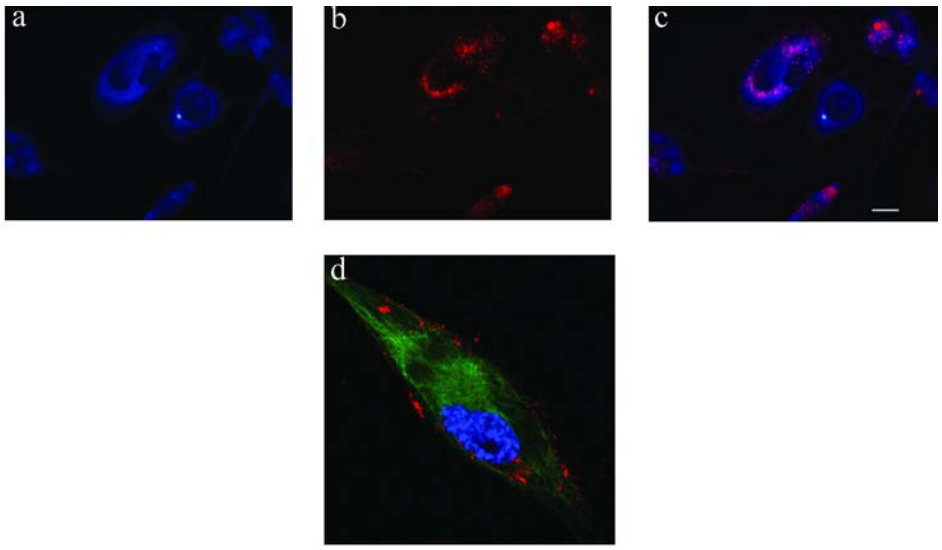

Figure 6. ***ER-colocalization and microtubules traffic of HLf.*** Cells were incubated with 0,1 mg/ml HLf -Texas Red for 30 min./ 37 °C, washed and chased in fresh medium for 3,5 h/ 37 °C. Before visualization, living cells were labeled for 30 min at 37 °C with ERtracker. (a) Image through a blue filter representing ER compartment; (b) Image through a red filter representing HLf; (c) Merged image of panels (a)-(c) representing colocalization of HLf with the ER compartment. Scale bar 10 μm; (d) Confocal imaging of microtubules driven traffic of HLf in THP-1 cells. Cells were treated with 0.1 mg/ml HLf-Texas Red (red) for 30 min/ 37 °C, fixed and then immunolabeled for microtubules (green) by incubation with mouse anti-α-tubulin IgG and then goat anti-mouse Alexa Fluor 488-conjugated IgG. Nuclei were visualized with DAPI (blue).

It is documented that some viruses reach ER compartment by direct or retrograde pathway in order to escape the degradative pathway (Lilley *et al.* 2006). However, this action involves activated caveosomes routes. In our case Lf may target ER compartment in order to bypass the degradative cytoplasmic pathway.

Lf is transported via endosomes along microtubules

Confocal imaging of the microtubules using mouse anti-alpha-tubulin antibodies and goat anti mouse Alexa Fluor488-conjugated antibodies for staining (Fig. 6d) revealed that HLf trafficking appears to be vesicular and dependent on intact microtubule network. In order to understand the biological properties of Lf which are not related to its iron-binding capacity, the trafficking and intracellular localization of Lf proved to be an important research topic. It was reported that in *Vero* cells Lf is taken up by endocytosis and transported *via* endosomes along microtubules towards the nucleus periphery (Marr *et al.* 2009). In this way, Lf intracellular transport manages to delay the intracellular trafficking of the virus and finally affects the replication mechanism by competing with the microtubule transport of HSV-1.

Conclusion

Lf is internalized mainly by the clathrin-mediated pathway in THP-1 cells, reaches the ER and is transported via endosomes along microtubules. The data presented may prove useful for new therapeutic approach involving Lf treatment, taking into account its potential trafficking to the ER compartment and the mechanism of Lf entry into different target cells.

Antiviral activity of lactoferrin

Human and bovine Lf has been shown to possess the capacity to inhibit both naked and enveloped viruses (Berlutti *et al.* 2011). The binding to host cell-surface molecules, such as asialoglycoprotein receptor and heparin, and prevention of binding and accumulation at target cell surface of viral particles was one of the antiviral mechanism proposed (Treichel *et al.* 1997). It was reported that GAGs mediate the binding of bovine Lf to plasma membrane but at the same time mediate the attachment of the adenoviral particle to target cells, and thus the competition that might occur between protein and viral particle for the same receptor molecule could account for the inhibition of the infection of the target cell (Di Biase *et al.* 2003). It was suggested that for the inhibitory effect on adenoviruses infection the cationic N-t of Lf, which contains its major GAG binding region, is required and that the C-t lacks any anti-adenovirus activity. Interestingly, the iron-binding capacity of Lf is not involved in the antiviral mechanism. However, for some viruses such as rotaviruses, apo-bovine Lf exhibits an enhanced capacity of inhibition of the viral infection than the iron-saturated protein (Superti *et al.* 1997). An enhanced antiviral activity was reported in the case of poliovirus, HIV, and herpes simplex virus (HSV) infection when Zn- or Mn-loaded bovine Lf was used (Marchetti *et al.* 1998; Siciliano *et al.* 1999). It has been shown recently that in Vero cells Lf may delay the HSV-1 intracellular traffic to the nucleus along the microtubules, thus inhibiting viral replication, a mechanism which is completely different than interfering with viral entry (Marr *et al.* 2009).

Lactoferricin (Lfcin) is a 47 amino acids long-peptide (residues 1–47) from the N-t region of HLf which is released after pepsin digestion. Bovine Lfcin is a shorter peptide of only 25 amino acids (residues 17–41 of bovine Lf) (Bellamy *et al.* 1992). Many functions of the parental protein have been shown to be preserved in Lfcin, some being even enhanced as compared with the parental molecule in some cases (Gifford *et al.* 2005).

Lfcin proved to be ineffective against hepatitis C virus (HCV), as opposed to Lf. It was reported recently that a 33-residue peptide from C-t region of Lf, known as the Nozaki peptide, was able to bind to the E2 protein of HCV and consecutively prevent HCV infection in a in human liver cells. As compared to the parental protein, the E2-binding capacity and anti-HCV activity of the Nozaki fragment was weaker. The helical secondary structure is required for E2-peptide interaction and efficient viral inhibition, the affinity of binding increasing with the helicity, as demonstrated by Beleid using synthetic helical peptides derived from

the Nozaki fragment (Beleid *et al.* 2008). Unlike Lf, Lfcin was observed to inhibit HIV infection only at a very low level. These results suggests that for the inhibition of the viral entry step other domains from the parental protein may be required (Berkhout *et al.* 2004). Other fragments were shown to inhibit rotaviruses infection although to a lower extent than full-length Lf, such as tryptic fragments of Lf identified as a large fragment (residues 86–258) and a small peptide (residues 324–329: YLTTLK) (Superti *et al.* 2001).

For the enhanced activity of Lf as compared to LFcin peptide, three hypotheses could be proposed: (ii) the role of the interactions in adenovirus infection between various cellular and viral components, in such a way that the inhibition of this event could have several targets; (i) a minor steric hindrance exerted by the polypeptide in the competition with viruses for GAGs binding; (iii) the role of other domains, in addition to those involved in GAG binding, which could be required for the inhibitory capacity of Lf (Seganti *et al.* 2004).

Interestingly, a synergistic effect of common antiviral drugs in combination with Lf or Lfcin was reported in many clinical studies. For example, the anti-HSV activity of acyclovir (ACV), a nucleoside analogue used for inhibition of viral replication, is potentiated by the use of Lfcin. Lf and Lfcin inhibit HSV 1 and 2 infection and exhibit synergy when combined with ACV (Andersen *et al.* 2003).

A schematic representation of the possible mechanisms of antiviral activity of Lf is depicted in Fig. 7.

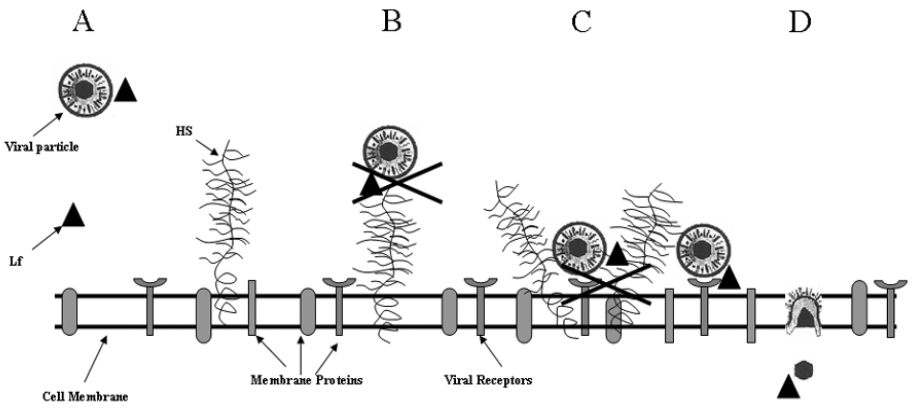

Figure 7. ***Representation of different antiviral modes of action of Lf.*** Lf could prevent viral infection of the host cells either by the direct binding to virus particles (A), or by competing with virus for common receptors/co-receptors (B, C) at the surface of the target cells. Finally, an intracellular activity of Lf has been postulated (D). (Adapted from (van der Strate *et al.* 2001)

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family, and contains a partially double-stranded DNA molecule. A lipid bilayer envelope derived from the host cell, bearing the transmembrane viral surface proteins surrounds the genome (Summers *et al.* 1975; Robinson 1977). For translation of the viral proteins the virus has developed an efficient system: the translation is made from four overlapping genes coding for polymerase, X, surface, and core proteins (Galibert *et al.* 1979). More than 50 million new cases of HBV infection are diagnosed annually and it is reported that around 350 million patients are currently infected, despite the existence of an anti-HBV vaccine in therapeutic programs (Lee *et al.* 1997). However, there is a search for a much needed, alternative treatment since the replication inhibitors which are

the drugs currently used to treat chronic infections with HBV, may induce selection of drug-resistance mutations and were demonstrated to have limited efficacy (Ono-Nita *et al.* 1999).

It was first described in PH5CH8 cells, a non-neoplastic human hepatocytes cell line the ability of Lf to inhibit HBV adsorption on target cells by preventing its interaction with cell-surface molecules (Hara *et al.* 2002). Recently, it was reported that zinc- and iron-saturated bovine Lf, but not a Lf hydrolysate inhibited the HBV-DNA amplification in hepatoma-derived HepG2 cells infected with the virus (Li *et al.* 2009). However, the experiments were performed in HepG2 cells, which possess very low infection efficiency, thus making the conclusions difficult to interpret. The observations could not accurately define the mechanism of the inhibition of HBV infection or if the antiviral capacity of Lf is restricted to some domains or is dependent on the structural integrity of the parental molecule.

In this study, human Lf (HLf) and seven HLf-derived synthetic peptides (HLP) corresponding to the N-t part of the parental protein (1-47 amino acids sequence) were tested for their capacity to prevent hepatitis B virus (HBV) infection and replication using the HepaRG and HepG2.2.2.15 cell lines.

Results and Discussions

Screening of Lf-derived peptides on HBV infection

Whether or not the ability of Lf to inhibit viral infections depends only on its structural integrity it is still controversial. The cationic N-t (1–333 amino acids) of Lf is the most important region enriched in positive charges of the polypeptide chain and this gives it some unique properties. This region is important for the ability of LF to bind to many cell types, since it provides the binding site for heparin and glycosaminoglycans (GAGs) (Mann *et al.* 1994; Wu *et al.* 1995).

All synthetic peptides used in the antiviral screening were designed within the N-t lobe. Single mutations were also introduced in the wild-type sequence of some peptides in order to better understand the structure-function relationship (Fig. 8).

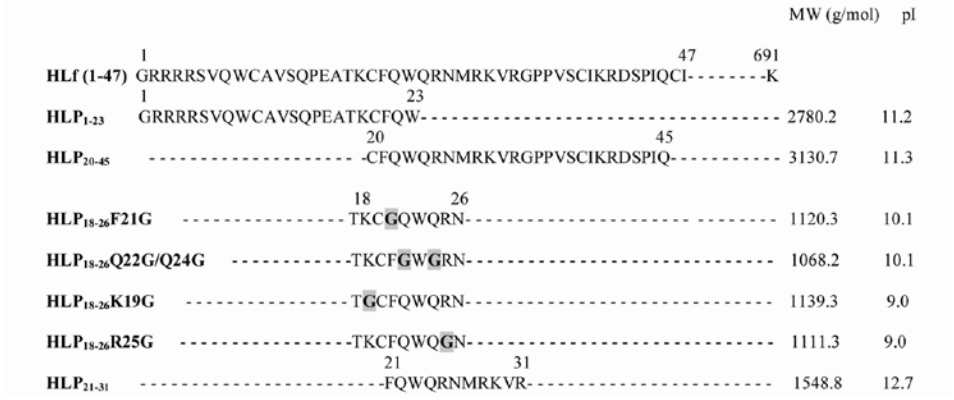

Figure 8. ***Primary structure of HLf (sequence 1-47) and HLf-derived synthetic peptides (HLP) used in the anti-viral experiments.*** The substituted amino acids are highlighted in gray. The molecular weight (MW) and iso-electric point (pI) of the peptides are presented.

The N-t region was first split in two sequences, HLP_{1-23} and $HLP_{20-45,}$ aiming to separate the two cationic clusters (residues 2–5 and 28–31) responsible for most Lf beneficial properties. Based on a report published previously, demonstrating an important antimicrobial activity of this second region in bovine Lf (Tomita *et al.* 1994), a shorter peptide (HLP_{21-31}) was further used in the antiviral screening. Mutant sequences of some specific amino acids were also taken into account (HLP_{18-26}F21G, HLP_{18-26}Q22G/Q24G, HLP_{18-26}K19G, HLP_{18-26}R25G), since it was shown previously that the replacement of either aromatic or charged residues with glycine within the 18–26 region may alter the hydrophobicity and flexibility of the peptides and modulate their activity (Farnaud *et al.* 2004; Moriarty *et al.* 2004).

Using the highest, nontoxic concentration of peptides, a primary screening of the anti-HBV activity of these Lf-derived peptides was conducted in HepaRG cells (Fig. 9).

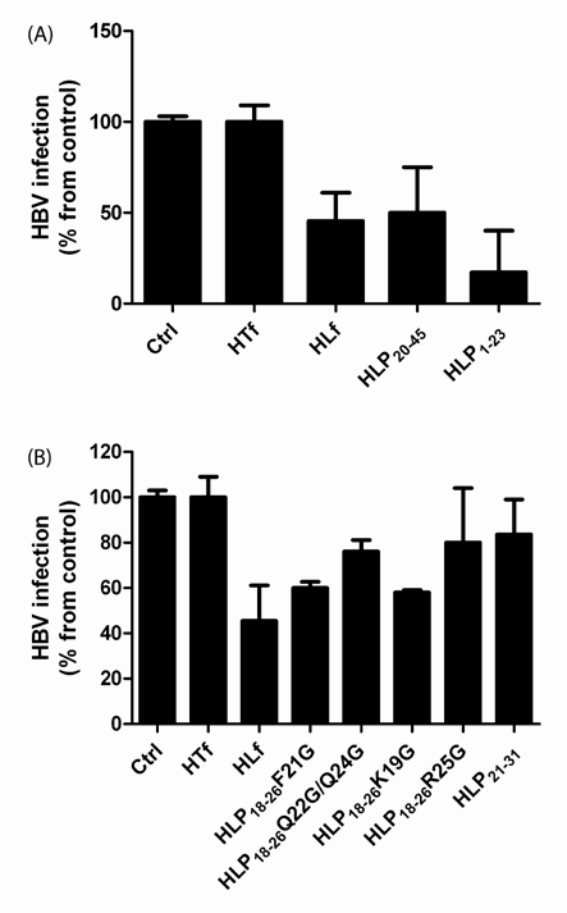

Figure 9. ***Effect of HLP against HBV infection in HepaRG cells.*** Differentiated cells were infected with 50 µl of HBV inoculum containing 1 x 10^8 GEq. HLP were added together with the viral inoculum at either 250 (A) or 500 µM (B) and incubated for 16 h/ 37 °C. The amount of HBV-specific RNA or encapsidated DNA in cells was quantified by reverse transcription (RT) real time-PCR (A) or real-time PCR (B), respectively, at day 11 p.i.. The results were normalized to a β- actin internal control and expressed as percentages of HBV replication from control (Ctrl). Ctrl refers to untreated, HBV-infected HepaRG cells. Data are means ± standard deviation of triplicate samples. Each experiment was repeated at least three times (Florian *et al.* 2013).

Peptides were added together with the viral inoculum, maintained during infection, and then removed in order to evaluate the overall antiviral effect comprising both an interaction with the target cell or the virus. The longer peptides (23–25 amino acids) tended to form a fine precipitate when used at a concentration of 500 µM, unlike the short ones (9–11 amino acids); therefore, their concentration was lowered to 250 µM (Fig. 9A). HTf, a member of the transferrin family with no antiviral activity, and HLf, were also included as

negative and positive controls, respectively, at concentrations normally used in other antiviral assays (1 mg/ml, the equivalent of 12,5 µM) (Lazar *et al.* 2007).

The level of HBV replication was monitored in infected HepaRG cells, as a direct measure of viral entry, at 11 days p.i. Within the first series of peptides investigated, HLP_{1-23} proved to be a potent peptide in the terms of antiviral activity (about 80 %), while HLP_{20-45} showed a more moderate effect (50 % inhibition) (Fig. 9A). HLf inhibited HBV infection by about 55% (Fig. 9A) and HTf had no antiviral activity, as expected, confirming the specificity of the infectivity assay. No relevant antiviral effect was detected from the peptides of the second series of shorter sequences, despite being used at a higher concentration of 500 µM (Fig. 9B).

Of the series tested, four peptides showed 40 to 75% inhibition of HBV infection in HepaRG cells, HLP_{1-23}, containing the GRRRR cationic cluster, one of the two glycosaminoglycan binding sites of the native HLf involved in its antiviral activity being the most potent.

HLP_{1-23} as a candidate for anti HBV infection in early steps

Dose-dependence antiviral effect

The infection of HepaRG cells by HBV was inhibited by HLP_{1-23} in a dose-dependent manner, as presented in Fig. 10.

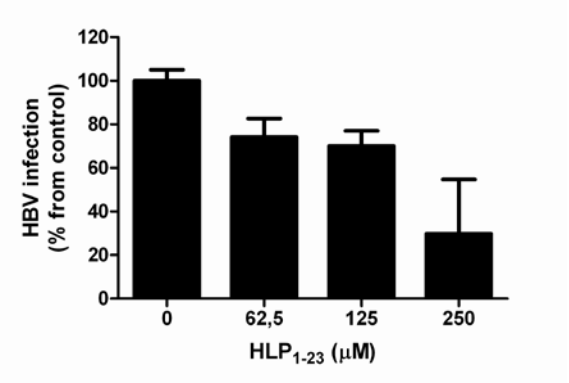

Figure 10. ***HLP_{1-23} inhibits HBV infection in a dose-dependent manner.*** HLP1-23 was added to differentiated HepaRG cells at a final concentration of 62.5, 125 and 250 µM and incubated together with the virus inoculum for 16 h/ 37 °C. The level of HBV-specific encapsidated DNA in cells was quantified by real-time PCR at day 11 p.i. The results were expressed as percentages of HBV replication from control (no treatment). Data are means ± standard deviation of triplicate samples. Each experiment was repeated at least three times (Florian *et al.* 2013).

Time of addition assay on HLP_{1-23}

The antiviral activity of HLf depends on the time of treatment, being associated with its capacity to interact with cell surface GAGs, thus preventing the virus particles binding to these secondary, non-specific receptors, as it was previously reported (Berlutti *et al.* 2011). Briefly, differentiated HepaRG cells were treated with the peptide at a concentration of 250 µM either before viral inoculation, or at 24 h after removal

of the HBV inoculum. As a control for inhibition of HBV entry HLf was employed. The level of HBV replication was measured at day 11 p.i. (Fig. 11).

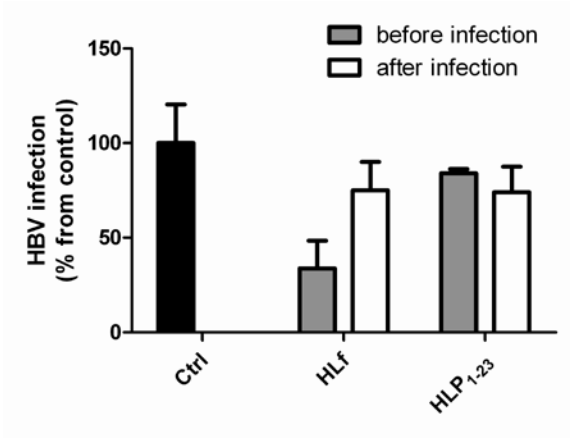

Figure 11. ***HLf and HLP$_{1-23}$ inhibit HBV infection through different mechanisms.*** Differentiated HepaRG cells were infected with 50 µl of HBV inoculum containing 1 x 10^8 GEq. HLf (1 mg/ml) and HLP$_{1-23}$ (250 µM) was added before viral infection (1 h/ 37 °C) or after viral infection (24 h/ 37 °C) and cells were further incubated with fresh medium for 11 days. The level of HBV-specific encapsidated DNA in cells was quantified by real-time PCR. The results were expressed as percentages of HBV replication from control (untreated sample). Data are means ± standard deviation of triplicate samples. Each experiment was repeated at least three times (Florian *et al.* 2013).

HLf treatment resulted in significant antiviral effect when the cells were treated with the protein before infection, as expected, confirming that plasma membrane molecules are its primary target (Fig. 11, grey bars). On the contrary, HepaRG cells treated with the protein after viral entry led to a nearly complete loss of the inhibitory effect (Fig. 11, white bars).This finding supports the hypothesis previously described concerning the anti-HBVmechanism of action of HLf in non-neoplastic human liver cells PH5CH8 (Hara *et al.* 2002).

Treatment of the cells with the peptide before viral inoculation had no effect on the infection outcome, unlike the full-length protein HLf providing evidence that the HLP$_{1-23}$ peptide does not act on cell-surface molecules. Moreover, addition of HLP$_{1-23}$ after virus entry step has no consequence on the efficiency of HBV infection, implying that the initial steps of the viral life-cycle are also not modulated by the peptide treatment (Florian *et al.* 2013).

The results strongly suggest that HLP$_{1-23}$ acts neither by preventing viral attachment to the HepaRG cells nor by inhibiting early post-entry steps (Florian *et al.* 2013**).**

HBV viral particle- HLP$_{1-23}$ interaction

A possible peptide-viral particle interaction was further investigated. by incubation of increased concentrations of HLP$_{1-23}$ with ten fold dilutions of purified HBV for 1 hr at 16 ^{o}C, to permit peptide attachment to the viral particles.

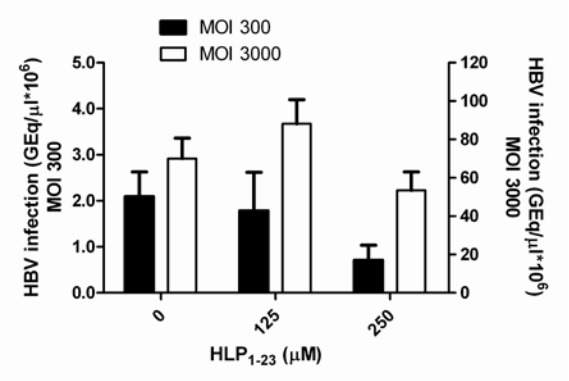

Figure 12. ***HLP$_{1-23}$ inhibits HBV infection by binding to HBV particle.*** HLP$_{1-23}$ at a final concentration of 125 and 250 µM was incubated together with the virus inoculum for 1 h before infection. The level of HBV-specific encapsidated DNA was detected in cells by real time PCR at day 11 p.i. The results were expressed as percentage of HBV infection from control (no treatment). Data are means ± standard deviation of duplicate samples and were obtained from two independent experiments (Florian *et al.* 2013).

HLP$_{1-23}$ succeeded in inhibiting the HBV infection by 75 %, at the highest concentration used when the HepaRG cells were inoculated with HBV at a multiplicity of infection (MOI) of 300 GEq/cell, as shown in Fig. 12. By increasing the MOI by 10-fold, a more moderate effect of 30 % inhibition at the same concentration of the peptide was recorded (Florian *et al.* 2013).

The data suggest that the peptide HLP$_{1-23}$ has a different anti-HBV mechanism of action as compared to the full-length molecule. Taken together, the results obtained point to viral particles being the target of the peptide, since the strong inhibition by HLP$_{1-23}$ was observed in two experimental settings: i) when the HBV inoculation occurred simultaneously with the peptide treatment and ii) when the peptide was incubated with the virus before infection. The lower efficiency of the HLP$_{1-23}$ as compared to the native molecule when used at the same concentration is explained by the fact that the peptide acts at a different step of the HBV life cycle.

It can be hypothesized that the presence of a cationic region of the HLP$_{1-23}$ peptide is sufficient for the peptide to act as positively charged ligand which interact stably with negatively charged residues on the virion envelope, while the absence of the second glycosaminoglycan binding domain in the peptide sequence prevents its attachment to the cell membrane. **It is suggested that HLP$_{1-23}$ prevents HepaRG infection by neutralizing virion sites involved in cell binding (Florian *et al.* 2013).**

Conclusion

The HLP$_{1-23}$ peptide may be part of a future non-toxic therapeutic approach for potential clinical applications in preventing HBV infection by neutralizing the viral particles. Further studies are needed to establish the molecular details of HBV inhibition by HLP$_{1-23}$ and how this activity can be improved.

Conclusions

1. HLf is using mainly a clathrin-dependent route for internalization in THP-1 macrophage like cells, a pathway also dependent on cholesterol depletion and endosome acidification.

2. Dominant negative experiments on THP-1 Dyn-2 K44A cell line showed that the inhibition of internalization of Lf in the transduced cell lines is obtained only in the presence of specific endocytic inhibitors, the level of inhibition being similar to that observed in parental cell line. Moreover, if clathrin-dependent endocytosis is blocked, cav-1 dependent-endocytosis of HLf can be induced.

3. HLf partially co-localize with EEA-1, a marker specific for early endosomes. Only minimal co-localization with HLf is observed, demonstrating that the two proteins have different distribution pattern. Partial overlapping with lysosomes compartment was detected suggesting that a small part of the internalized HLf is recycled and a small part is taken to a degradative pathway.

4. It is shown for the first time that Lf targets the ER and is transported *via* endosomes along microtubules. In addition, the data may prove useful for the design of new therapies involving Lf treatment, taking into account the mechanism of Lf entry into different target cells and its potential trafficking to the ER compartment.

Based on the results obtained, a mechanism for HLf endocytic process and intracellular targets in THP-1 macrophage-like cells is proposed (Fig. 13).

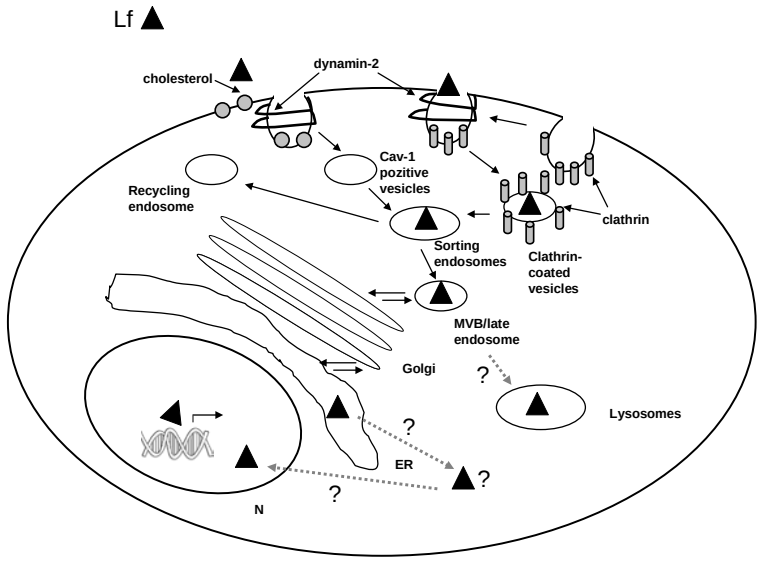

Figure 13. ***Schematic representation of HLf endocytosis and intracellular pathways in THP-1 cells.***

5. HLf is able to induce an increase in IL-6 expression in a dose-dependent manner in THP-1 cells differentiated to macrophages. The mechanism involves transactivation from IL-6 promoter *in vitro*, as revealed by reporter gene assay. Moreover, preliminary results on DNA binding analysis *in vivo* show that Lf binds poorly on nuclear fractions extracted from cells transfected with Lf. These findings suggest that Lf up-regulates transcription of IL-6 cytokine gene from IL-6 promoter and the process is DNA-binding dependent.

6. The results on antiviral properties of Lf and synthetic Lf-derived peptides on HBV infectivity reveal that four peptides show 40 to 75% inhibition of HBV infection in HepaRG cells, HLP_{1-23} containing the GRRRR cationic cluster, one of the two glycosaminoglycan binding sites involved in the antiviral activity, being the most potent. Infection of HepaRG cells is inhibited by HLP_{1-23} in a dose-dependent manner. HLP_{1-23} acts neither by preventing viral attachment to the HepaRG cells nor by inhibiting early post-entry steps. It is suggested that HLP_{1-23} prevents HepaRG infection by neutralizing virion sites involved in cell binding. However, HLP_{1-23} is not an inhibitor of HBV replication, as demonstrated on HepG22215 cells supernatants analysis.

Based on the results from the HBV experiments, an antiviral mechanism of action of the HLP_{1-23} peptide is proposed (Fig. 14).

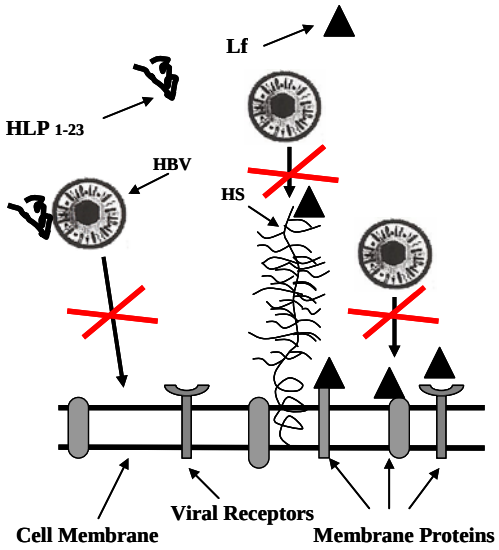

Figure 14. ***Schematic representation of the anti-HBV mechanism of HLP_{1-23}.***

List of published papers

1. **Florian PE**, Macovei A, Lazar C, Milac AL, Sokolowska I, Darie CC, Evans R W, Roseanu A, Branza-Nichita N. Characterization of the anti-HBV activity of HLP1-23, a human lactoferrin-derived peptide. *J Med Virol*.2013; 85(5): 780-788 Impact factor: 2.82
2. **Florian P**, Moisei M, Trif M, Evans RW, Roseanu A, Anti-inflammatory and anti-tumoral activity of human recombinant lactoferrin.*Rom J Biochem*. 2012; 49(2):2163-171
3. **Florian P**, Macovei A, Sima L, Nichita N, Mattsby-Baltzer I, Roseanu A. Endocytosis and trafficking of human lactoferrin in macrophage-like human THP-1 cells (1).*Biochem Cell Biol*. 2012 Jun;90(3):449-55. doi: 10.1139/o11-090. Epub 2012 Mar 1 Impact factor: 2.67
4. Roseanu A, **Florian PE**, Moisei M, Sima LE, Evans RW, Trif M. Liposomalization of lactoferrin enhanced its anti-tumoral effects on melanoma cells. *Biometals*. 2010 Jun;23(3):485-92. doi: 10.1007/s10534-010-9312-6. Epub 2010 Feb 27 Impact factor: 2.82
5. Roseanu A, **Florian P**, Condei M, Cristea D, Damian M. Antibacterial activity of Lactoferrin and Lactoferricin against oral Streptococci. *Rom Biotech Lett***.** 2010; 15(6):113-120 Impact factor: 0.349
6. **Florian P**, Trif M, Evans RW, Roseanu A. An overview on the antiviral activity of lactoferrin. *Rom J Biochem*. 2009;46(2):187-197
7. Constantinescu C, Papavlu A, Rotaru A, **Florian P**, Chelu F, Dinca V, Roseanu A, Dinescu M. Multifunctional thin films of lactoferrin for biochemical use deposited by MAPLE technique. *Appl Surf Sci***.** 2009;255(10):5491-5495 Impact factor: 2.103

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