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Differential Modulation of Human Lactoferrin Activity against Both R5 and X4-HIV-1 Adsorption on Epithelial Cells and Dendritic Cells by Natural Antibodies

Héla Saidi, Jobin Eslaphazir, Cédric Carbonneil, Laetitia Carthagena, Mary Requena, Nadine Nassreddine, and Laurent Belec

Human lactoferrin (Lf) is an iron binding glycoprotein that is present in several mucosal secretions. Many biological functions have been ascribed to Lf. In the present study, we showed that Lf limited specifically adsorption of R5- and X4-HIV-1-free particles on endometrial epithelial HEC-1A cells, by inhibiting virus adsorption on heparan-sulfated proteoglycans. But, Lf did not interfere with both R5 and X4-HIV transcytosis. We showed also the efficacy of Lf in preventing R5 and X4-HIV capture by dendritic cells. Conversely, we demonstrated that Lf-reacting natural Abs (NAbs) present within i.v. Ig-enhanced HIV attachment on dendritic cells by forming HIV-Lf-NAbs. HIV particles were able to directly interact with Lf following its interaction with NAbs. We also found Lf-reacting natural Abs within cervicovaginal secretions, suggesting the existence of Lf-NAbs complexes in women genital tract in vivo. In conclusion, this study highlights Lf as a potent microbicide and reports new function for NAbs within the genital compartment that may compartment that may abolish the inhibitory activity of microbicidal compounds. Thus, we proposed a model in which Lf would appear as a double-edged sword that could have beneficial or detrimental effects depending on both cellular and molecular environments. This study highlights the use of Lf derivates as microbicide candidates to limit such interferences.

Sexual transmission of HIV type 1 (HIV-1) is the major way of HIV transmission worldwide. For sexual transmission to occur, HIV-1 must cross the pluristratified or monostratified genital epithelia (1). This passage can occur by physical breaches, transcytosis (2), or after capture by intraepithelial dendritic cells (DC). Subsequently, to the HIV capture, the DC transfer the virus to CD4+ T cells, simultaneously activating these CD4+ T cells to produce high levels of HIV (3, 4). Such transfer can occur locally in inflamed mucosa or following DC maturation and migration toward local lymph nodes.

Since HIV is transmitted mainly during heterosexual intercourse through the vaginal mucosa; new methods of HIV prevention that can be controlled by women are urgently needed. These methods include the use of microbicidal molecules able to prevent HIV entry through mucosa (5). Topical HIV-1 inhibitors may target the incoming virus at several steps of molecular events driving viral entry (6). Inhibitors interfere with the interaction between the HIV envelope glycoprotein gp120 and its host cell receptors and/or with the fusion stage. Some HIV inhibitors can disturb viral membrane. However, viral membrane is derived from the host cells from which it buds. Thus, the cytotoxicity for host primary cells of a topical microbicide molecule and its effect on the maintenance of the integrity of a tight epithelial cells monolayer are an important issue (7–9).

Human lactoferrin (Lf) is an 80-kDa cationic glycoprotein belonging to the transferrin family of Fe3+-transporting protein (10). Lf is produced by epithelial cells and, as a result, is present in mucosal secretions (11) such as seminal and vaginal fluids (12). One of the functions (13, 14) of Lf is the transport of metals, but Lf is also an important component of the nonspecific immune system since Lf has a broad spectrum of activity against a variety of bacteria, fungi, and viruses (15). The antiviral effect of Lf lies in the early phase of infection by inhibiting attachment of viruses as alphavirus (16) to heparan sulfate glycosaminoglycans expressed on cell surface. Taken together, Lf would be evaluated as candidate microbicidal molecule.

The female genital tract possesses various systems of defenses against the infectious risk, which appear complementary, additive, and even synergistic (17). These defenses comprise first nonimmune strategies, passive (synthesis of protective mucus; pH; epithelial barrier) or active (inflammatory reaction; secretion of soluble factors such as lactoferrin), which are likely very efficient to limit the infectious inoculum before antigenic stimulation. When these initial lines of defenses have failed, a third strategy, acquired and specific of the pathogen, occurs progressively (18–20). Irrespective of any immunization procedure, female genital secretions contain very high amount of IgG, at levels >10-fold those of IgA, and originating in part from plasma by transudation (19). These Abs are polyreactive molecules that can recognize different unrelated epitopes. Thus, natural polyreactive Abs lining the female
genital mucosae may hamper the penetration of a pathogen just before that acquired defenses specific of the pathogen may be involved (18, 21, 22).

In this study, we showed that Lf limited specifically attachment of R5- and X4-HIV-1-free particles but not viral transcytosis across a monolayer of endometrial HEC-1A cells, probably by inhibiting virus attachment on heparan sulfate proteoglycans (HSPG). We showed the efficacy of Lf in preventing HIV uptake by DC. Conversely, we demonstrated that Lf-reacting natural Abs (NAbs) present within i.v. Ig (IV Ig)-enhanced HIV attachment on DC by forming HIV-LF-interacting immune complexes. We also found these Lf-reacting NAbs within cervicovaginal secretions (CVS). This study highlights Lf as a potent natural microbicide and reveals a new function for NAbs within the genital compartment, which are able to limit the activity of microbicide compounds.

Materials and Methods

Cells lines and virus strains

Epithelial endometrial cell line HEC-1A was maintained in RPMI 1640 containing FCS and antibiotics (100 μg of streptomycin/ml and 100 U of penicillin/ml; Invitrogen Life Technologies). The primary X4-HIV-1b simplified and the laboratory adapted strain X4-HIV-1bLA (gifts from Prof. F. Barré-Sinoussi, Institut Pasteur, Paris, France) were grown in PBL of healthy donors stimulated with PHA-P and IL-2. Primary R5-HIV-1bCSF and R5-HIV-1bLA were amplified in monocyte-derived macrophages of healthy donors. Tropism of viruses was determined using U87 cells (provided by Dr. E. Menuet, Institut Pasteur) transinfected with DNA encoding for human CD4 and CCR5 or CXCR4. HIV was quantified in cell culture supernatants by using the p24 Ag capture ELISA (HIV-1 core profile ELISA; DuPont de Nemours, Les Ulis, France, detection limit 5 pg/ml).

Therapeutic IV Ig

Octagam (Octapharma), a therapeutic preparation of pooled normal IgG from plasma of healthy donors, was used in this study. A stock solution of IV Ig (50 mg/ml; 0.3 mM) was prepared in PBS and was dialyzed twice against large volume of PBS at 4°C to remove the stabilizing compounds. Concentration of IV Ig reacts with metabolically active cells. The formazan crystals were solubilized 30 min at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well was measured in a microtiter reader at 490 nm. To translate the OD490 values into the number of live cells in each well, the OD490 values were compared with those of standard OD490 vs cell number curves generated for each cell type. The percentage survival was calculated using the formula: percentage of survival = (live cell number (test)/live cell number (control)) × 100.

Epithelial monolayer integrity

The effect of Lf on its ability to maintain an intact epithelium was determined by measuring transepithelial resistance (TER). HEC-1A was grown as a tight polarized monolayer on a permeable support of 0.4-μm pore diameter polycarbonate (Transwell; Costar) (2). Apical and basolateral media were replaced, and TER was measured daily with a Millicell-ERS (Electrical Resistance System) instrument (Millipore). When plateau TER was reached, Lf or media alone was added in duplicate wells, and the TER was measured at 30 min and 2, 4, 9, and 24 h. The epithelial resistance was expressed as follows: epithelial resistance = (Ω × cm) − the resistance of Transwells without cells.

HIV adsorption assay on epithelial cells

HEC-1A cells, seeded at confluence in 48-well plates, were incubated with increasing concentrations of Lf and HIV-1 (5 ng of p24 Ag). Each sample was performed in duplicate. After 1 h of incubation, four washes removed unattached virus, and cells were lysed by adding 1% Triton X-100 for 45 min at 37°C, and the concentration of HIV p24 Ag was measured by p24 Ag capture ELISA.

HIV cell-free particles transcytosis assay

Epithelial cell line HEC-1A was grown as a tight polarized monolayer on a permeable support of 0.4-μm pore diameter polycarbonate (Transwell; Costar) as described previously (2). The tightness of the monolayer of HEC-1A cells was monitored by measuring resistivity above 300 Ohm·cm². HIV-1 (5 ng of p24 Ag) was added together with increasing concentrations of Lf on the apical side of the HEC-1A monolayer at 37°C for 1 h. HIV-1 transcytosis was assessed by detecting the presence of p24 Ag (HIV-1 core profile ELISA) in the basolateral chamber of the Transwell.

Generation and culture of immature monocyte-derived DC (iMDDC)

iMDDC were generated from monocytes as described. Briefly, PBMC were isolated from cryopreservation of healthy adult donors by Ficoll density gradient centrifugation on medium for separation of lymphocytes (Eurobio). The percentage of monocytes was determined by flow cytometry using forward scatter and side scatter properties. PBMC were resuspended in RPMI 1640 medium supplemented with glutamine (BioWhittaker Europe), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen Life Technologies). Cells were seeded into 24-well-plates (Costar) at the concentration 1 × 10⁶ adherent cells/ml. Cells were incubated at 37°C for 45
min. Nonadherent cells were removed by four gentle washes. Adherent monocytes were incubated in RPMI 1640 medium with 10% FCS, glutamine, and antibiotics in the presence of recombinant human IL-4 and recombinant human GM-CSF (10 ng/ml) to differentiate to iMDDC. Half the medium, including all supplements, was replaced every 2 days. After 6 days of culture, nonadherent cells corresponding to the DC-enriched fraction were harvested, washed, and used for subsequent experiments. Flow cytometry analysis (BD Biosciences) demonstrated that iMDDC were 90% or more pure.

**HIV adsorption on iMDDC**

iMDDC cells were washed twice after 6 days of differentiation and seeded into 96-well culture plates (10^5 cells/well). In some experiments cells were pretreated using FcR blocking reagent (Miltenyi Biotec). HIV-1 (1 ng of p24 Ag) and increasing Lf concentrations with or without anti-Lf Abs or total IVIg were coincubated with cells at 37°C for 1 h. Each sample was performed in triplicate. After four washes to remove the unattached virus, cells were lysed by incubation for 45 min at 37°C with PBS and 1% Triton X-100. The amount of cell-associated HIV was evaluated using p24 Ag capture ELISA.

**Reactivity of Lf or Lf-immune complexes with HIV cell-free particle**

Microtiter plates (Maxisorp; Nunc) were left uncoated or were coated with either 50 μg of Lf or with 50 μg/ml rabbit anti-human Lf polyclonal Abs (Ab anti-Lf) at 4°C for 16 h. Following four washes with 1% PBS and 0.5% Tween 20, wells were further incubated with or without 50 μg of Lf in the presence or absence of anti-Lf Ab at 37°C for 1 h. Wells were washed four times, and R5 or X4-tropic HIV (1 ng p24 Ag) was added at 37°C for 1 h. After four washes to remove excess virus, HIV particles were lysed by incubation for 45 min at 37°C with PBS and 1% Triton X-100. The concentration of HIV was measured by p24 capture ELISA. For positive control, wells were first incubated with anti-HIV gp120 2G12 (10 μg/ml) (National Institutes of Health AIDS Reagent Program).

**Assessment of infectiousness of Lf treated HIV-particles**

HIV (1 ng of p24 Ag) was coated on poly-l-lysine precoated 96-well plates (Greiner Bio-One) at 4°C overnight. After washes, plates were incubated or not with 50 μg of Lf at 37°C for 1 h. In positive and negative control wells, PBS and 1% Triton X-100 and PBS were added, respectively. After four washes, 10^5 stimulated peripheral blood lymphocytes were added per well. After 6 days, viral production was assessed by p24 Ag capture ELISA.

**Reactivity of IVIg and CVS Ig with Lf**

Microtiter plates (Maxisorp; Nunc) were coated with 10 μg of Lf in PBS at 4°C overnight. Wells were washed with four times with PBS and 0.5% Tween 20 before saturation with PBS containing 1% gelatin. Plates were then incubated with sequential dilution of IVIg, concentrated CVS, Lf-reactive IVIg, or Lf-reactive-depleted IVIg were added for 1 h at 37°C. After washing, plates were incubated with secondary biotinylated mouse-anti-human Fab Abs at 37°C for 1 h. Three negative controls were used: 1) in uncoated wells, the highest dilution of IVIg or CVS followed by the secondary biotinylated mouse-anti-human Fab Abs were added; 2) in uncoated wells, the secondary biotinylated mouse-anti-human Fab Abs were added; and 3) wells were coated with Lf and secondary biotinylated mouse-anti-human Fab Abs were then added. Streptavidin was further added at room temperature for 30 min. The substrate o-phenylenediamine was finally added, and the OD was read at 490–650 nm.

**Determination of avidity of purified Abs to lactoferrin**

The relative avidity of anti-Lf Abs purified from IVIg of seronegative women was assessed using a potassium thiocyanate (KSCN) dissociation assay. We have chosen to compare their avidity to the one of anti-CCR5 Abs purified from IVIg (26). We used the CCR5 peptide CSSHF PYSQYQFWKNQTLK that corresponds to the second extracellular loop of CCR5 (II/E/CCR5). Similarly to the anti-Lf Abs, anti-CCR5 Abs were purified from IVIg by immunoaffinity, as described previously (26). Briefly, 100 μl per well of a Lf solution at 10 μg/ml or 25 μg of the CCR5 peptide were coated on 96-well plates (Maxisorp; Nunc) overnight at 4°C. After saturation with PBS containing 1% gelatin for 1 h at 37°C, the plates were further incubated with 40 μg/ml purified Abs for 2 h at 37°C. After several washes, the plates were treated with KSCN at increasing molarities from 0.1 to 2 M for 30 min at room temperature. The plates were washed and incubated with peroxidase-labeled goat anti-human F(ab’)2, for 1 h at 37°C. After extensive washes, substrate was added, and the peroxidase activity was revealed at 490 nm. The relative avidity (avidity index) was expressed as the molar concentration of KSCN inducing a decrease of 50% in the OD of the control value obtained in the absence of KSCN.

**Immuoaffinity purification of Lf reactive Abs from IVIg or CVS**

Lf (2 ng) was coupled to activated Sepharose 4B according to the manufacturer’s instructions. Pools of CVS from healthy HIV− PSA− women (n = 22) or IVIg were allowed to interact with the matrix overnight at 4°C before extensive washing of the column with PBS until the OD of the effluent reached 0.001. The column was then eluted with 0.2 M glycine-HCl (pH 2.5). The pH of eluted material was rapidly neutralized with 1 M Tris-HCl (pH 8.3) and further dialyzed against PBS overnight.

**Statistical analysis**

A paired Student’s t test was used to determine the statistical significance of the data. A value of p < 0.05 was considered the level of statistical significance.

**Results**

**Binding of Lf is mostly mediated by HSPG on endometrial epithelial cells**

We first checked the ability of endometrial epithelial cell line HEC-1A to bind with Lf. As observed by immunofluorescence confocal laser microscopy, Lf could be efficiently adsorbed on the surface of epithelial cells. Interestingly, when epithelial cells were incubated with anti-Lf Abs without exogenous Lf, weak fluorescence intensity was observed on the cells suggesting a production of endogenous Lf by these endometrial cells (Fig. 1A). These results were further confirmed using flow cytometry (Fig. 1B).

It was previously reported that Lf has an affinity for heparin sulfate moieties. We thus assessed the attachment of Lf on heparininate III-treated epithelial cells. In treated cells, the attachment...
of Lf is inhibited by 88% (Fig. 1B), suggesting a preponderant attachment of Lf on HSPG.

**Lf efficacy against HIV-1 adsorption on epithelial cells**

It was shown that HSPG are absolutely required for HIV-1 attachment to macrophages and endothelial cells (25). The activity of Lf was then evaluated in HIV-1 capture experiments on epithelial endometrial cells. HEC-1A cells were exposed to cell-free CCR5-tropic (HIV-1Ba-L), CXCR4-tropic (HIV-1NDK, HIV-1Lai), or dual tropic (HIV-1_89.6) isolates in the presence of increasing concentrations of Lf. Whatever the tested viruses, Lf inhibited attachment of HIV-1 on epithelial cells in a dose-dependent manner (Fig. 2A).

**FIGURE 2.** Evaluation of Lf inhibitory activity on HIV adsorption on epithelial cell line (HEC-1A). A, HEC-1A were coincubated with increasing concentrations of Lf and one of the four HIV-1 viral strains for 1 h. Cells were lysed, and the concentration of HIV-p24 associated to cell lysates was evaluated by ELISA. Results are expressed as percentage ± SD of the mean of inhibition of HIV adsorption on HEC-1A cells. B, HEC-1A were incubated with Lf (100 µg/ml) and one of the four HIV-1 viral strains in the presence or absence of increasing concentrations of polyclonal anti-Lf Abs for 1 h. Cells were lysed, and the concentration of HIV-p24 associated to cell lysates was evaluated by ELISA. C, Evaluation of Lf toxicity on HEC-1A. HEC-1A cells were cultured with increased concentrations of Lf (ranged from 1 to 200 µg/ml) for 24 h. After washing, culture viability was determined by using the MTT-cytotoxicity assay according to the manufacturer’s instructions. The values given are the percentage of viability ± SD of epithelial cells. All results are representative of three independent experiments, and assays were performed in duplicates.

In addition, the inhibitory effect of Lf reached a plateau at 100 µg/ml for all tested viruses. Interestingly, Lf had a better inhibitory activity on X4-HIV$_{LAI}$ and -HIV$_{NDK}$ viral strains (73.8 ± 0.9% and 58.3 ± 2.4% of inhibition, respectively) than R5-HIV$_{Ba-L}$ strain (54.6 ± 0.9% of inhibition). The lower inhibition was observed in the presence of dual tropic HIV$_{89.6}$ strain (42.8 ± 1.1% of inhibition). When rabbit anti-human Lf Abs were added before addition of Lf, we did not observe any inhibition (Fig. 2B). This result suggests that inhibition of adsorption on epithelial cells by Lf was specific.

To evaluate if this inhibitory effect is due to Lf-induced toxic effect toward epithelial cells, a standard cytotoxicity assay was performed. As depicted in Fig. 2C, no cytoxicity on HEC-1A was induced by Lf whatever the tested concentration (below 200 µg/ml). In addition, Lf did not change the pH of the medium significantly up to a concentration of 500 µg/ml (data not shown).

**Lf does not inhibit HIV transcytosis through a tight epithelial cells monolayer**

Although HEC-1A cells do not become productively infected, they have the capacity to transfer HIV-1 through the monolayer epithelium. To evaluate the capacity of Lf to inhibit this transfer, a dual-chamber model was used. The apical chamber of this model consisted of a confluent monolayer of HEC-1A, whereas the basal chamber contained fresh medium. Cell-free virus (HIV-1JR-CSF or HIV-1NDK) was added to the apical surface of HEC-1A cells with increasing concentrations of Lf. HIV-transverse through the monolayer was assessed by quantification of p24 Ag present in the basal chamber after 24 h of incubation. As shown in Fig. 3, A and B, Lf did not inhibit transcytosis of cell-free HIV-1 whatever the tropism of the viruses.

In addition, to control that Lf did not alter the integrity of the epithelial barrier, the conductivity between apical and basal chambers was measured before and after Lf incubation. Conductivity remained constant (200 Ω/cm$^2$) during all the experiment, thus excluding any effect of Lf on epithelial barrier integrity (Fig. 3C).

**Efficacy of Lf against HIV-1 capture by DC**

As DC are reported to be involved in HIV mucosal transmission, we further investigated the effect of Lf on HIV-1 capture by iMDDC. Similarly to epithelial cells, absence of toxicity of Lf toward iMDDC was firstly confirmed (Fig. 4A). iMDDC were then incubated with cell-free HIV-1$_{Ba-L}$ with or without Lf. As depicted in Fig. 4B, results show that Lf significantly reduced the amount of HIV-1 particles captured by iMDDC by 63.2 ± 1.8%. Surprisingly, coincubation of anti-Lf Abs with Lf and HIV dramatically increased, in a dose-dependent manner, the adsorption of HIV on iMDDC, reaching 3.9 ± 0.3- and 7.3 ± 0.3-fold increase for 5 and 10 µg/ml rabbit anti-human Lf Abs, respectively, as compared with HIV adsorption on Lf-treated iMDDC. Thus, these data suggest that Lf-immune complexes can facilitate HIV binding on iMDDC as suggested by the 45.9 ± 1.1% and 167.5 ± 0.4% increase of HIV attachment on Lf-immune complexes-treated iMDDC, as compared with HIV attachment on untreated iMDDC.

**Modulation of Lf antiviral activity by interaction with Abs**

To explain how anti-Lf Abs enhanced attachment of HIV in presence of Lf, we developed an ELISA to evaluate the affinity of Lf to a whole HIV particle. Thus, Lf was coated on plates and virus was added. After washes to remove unattached virus, Lf-bound virus was evaluated by p24-ELISA. As depicted in Fig. 5, coated Lf has no affinity for HIV, whatever the tropism of the virus. Conversely, when anti-Lf Abs were coated prior Lf addition and HIV
incubation, adsorbed virus was detected, suggesting a direct binding between HIV and Lf when this molecule interacted with specific Abs. However, another explanation for the absence of detected interactions between HIV and Lf without specific Abs could be a disruption of HIV particles induced by Lf leading to an inactivation of HIV particles infectiousness induced by Lf. HIV was thus adsorbed on poly-L-lysin-precoated wells and further incubated with or without Lf, as described in Materials and Methods.

To assess the infectiousness of these Lf-treated HIV particles, activated peripheral blood lymphocytes, well-known producers of high levels of HIV (27), were incubated with Lf-treated or untreated HIV particles. The levels of viral production by activated peripheral blood lymphocytes were similar in wells containing cells cocultured with virus treated or untreated with Lf. In contrast, no viral production was detected in wells containing Triton X-100-treated HIV and activated peripheral blood lymphocytes (data not shown). Taken together, these data strongly suggest that Lf does not alter the infectiousness of the virus and Lf can only interact with HIV following immune complex formation.

Detection of Lf interacting NAbs within IVIg and CVS

Considering these previous results, we hypothesized that Lf-immune complex formation could occur in vivo, in the presence of Lf and endogenous NAbs. We thus aimed at detecting the presence of Lf-interacting NAbs in two biological sources of NAbs: IVIg and CVS. Indeed, IVIg could mimic Abs from serum and CVS contain genital Abs. Thus, both are of interest in a context of a genital microbicide development based on Lf.

As depicted in Fig. 6A, we found Lf-reactive Abs in both IVIg and CVS. Natural polyreactive Abs found in pooled CVS (n = 22) contained similar concentrations of IgA and IgG and fewer IgM (37, 36, and 4 μg/ml, respectively) (data not shown).

We further purified Lf-reactive Abs from IVIg by affinity chromatography. Unfortunately, due to lack of material, this could not be performed on CVS. As shown on Fig. 6B, purified Abs reacted with Lf, whereas no binding to Lf was observed in the presence of anti-Lf-depleted IVIg.
We also compared the relative avidity of these purified anti-Lf Abs from IVIg to purified anti-CCR5 NAbS from IVIg using a KSCN assay (26). The molarities of KSCN required to dissociate 50% of total anti-Lf Abs from the Lf and total anti-CCR5 Abs from the CCR5 peptide were 0.74 and 0.81 M, respectively (Fig. 5C). Therefore relative avidity of anti-Lf Nabs was similar compared with relative avidity of anti-CCR5 Nabs.

**IVIg facilitate HIV adsorption on iMDDC via Lf-immune complex formation**

We further investigated the relative ability of total and purified Lf-reactive IVIg to induce enhancement of HIV attachment on iMDDC, as observed with rabbit anti-human Lf polyclonal Abs. We first confirmed that HIV \(_{\text{Ba-L}}\) and HIV \(_{\text{NDK}}\) viruses adsorbed on iMDDC expressing CD4, the initial multistep interaction between HIV-1 and host cell surface could involve others surface molecules such as HSPG (25, 28–30) or mannose receptors (31). Our results showed inhibitory effect of Lf on HIV binding on epithelial cells in a dose-dependent manner. We have also shown that, endometrial epithelial cells can bind Lf mostly via HSPG as demonstrated by heparitinase III treatment. Therefore, inhibitory effect of Lf on HIV attachment may mostly act by preventing interaction between HIV and HSPG expressed by endometrial cells. These data are in agreement with other studies demonstrating the inhibitory effect of Lf on adsorption of HSPG-interacting viruses such HSV-1, -2, or HPV (16, 32). The mechanism of Lf inhibition is thought to be due to relatively nonspecific charge-charge interactions between Lf and sulfated residues of HSPG, which thus are no more available to interact with positively charged-V3 loop of HIV-gp120 (33).

Taken together, all these data demonstrate that 1) Lf limits HIV-1 adsorption on iMDDC and that 2) IVIg could reverse this inhibition and moreover facilitate HIV adsorption on iMDDC via formation of immune complexes with Lf.

**Discussion**

In the present study, we showed that Lf limits specifically attachment of R5 and X4-HIV-1-free particles but not viral transcytosis across a tight monolayer of epithelial cells, probably by interfering with attachment of virus on HSPG. We have also shown efficacy of Lf in limiting HIV uptake by DC. Conversely, we have also demonstrated that Lf-reacting NAbS present within IVIg-enhanced HIV attachment on DC by forming HIV-interacting immune complexes.

HIV entry into target cells is mediated by the successive interactions of the envelope glycoprotein gp120 with CD4 and a coreceptor, CXC4 or CCR5. However, in cells lacking or weakly expressing CD4, the initial multistep interaction between HIV-1 and host cell surface could involve others surface molecules such as heparan sulfate (25, 28–30) or mannose receptors (31). Our results showed inhibitory effect of Lf on HIV binding on epithelial cells in a dose-dependent manner. We have also shown that, endometrial epithelial cells can bind Lf mostly via HSPG as demonstrated by heparitinase III treatment. Therefore, inhibitory effect of Lf on HIV attachment may mostly act by preventing interaction between HIV and HSPG expressed by endometrial cells. These data are in agreement with other studies demonstrating the inhibitory effect of Lf on adsorption of HSPG-interacting viruses such HSV-1, -2, or HPV (16, 32). The mechanism of Lf inhibition is thought to be due to relatively nonspecific charge-charge interactions between Lf and sulfated residues of HSPG, which thus are no more available to interact with positively charged-V3 loop of HIV-gp120 (33). Consistently with this idea, we showed that Lf inhibited more efficiently attachment of strongly positively charged X4-tropic strains than dimly positively charged R5-tropic strains on epithelial cells, similarly to other polycationic compounds (34). The removal of heparan sulfate moieties of cell surface proteoglycans (HSPG) from the apical pole of HEC-1A induced for at least a 60% decrease of both R5- and X4-HIV-1 attachment, without inhibiting the transcytosis of the virus, showing their important implication in viral attachment and not in transcytosis (H. Saidi, submitted for publication). Our results are in agreement with these data, showing that the Lf clearly limited the adsorption of the virus on the HSPG expressed on the epithelial cells without interfering with the transcytosis of the virus.

However, several studies have also reported direct interactions between Lf and the GPGRAF sequence within the V3 loop of HIV-gp120 (35). Our results demonstrated that Lf could bind HIV only in certain conditions. Lf consists of a polypeptide chain, folded into two globular lobes designated N- and C-lobes (28, 29, 36, 37). Most of biological activities of Lf do not depend on the sequestration of free iron, but relate to the binding of Lf to negatively charged molecules such as bacterial LPS, heparin, human lysozyme, and DNA (38). First basic cluster of Lf, Arg\(^\text{2}-\text{Arg}^\text{2}\)-Arg\(^\text{2}-\text{Arg}^\text{2}\), is essential for these interactions (38, 39). In addition, a second highly positively charged region also located in the N-terminal region in position 25–40 has been implicated in Lf biological effects. Therefore, proximal N-terminal region can easily bind to a wide range of molecules and likely to HIV or plastic. Indeed, our results demonstrated that coated Lf did not bind to HIV whereas immune complexed-Lf strongly bound to HIV. Recently, it was reported that the majority of anti-human Lf Abs produced in

**FIGURE 5.** Evaluation of direct interaction of HIV with Lf or with Lf-interacting Abs. On 96-well plates, Lf were coated (C) or not (D and E), and HIV \(_{\text{Ba-L}}\) or HIV \(_{\text{NDK}}\). Viruses were added for 1 h at 37°C. For positive control, wells were coated with anti-gp120 2G12, and HIV \(_{\text{Ba-L}}\) or HIV \(_{\text{NDK}}\) viruses were added for 1 h at 37°C (A). For negative controls, wells were coated or not with polyclonal anti-Lf and HIV \(_{\text{Ba-L}}\) or HIV \(_{\text{NDK}}\) viruses were added at 37°C for 1 h (B). For some wells with coated polyclonal anti-Lf, Lf was further added at 37°C for 1 h, and HIV \(_{\text{Ba-L}}\) or HIV \(_{\text{NDK}}\) viruses were subsequently added for 1 h at 37°C (E). Bound HIV particles were lysed, and their concentrations were evaluated by HIV-1 p24 Ag capture ELISA. Results were expressed as mean ± SD of the values obtained from three independent experiments. ***, p ≤ 0.01 using paired Student’s t test between A and D or (E) experimental conditions.
mouse targeted the C-lobe, and rarely the proximal N-terminal region (40). Thus, we propose a model where HIV directly interacts with Lf via its proximal N-terminal region, only if this region does not already interact with other ligands, such as plastic, HSPG, heparin, DNA, LPS, or other pathogens. This hypothesis is sustained by the observation of a weak direct binding of Lf and HIV in solution, before interaction with anti-Lf-coated plates (data not shown). To confirm these data, other experiments using Lf peptides need to be performed. However, we cannot exclude that interaction between Lf and specific Abs may also induce a conformational change of Lf, thus unmasking an HIV-binding site.

One of the cell types first encountered by HIV-1 are intraepithelial and submucosal DC strongly implicated in HIV transmission (41). Our data demonstrated that Lf also limited HIV adsorption on DC. HIV adsorption on DC have been reported to mostly result from Lf immune complex formation. We have further suggested that this phenomenon may be physiologic considering that 1) CVS from healthy donors and IVIg contained Lf-reactive NAbs and that 2) such purified Abs from IVIg also enhanced attachment of virus on DC by partially implicating FcγR. In addition, concentration of IVIg used in that study are fully physiologic, considering that therapeutic concentrations of IVIg are between 12.5 and 25 mg/ml, as reported previously (32–54).

Notably, HEC-1A cells don’t express FcγR, and we did not observed this enhancement of HIV attachment on epithelial cells by anti-Lf Abs. Importantly, we cannot exclude that the anti-Lf Abs could inactivate Lf by making multimeric immune complexes. But, this phenomenon seems to be minor since enhancement of HIV attachment on epithelial cells by anti-Lf Abs would result in the inhibition of transmission of HIV-1 by these Lf-treated DC (submitted for publication). In addition, in the chimpanzee model, HIV trapping was detected in germinal centre of animals with the highest viral load and the attachment of HIV-immune complexes to DC network was showed to be crucial to progression of disease (48–51). Our data also demonstrated Lf-specific IgG Abs enhanced HIV-1 attachment on DC via Lf immune complex formation. We have further suggested that this phenomenon may be physiologic considering that 1) CVS from healthy donors and IVIg contained Lf-reactive NAbs and that 2) such purified Abs from IVIg also enhanced attachment of virus on DC by partially implicating FcγR. In addition, concentration of IVIg used in that study are fully physiologic, considering that therapeutic concentrations of IVIg are between 12.5 and 25 mg/ml, as reported previously (52–54).

Notably, HEC-1A cells don’t express FcγR, and we did not observed this enhancement of HIV attachment on epithelial cells by anti-Lf Abs. Importantly, we cannot exclude that the anti-Lf Abs could inactivate Lf by making multimeric immune complexes. But, this phenomenon seems to be minor since enhancement of HIV attachment on DC is observed in the presence of increasing concentrations of anti-Lf Abs. More interestingly, enhancement of HIV adsorption on DC is still observed in the presence of Lf and total unfraccionated IVIg, highlighting the physiological relevance of this phenomenon. In addition we have confirmed a weak endogenous secretion of Lf by HEC-1A cells, as described previously (55). It is well known that the female genital secretions contains a very high amount of IgG, at levels > 10-fold those of IgA,
Cells were further coincubated or not with Lf (100 μg/ml) with HIVBa-L, IVIg, or anti-Lf IVIg. In other control wells, cells were coincubated with increasing concentrations of total IVIg, or with a fixed concentration (450 μg/ml) of IVIg or anti-Lf IVIg-treated cells and Lf-treated cells. The concentration of attached virus was measured by p24 Ag capture ELISA. Mean values of duplicates are reported. Error bars represent SDs. One experiment representative of two is presented. +, p ≤ 0.05 and **, p ≤ 0.01 (paired Student’s t test) between total IVIg or anti-Lf IVIg-treated cells and Lf-treated cells.

FIGURE 7. IVIg facilitate HIV adsorption on IMDDC by inducing the formation of Lf-immune complex. IMDDC were pretreated (+ BR) or not (− BR) with FcR-blocking reagent as indicated in Materials and Methods. Cells were further coincubated or not with Lf (100 μg/ml), HIV-1Ba-L (A), or HIVNDK (B), in the presence or absence of various concentrations of purified anti-Lf Abs. In controls wells, cells were coincubated with Lf (100 μg/ml), HIV-1Ba-L (A), or HIVNDK (B), in the presence of increasing concentrations of total IVIg, or with a fixed concentration (450 μg/ml) of mAbs against IgG1 (M Abs). In other controls wells, cells were coincubated with HIV-1Ba-L (A) or HIVNDK (B), in the presence of a fixed concentration (1000 μg/ml) of anti-Lf purified. The concentration of attached virus was measured by p24 Ag capture ELISA. Mean values of duplicates are reported. Error bars represent SDs. One experiment representative of two is presented in this data. +, p ≤ 0.05 and **, p ≤ 0.01 (paired Student’s t test) between total IVIg or anti-Lf IVIg-treated cells and Lf-treated cells.

and originating in part from plasma by transudation. Since Lf is mainly produced in women’s genital tract by epithelial cells, the formation of Lf-immune complexes may occur within the women’s genital tract in vivo, and the facilitation of HIV adsorption on DC could happen if such immune complexes encounter subepithelial DC. Nevertheless, we have shown that these anti-Lf Abs have a modest avidity for the Lf. So, we cannot exclude that these polyreactive Abs would interact with other proteins in CVS, which could potentially reduce their in vivo concentration as anti-Lf Abs. However, we have shown that weak concentration of these anti-Lf Abs is sufficient to abrogate the antiviral activity of the Lf, which could in fine facilitate the viral transmission. We thus propose a model where NAbAbs react with Lf outside the proximal N-terminal region of the molecules. Such immune complexes are further adsorbed into DC surface, partially via FcR. Thus, HIV can finally interact with 1) its cellular targets without any hindrance from Lf and 2) with the proximal N-terminal region of Lf, therefore explaining the increased adsorption of HIV on DC in such conditions. Lf and NAbAbs belong to innate immunity, such as the complement system. Our group has previously reported relevance of HIV opsonization by seminal compound complement in HIV mucosal transmission (56). We report here a model in which Lf would appear as a double-edged sword that could have beneficial or detrimental effects depending on the environment.

In addition to its anti-HIV activity, Lf possesses other advantages such as its ability to be produced in large quantities and its limited toxicity and immunogenicity. It is therefore a potent microbicide to prevent sexual HIV-1 transmission, possibly in combination with other potential compounds (57). Sexually transmitted diseases have a marked effect on both viral shedding in the genital tract and the risk of acquiring HIV-1 infection, since they increase HIV target cells recruitment and create lesions in the genital tract. Hence, an ideal microbicide should also have a broad spectrum of activity against a variety of sexually transmitted microbes within genital secretions (58, 59). And it’s the case of Lf, which is active against a large spectrum of bacteria (60), parasites (61), and viruses such as herpes simplex virus type 1 and 2 (28), adenovirus (62), HIV (63–65), hepatitis B (66) and C (67) virus, human CMV (63, 68), hantavirus (69), retrovirus 71 (70), and alphavirus (16).

In conclusion, using a natural microbicide that interferes at the same time with HIV-1 capture, transmission, and even replication might be a promising protective strategy. However, interactions with innate immune system and especially NAbAbs must be taken account to develop technical approach minimizing such interferences. Regarding Lf, a peptidic approach could be followed. Indeed, designing peptides able to interact with HIV and cellular targets without interacting with NAbAbs would be of particular interest. Also, the notion of combining two or more microbicides that have different mechanisms of action within a single product is an attractive objective, and more work on this is highly desirable. Combination products could in principle provide a greater degree of protection than single agents, a broader spectrum of activity against various pathogens and a lower risk of adverse reactions. Since high concentration of Lf are necessary to inhibit the adsorption on the HIV target cells, the combination of Lf peptide with one or two other component(s) would allow a lower dose of this component.

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Disclosures

The authors have no financial conflict of interest.

References


In Results, in sentence 16 under the heading *A Cullin-based ubiquitin ligase pathway is involved in host cell-mediated IRF-3 degradation following SeV infection*, reference to CREB coactivator is incorrect. In sentence ten, under the heading *Degradation of IRF-3 is dependent of the TBK1/IKKi-signaling pathway*; reference to RNA interference silencing technology is incorrect. The corrected sentences are shown below.

Interestingly, this increase in the stability of the hyperphosphorylated forms of IRF-3 was also associated with a sustained activation of IRF-3 as verified by the presence of dimers or its association to CREB binding protein (CBP) coactivator after infection with SeV (Fig. 3E).

We next directly examined the contribution of the IKK-related kinases in IRF-3 degradation by first using RNA interference (RNAi) technology.

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The second author’s last name is misspelled. The correct name is Jobin Eslahpazir.