Liposomal Lactoferrin Effect in Preventing SARS-CoV-2 Binding in HACAT Keratinocytes

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ABSTRACT
SARS-CoV-2 uses ACE2 receptors (Angiotensin-Converting Enzyme) for entry into the host cell and the transmembrane serine protease 2 (TMPRSS2) for S protein priming. Lactoferrin is endowed with potent antiviral activity, related to its ability to block viral entry into host cells by interacting with viral and/or cell surface receptors. SARS-CoV-2 uses its spike proteins to invade cells via ACE2 receptor. The ability of free and encapsulated LF to prevent binding of SARS-CoV-2 spike protein to HaCaT keratinocytes receptors was evaluated (fluorescent microscopy) through immunostaining with lactoferrin antibody (Cy3-red) and spike protein labeling with Atto48 (green). Untreated control HaCaT cells incubated with the labeled spike protein did not present any red fluorescence due to lactoferrin antibody staining, but a green fluorescence owing to labeled spike protein was observed. Cells treated with 0.001% lactoferrin showed lactoferrin (red) bound cells although Spike protein (green) was not detectable. % 0.001 LF did not bind most cells (red), whereas spike protein bound cells (Green), but less than the control without product treatment. 0.001% liposomal lactoferrin (LLF) did not show spike binding signal (Green) and high lactoferrin staining was observed (red). LF and LLF prevented the binding of SARS-CoV-2 spike protein to HaCaT cell receptors although at the same concentration LLF was more effective than the free form. LF encapsulation improves the delivery of LF. The liposome by its own structure and composition interact with lipoprotein components of the virus or from cell membranes (TMPRSS2). Oral administration of LF may be useful in the care of patients with COVID-19 infections.

Key words: Lactoferrin, COVID-19, SARS-CoV-2, Spike protein, ACE2.

INTRODUCTION
A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, to cause an epidemic of severe pneumonia, now called coronavirus disease (COVID-19), which spread to other parts of China and to the rest of the world.[4] The disease is now a pandemic with, as at May 15, 2020, more than 4.4 million cases and more than 302,000 deaths worldwide. In this regard, the United States, Russia, the United Kingdom, Spain, Italy, Brazil, Germany, Turkey, France and Iran are the countries most affected by the pandemic in terms of the number of confirmed contagions and the only ones where the 100,000-case barrier has been exceeded (WHO, 2020). While there are estimates of disease severity and infection attack rates, the true severity of disease remains a major knowledge gap because mild or asymptomatic infections are difficult to estimate.[2] Coronaviruses (CoVs) are a group of enveloped positive-stranded RNA viruses that can cause respiratory, intestinal, and central nervous system infections in humans and animals.[3] Until 2019, six strains of coronaviruses that can infect humans had been identified.[4] Among them, four human coronaviruses, including HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1, are not highly pathogenic and only cause mild respiratory diseases. However, two other coronaviruses, the severe acute respiratory syndrome coronavirus (SARS-CoV)[5] and the Middle East respiratory syndrome coronavirus (MERS-CoV),[6] have caused two large-scale pandemics and resulted in more than 8,000 cases, including nearly 800 related deaths and about 2,500 cases, including about 860 related deaths, respectively. The outbreaks of SARS-CoV and MERS-CoV indicated that some coronaviruses can be highly pathogenic when they transmit from animals to humans.[7] Therefore, it is urgent to develop antiviral treatments or vaccines targeting such high-risk coronaviruses like SARS-CoV and MERS-CoV and SARS-CoV-2. Virus infections initiate with binding of viral particles to host surface cellular receptors. Receptor recognition is therefore an important determinant of the cell and tissue tropism of a virus. In addition, the gain of function of a virus to bind to the receptor counterparts in other species is also

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a prerequisite for inter-species transmission.\[8\] Coronaviruses use the surface spike (S) glycoprotein on the coronavirus envelope to attach host cells and mediate host cell membrane and viral membrane fusion during infection.\[9\] The spike protein includes two regions, S1 and S2, where S1 is for host cell receptor binding and S2 is for membrane fusion. The S1 region also includes an N-terminal domain (NTD) and three C-terminal domains (CTD1, CTD2, and CTD3).\[9,10\] For SARS-CoV, the receptor binding domain (RBD) is in the CTD1 of the S1 region. SARS-CoV attaches the human host cells through the binding of the RBD protein to the angiotensin-converting enzyme II (ACE2).\[9,10\] Therefore, the interaction between RBD and ACE2 is a prerequisite for the human infection with SARS-CoV, even though, the region in SARS-CoV-2 S protein that is responsible for ACE2 interaction remains unknown. From a phylogenetic point of view, the genetic similarities shared by SARS-CoV-2 and the 2002 SARS coronavirus strain (SARS-CoV; they both belong to the so-called beta CoV group) and other bat-isolated coronaviruses strains have already been underlined, with more than 96% gene identity.\[11-13\] The alignment of spike protein and protease showed an analogy in primary sequences of more than 75% and 96% respectively.\[14,15\] Spike proteins and proteases are targets of choice for inhibition of SARS and MERS and several efforts have been made to develop inhibitors of their activities. LF has been found to experimentally inhibit viral entry via binding to host cell surface heparan sulphate proteoglycans (HSPGs) in murine coronavirus,\[16\] and human coronaviruses hCoV-NL63\[17\] and pseudo typed SARS-CoV.\[18\] There are yet no published studies on LF effects on SARS-CoV-2 and its entry into host cells. LF was able to block the interaction of spike protein and ACE2, the functional receptor of SARS-CoV.\[18\] Previous studies demonstrate that LF colocalizes with the widely distributed cell-surface heparan sulphate proteoglycans (HSPGs) and treatment of the cells with heparinase or exogenous heparin prevented binding of spike protein to host cells and inhibited SARS pseudovirus infection, demonstrating that HSPGs provide the binding sites for SARS-CoV invasion at the early attachment.\[18\] Both SARS-CoV and SARS-CoV-2 spike protein structures are very similar and depends on the same ACE2 receptor for cell entry.\[19\] HSPGs serve as attachment sites that congregate the virus on the cell surface and facilitate specific entry receptors such as ACE2. It is thus likely that LF can inhibit SARS-CoV-2 invasion at micromolar concentrations and in a dose dependent manner just as in the case of SARS-CoV.\[14\] SARS-CoV-2 uses ACE2 receptor for entry and the serine protease transmembrane (TMPRSS2) for S protein priming. Inhibition of these two targets may constitute a good prophylaxis and treatment option.

Here, we analyzed the in vitro ability of free and PC-encapsulated lactoferrin to prevent the binding of SARS-CoV-2 spike protein to HaCaT cell receptors, using immunostaining with lactoferrin antibody (Cy3-red) and SARS-CoV-2 spike protein labeled with Atto 48 (green) (Figure 1).

**MATERIAL AND METHODS**

**Products**

The following products were tested: free lactoferrin and LF encapsulated in a phosphatidyl choline liposome (liposomal lactoferrin). Samples were stored refrigerated (4ºC) until the start of the experiment and dilutions were freshly prepared each time.

Lactoferrin was encapsulated in positively charged PC-liposomes at the mentioned concentrations. The liposome preparation presented a unimodal size distribution with a diameter between 80 and 150 nm, a polydispersity index below 0.20, and a zeta potential of (30-150) mV.

**Platform**

Human keratinocytes (HaCaT line, AcceGen Biotechnology, Fairfield, New Jersey).

**Analytical equipment**

Leica fluorescence Microscope, incubator, statistical analysis software, laminar flow hood, micropipettes, pipettes, propipette, rack, thermo block, vortex, refrigerated centrifuge, 20x objective, 24-wells plates suitable for cell culture, coverslips, consumables.

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**Figure 1:** Lactoferrin and viral spike protein compete for the ACE2 receptor in the host cell membrane; the liposome by its own structure and composition (fatty acids, surfactants, alcohol) may induce changes in the lipids and proteins of the cell and the virus (TMPRSS2).
Reagents
Distilled water (Braun), DMEM medium, fetal bovine serum (Fisher), Penicillin, Streptomycin, Phosphate buffered saline (Sigma), Trypan Blue Solution (Bio-Rad), Trypsin-EDTA (Gibco), paraformaldehyde, Recombinant SARS-CoV-2 Spike Protein, S1 Subunit (Raybiotech), Atto 488 protein labeling kit (Sigma-Aldrich), gel filtration column (Sigma-Aldrich), Mouse monoclonal [2B8] to Lactoferrin antibody (Abcam), Cy3 (red fluorescence) anti-mouse, fluorescence preserving medium, and DAPI.

Atto 488 is a labeling dye with high molecular absorption (90,000) and quantum yield (0.80) as well as enough Stokes shift between excitation and emission maximum.

Procedure
Recombinant SARS-CoV-2 Spike Protein, S1 Subunit was first precipitated and resuspended in sodium bicarbonate buffer solution for 488 protein labeling process using Atto 488 protein labeling kit. After labeling, protein-dye-conjugates were separated from free dye using a gel filtration column. HaCaT cells were seeded on coverslips and treated with products (Lactoferrin and Lactoferrin Liposomes) at 2 different not cytotoxic concentrations (0.0001% and 0.001%) for 24 hours. Untreated controls were included in the experiment. After that, 488-labeled SARS-CoV-2 Spike Protein was added to cells for 2 hours. After the incubation period cells were fixed with 4% paraformaldehyde in cell medium and blocked with 10% Fetal bovine serum in PBS. Next, cells were incubated over night with Mouse monoclonal [2B8] to Lactoferrin antibody (Abcam) and finally treated with Cy3 (red fluorescence) anti-mouse and DAPI (cell nucleus staining) for 1 hour. Coverslips were mounted on slips using fluorescence preserving medium. 5 images per treatment were obtained in a Leica fluorescence microscope using 20x objective. Results showed a significant increase in the intensity, as shown in Figures 5 and 6.

RESULTS
According to previous assays, 0.0001% and 0.001% concentrations were selected for this assay as non-toxic concentrations. Immunostaining with lactoferrin antibody (Cy3-red) and SARS-CoV-2 spike protein labeled with Atto48 (green), was used to assess the capacity of Lactoferrin and Lactoferrin Liposomes to prevent the binding of SARS-CoV-2 spike protein to HaCaT keratinocytes cell receptors, after 24 hours of treatment. 5 images per condition were obtained in a Leica fluorescence microscope using 20x objective. Results showed that untreated control HaCaT cells incubated with the labeled spike protein of SARS-CoV-2 did not present any red fluorescence due to lactoferrin antibody staining, but green fluorescence owing to labeled spike protein was observable as shown in Figure 2. When cells were previously treated during 24 hours with 0.001% LF, results indicated LF (red) bound cells even though spike protein (green) was not detectable (Figure 3), suggesting LF treatment is preventing from spike protein binding to HaCaT cells. When Lactoferrin at 0.0001% concentration was used, lactoferrin was too diluted and did not bind most cells (red), whereas spike protein bound cells (green), but less than the control without product treatment (Figure 4). When liposomal lactoferrin was applied on cells at 0.0001% and 0.001% concentrations, results showed no spike protein binding signal (green), whereas lactoferrin staining (red) was clearly observed, binding cells in a dose-dependent intensity, as shown in Figures 5 and 6.

DISCUSSION
Recently, an in vitro study from Brazil showed the inhibitory effect of bovine lactoferrin on SARS-CoV-2 infection in African green monkey kidney epithelial cells (Vero E6) and in adenocarcinomic human alveolar basal epithelial cells (A549) where LF reduced progeny virus yield by 84.6% and 68.6% respectively.[20] This study demonstrate that LF is able to interfere with SARS-CoV-2 replication in vitro at 1 mg/ml. Regarding in vivo clinical studies, a very recent prospective observational study with COVID-19 patients revealed that the intake of liposomal lactoferrin allows a complete and fast recovery from the disease within the first 4-5 days.[21] The SARS-CoV-2 is an enveloped, RNA virus with a genome of about 30000 nucleotides in length and encodes a nonstructural replicate complex and structural proteins, including spike (S), envelope (E), and S2, membrane (M) and nucleocapsid (N) proteins.[16] SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) and the cellular transmembrane protease serine 2 (TMPRSS2) to enter target cells.[22] Priming of S proteins by host cell proteases is important for viral entry into cells and includes S protein cleavage at the S1/S2 and the S2 sites. This protease enhances the transmissibility of the
Figure 3: HaCaT cells treated with 0.001% lactoferrin and SARS-CoV-2 spike protein labeled with Atto48 (green). Cells were immune stained with lactoferrin antibody (Cy3-red) and DAPI (blue).

Figure 4: HaCaT cells treated with Lactoferrin at 0.0001% concentration, and SARS-CoV-2 spike protein labeled with Atto48 (green). Cells were immune stained with Lactoferrin antibody (Cy3-red) and DAPI (blue).

Figure 5: HaCaT cells treated with Liposomal Lactoferrin 0.0001% and SARS-CoV-2 spike protein labeled with Atto48 (green). Cells were immune stained with Lactoferrin antibody (Cy3-red) and DAPI (blue).
SARS-CoV and the new coronavirus (SARS-CoV-2) which is a metallopeptidase, is the cellular receptor for both SARS coronavirus and SARS-CoV-2 spike protein to host cells and inhibited SARS pseudovirus infection, demonstrating that HSPGs provide the binding sites for SARS-CoV spike protein to host cells and inhibited SARS pseudovirus infection, and S2, which triggers virus and host cell membrane fusion.\[18\] A segment with amino acids 318-510 of the protein S1 has been identified as the receptor binding domain for the ACE2 receptor.\[17\] ACE2, a metallopeptidase, is the cellular receptor for both SARS coronavirus (SARS-CoV) and the new coronavirus (SARS-CoV-2) which is responsible for the current epidemic COVID-19.\[14\] SARS-CoV-2 spike protein was 10 to 20 times more likely to bind ACE2 on human cells than the spike from the SARS virus from 2002, which enable SARS-CoV-2V to spread more easily from person to person than the earlier virus.\[23\] A segment with amino acids 318-510 of the protein S1 has been identified as the receptor binding domain for the ACE2 receptor.\[17\] ACE2 is highly expressed in the nose, mouth, epithelial respiratory tract, on human lung alveolar epithelial cells, enterocytes of the small intestine, brain, prostate, testis, bladder, liver, pancreas, and the brush border of the proximal tubular cells of the kidney.\[20-31\] LF has been found to experimentally inhibit viral entry via binding to host cell surface HSPGs in murine coronavirus,\[15\] and human coronaviruses hCOV-NL63\[20\] and pseudo typical SARS-CoV.\[14\] ACE2 and HSPGs are essential cell-surface molecules involved in SARS-CoV-2 cell entry. LF may play a protective role in host defense against SARS-CoV-2 infection through binding to HSPGs and blocking the preliminary interaction between SARS-CoV and host cells.\[14\] SARS-CoV and SARS-CoV-2 spike protein structures are similar, as well as both viruses depends on the same ACE2 receptor for cell entry.\[19\] so we can say that HSPGs serve as SARS-CoV-2 attachment sites that congregate the virus on the cell surface and facilitate specific entry receptors such as ACE2. It is thus likely that LF can inhibit SARS-CoV-2 invasion at micromolar concentrations and in a dose dependent manner just as in the case of SARS-CoV.\[18\] LF was able to block the binding of spike protein to host cells at 4°C, indicating that LF exerted its inhibitory function at the viral attachment stage but without disrupting the interaction of spike protein with angiotensin-converting enzyme 2 (ACE2), the functional receptor of SARS-CoV. LF was observed to colocalize with the widely distributed cell-surface heparan sulfate proteoglycans (HSPGs) and treatment of the cells with heparinase or exogenious heparin prevented binding of spike protein to host cells and inhibited SARS pseudovirus infection, demonstrating that HSPGs provide the binding sites for SARS-CoV invasion at the early attachment phase. Until now no in vitro studies on LF effects on SARS-CoV-2 and its entry into host cells has been published. The advent of fluorescence microscopy has made it possible to visualize proteins inside cells. This is particularly useful when studying the location of signaling pathways and binding partners. Here, we analyzed the in vitro ability of two lactoferrin products (Lactoferrin and Lactoferrin Lisosomes) to prevent the binding of SARS-CoV-2 spike protein to HaCaT cell receptors, using immunostaining with lactoferrin antibody (Cy3-red) and SARS-CoV-2 spike protein labeled with Atto48 (green). Results showed that untreated control HaCaT cells incubated with the labeled spike protein of SARS-CoV-2 did not present any red fluorescence due to lactoferrin antibody staining, but green fluorescence owing to labeled spike protein was observable. Cells treated with Lactoferrin 0.001% showed lactoferrin (red) bound cells although Spike protein (green) was not detectable. However, when Lactoferrin 0.0001% was used, lactoferrin was too diluted and did not bind most cells (red), whereas Spike protein bound cells (green), but less than the control without product treatment. Lactoferrin Lisosomes at 0.001% did not show spike binding signal (Green) and high lactoferrin staining was observed. Finally, Lactoferrin Lisosomes at 0.0001% showed no spike binding (green) but lactoferrin bound cells were detected (red). Encapsulation in phosphatidyl choline liposomes allows a better penetration of LF into the cell membranes. Liposomes contain fatty acids such as linoleic acid, palmitic acid and oleic acid which may interact both with the viral structures or with the host cell membranes. Nonionic surfactants such as Tween 20 which are in the liposome in concentrations up to 5%, can alter the physical properties of the cell membranes, change its phospholipid content, solubilize their own membrane or remove its peripheral protein content.\[24\] This detergent has a hydrophilic character due to the hydroxyl group. Unlike ionic detergents, they solubilize membrane proteins in a very gentle way. Non-ionic detergent dissolves type lipid-lipid and lipid-protein compounds, while not influencing interactions protein-protein. Thus, the native structure of proteins is preserved. In addition, the detergent replaces lipids that are normally attached to the hydrophobic part of proteins, creating a lipid-like environment and thus they can stabilize the solubilized proteins.\[21\] Liposomes content also organic solvents like ethyl alcohol at concentrations up to 5% which are used in the creation of liposomes as well as preservatives.\[25\] In conclusion, Lactoferrin and liposomal lactoferrin block the binding of SARS-CoV-2 spike protein to HaCaT cell receptors. At the same concentration, encapsulated LF is more effective in diminishing spike protein binding to cells. These findings, as well as the in vitro studies showing the inhibitory effect of bovine lactoferrin on SARS-CoV-2 infection in Vero and A549 cell lines, provides additional evidence for the use of oral liposomal lactoferrin, in the prophylaxis and care of patients affected with COVID-19 infections along or combined to conventional antiviral drug treatments.\[26\] LF is not a drug but a food supplement used safely for...
years to enhance our innate immune system by increasing natural killer cell activity, and neutrophil aggregation and adhesion. Since LF is already developed, larger clinical trials are needed to confirm its beneficial effects in COVID-19 infections which may represent a shortcut in comparison with the development of new products or vaccines, saving time, money and mainly lives.

REFERENCES
