COVID-19 during Pregnancy and Postpartum:

Antiviral Spectrum of Maternal Lactoferrin in Fetal and Neonatal Defense

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COVID-19 during Pregnancy and Postpartum: Antiviral Spectrum of Maternal Lactoferrin in Fetal and Neonatal Defense

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ABSTRACT

As the COVID-19 pandemic intensified the global health crisis, the containment of SARS-CoV-2 infection in pregnancies, and the inherent risk of vertical transmission of virus from mother-to-fetus (or neonate) poses a major concern. Most COVID-19-*Pregnancy* patients showed mild to moderate COVID-19 pneumonia with no pregnancy loss and no congenital transmission of the virus; however, an increase in hypoxia-induced preterm deliveries was apparent. Also, the breastmilk of several mothers with COVID-19 tested negative for the virus. Taken together, the natural barrier function during pregnancy and postpartum seems to deter the SARS-CoV-2 transmission from mother-to-child. This clinical observation warrants to explore the maternal-fetal interface and identify the innate defense factors for prevention and control of COVID-19-*Pregnancy*. Lactoferrin (LF) is a potent antiviral iron-binding protein present in the maternal-fetal interface. In concert with immune co-factors, maternal-LF modulates chemokine release and lymphocyte migration and amplify host defense during pregnancy. LF levels during pregnancy may resolve hypertension *via* down-regulation of ACE2; consequently, may limit the membrane receptor access to SARS-CoV-2 for cellular entry. Furthermore, an LF-derived peptide (*LRPVAA*) has been shown to block ACE receptor activity *in vitro*. LF may also reduce viral docking and entry into host cells and limit the early phase of COVID-19 infection. An in-depth understanding of LF and other soluble mammalian milk-derived innate antiviral factors may provide insights to reduce co-morbidities and vertical transmission of SARS-CoV-2 infection and may lead to the development of effective nutraceutical supplements.

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Introduction

Novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infections spiraled to a colossal magnitude in a short span, resulting in acute morbidity and mortality outcomes worldwide. Coronavirus disease 2019 (COVID-19) is the deadliest pandemic to have encountered in over 100 years with a catastrophic impact on public health and global economy. The clinical symptoms of COVID-19 are mainly fever (88.5%), cough (68.6%), myalgia or fatigue (35.8%), expectoration (28.2%), and dyspnea (21.9%). Blood reports indicate lymphocytopenia (64.5%), leukocytopenia (29.4%) and an increase in serum levels of C-reactive protein (44.3%) and lactic dehydrogenase (28.3%) (Li, Hunag, et al. 2020). Males are most affected (60%) in the gender distribution of COVID-19 patients, the overall discharge rate was 52%, and the case fatality rate (CFR) was 5% (Li, Huang, et al. 2020). The mean time from onset to death was 18.8 days (in China) and 24.7 days (out of China) (Verity et al. 2020). Asymptomatic SARS-CoV-2 carriage is common (Bai et al. 2020); however, the community prevalence of viral transmission and the duration of viral shedding among the dormant population is unknown. Screening and identification of asymptomatic carriers and serological assessment of herd immunity are unresolved. In addition to the presumably high number of asymptomatic SARS-CoV-2 carriers, the recently infected individuals prior to the onset of symptoms, the clinically recovered COVID-19 patients that still carry the virus, and the existence of potentially susceptible domestic and wild animals in close vicinity of the infected and dormant individuals – further confounds the preventive and control strategies for clinical management of COVID-19 (Azkur et al. 2020).

As the COVID-19 pandemic continues to spread, the containment of SARS-CoV-2 infection among pregnant women and the potential risk of mother-fetal vertical transmission is of major concern (Dashraath et al. 2020; Zaigham and Andersson 2020). Although pregnant women are at an immune-suppressive state due to gestation-related physiological changes, most COVID-19-*Pregnancy* patients suffered from mild or moderate COVID-19 pneumonia with no pregnancy loss (Schwartz and Dhaliwal 2020). The COVID-19-*Pregnancy* showed no indication of congenital transmission of the virus; however, an increased prevalence of preterm deliveries was observed (Dashraath et al. 2020; Li, Huang, et al. 2020). No evidence for perinatal transmission of COVID-19 from mother-to-newborn has been reported (Karimi-Zarchi et al. 2020; Peng et al. 2020). Preliminary observations indicated that the breastmilk from mothers with COVID-19 is free from SARS-CoV-2 (Lang and Zhao 2020; Martins-Filho et al. 2020). Whether breastfeeding could transmit the virus from previously infected and recovered mothers to their babies is unclear (Lamouroux et al. 2020). Taken together, pregnancy and postpartum seems to provide a natural physiological barrier to counteract congenital transmission of SARS-CoV-2 infection.

Syncytiotrophoblast (STB) lines the intervillous space of the placenta and provides the critical barrier function throughout gestation (Riquelme 2011). At the maternal-fetal interface, STB defends the fetus from a variety of infectious agents, in addition to its role in hormone synthesis to support pregnancy and in the regulation of placental transport of nutrients (Huppertz 2010; Göhner et al. 2017). STB also stimulates release of the iron-transport protein, 'lactoferrin (LF)', into the placental milieu and amniotic fluid (Thaler et al. 1999). LF is a potent antiviral agent, an effective modulator of
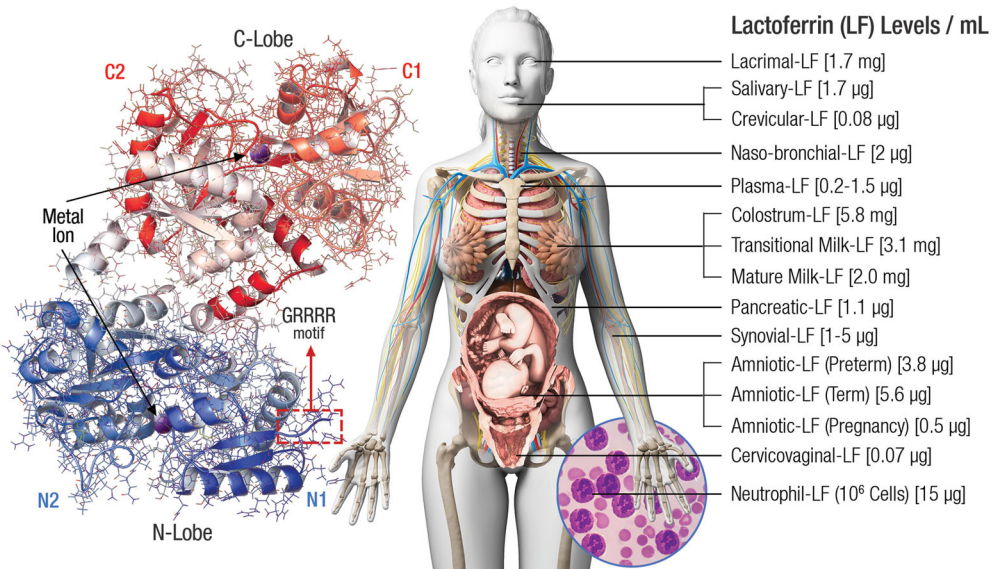
Figure 1. LF structure and distribution in the human body. Human LF (hLF) (PDB ID: 1LFG) is a poly-peptide chain folded into two symmetrical halves (N and C lobes) connected by a hinge region with an α-helix. The two lobes consist of two domains (N1 and N2, C1, and C2) and each lobe covalently binds one metal ion (Fe^{3+}, Cu^{2+}, Mn^{2+}, or Zn^{2+}) in a deep cleft between the two domains. LF is secreted by glandular epithelia with highest levels found in human colostrum. LF occurs in mature milk, most exocrine secretions, and in the secondary granules of mature neutrophils. LF levels are elevated during infection and/or inflammation due to the recruitment of neutrophils. LF is prominently found in both male and female reproductive systems.

immune responses, and a regulator of redox homeostasis in the body (Maneva et al. 2003; Wakabayashi et al. 2014). LF could interact with both maternal and fetal microenvironments to establish physical as well as immunological barriers to evade microbial pathogens. Maternal LF in colostrum and milk provides passive immune protection to the neonate from breast feeding (Woodman et al. 2018); thus, exogenous LF fortification of infant formula has been recommended worldwide for over two decades (Lönnerdal 2014). This review elucidates the multifunctional role of LF in various physiology pathways, including metal transport, oxidative stress, inflammatory response, innate and adaptive immunity to evade microbial pathogens. In the commerce-driven pharmaceutical pursuits, politically-motivated health legislations, humankind cannot afford to neglect one of its precious gifts from the 'Mother Nature' in the fight against the current COVID-19 and the future pandemics – the 'Innate Host Defense'!

Maternal lactoferrin (LF)

Lactoferrin (LF) is an iron-binding glycoprotein with a multi-functional role in various physiological pathways (Rosa et al. 2017). LF is a member of the transferrin family, with a molecular mass of ∼80-kDa. Its structure consists of a single polypeptide chain folded in two symmetric globular halves (N- and C-lobes), and each lobe is able to bind one ferric (Fe^{3+}) ion. LF is widely distributed in colostrum, milk as well as most exocrine secretions that bathe mucosal surfaces (Naidu 2000). LF appears to play a critical role

in the first line of host defense by modulating innate immune responses at mucosal surfaces. LF accelerates the maturation of T-cell precursors into competent T-helper (T_H) cells (Ando et al. 2010) and differentiates the immature B-cells into antigen-presenting cells (APCs) (Actor et al. 2009). LF secretion dramatically elevates during inflammation due to neutrophil degranulation and activation of microglial cells (Fillebeen et al. 2001). As one of the early inflammatory mediators, LF helps to combat pathogens and contributes to the activation of innate host defense *via* regulation of adaptive immune pathways (Siqueiros-Cendón et al. 2014). In concert with immune co-factors, maternal LF modulates chemokine release and lymphocyte migration to amplify host defense during pregnancy.

LF levels during pregnancy and postpartum

LF is one of the protective barriers in the maternal-fetal interface, as well as a multifunctional regulator of immune response and a broad-spectrum antimicrobial agent during pregnancy. Besides the mammalian lacteal secretions (milk and colostrum), where LF is present at a concentration of 5–7 g/L, it is the second most abundant milk protein after casein (Naidu 2000). LF is primarily found in exocrine secretions that bathe mucosal surfaces; it is present in tears, saliva, vaginal, seminal, nasal and bronchial secretions, bile, pancreatic, synovial, cerebrospinal, gastrointestinal fluids, and urine. It is also found in considerable amounts in secondary neutrophil granules (15 μg/10^6 neutrophils), where it plays a role in host defense (Figure 1). LF content in neutrophils markedly decline during viral infections compared to normal subjects, which suggests an acquired defect of neutrophil LF synthesis during viral infection (Baynes et al. 1988).

Cervical (or Endometrial)-LF appears in the endometrium at the early secretory phase of the menstrual cycle and these levels are elevated between *Days-23* to *-25* of the cycle. LF synthesis results from the effect of progestogens (Masson et al. 1968). In the female reproductive tract, LF has also been detected in the cervical mucus and endometrium of the secretory uterus (Tourville et al. 1969). LF in the cervical mucus is an integral part of the mucosal immune system and act as the first line of defense against infections (Masson and Ferin 1969). High levels of LF are detected in cervico-vaginal fluid (72.7 μg/mL), compared to the concentrations found in the other mucosal fluids (Bard et al. 2003). As a major estrogen-induced glycoprotein in the uterus, LF is up-regulated by physiological levels of estrogen at different stages of the estrous cycle. LF is secreted by the endocervical cells or shed from the endometrium during menses (Elass et al. 2002). Cervical-LF levels are elevated in vaginal mucus just after menstruation (63 to 218 μg/mg of protein) and lowest (3.8 to 11.4 μg/mg of protein) just before menses. Variation in vaginal-LF concentration may result in alterations and susceptibility to microbial pathogens (Cohen et al. 1987). In the infected cervix, elevated levels of LF appear to contribute to the regulation of inflammatory responses and the elimination of microbial pathogens or associated debris. Interestingly, LF levels in cervical mucus correlate with reproductive tract infections (if present) as a diagnostic marker for inflammatory disorders (Mania-Pramanik et al. 1999).

Follicular LF migrates into the oocyte from the serum and also produced by theca cells. The levels of serum-LF and follicular-LF are almost identical (Kelver et al. 1996). Follicular-LF levels are serum hormone-dependent and its concentration is estimated at ∼452 ng/mL. No correlation was found between follicular size and LF concentration (Sutton et al. 2003). Follicular-LF plays a prominent role in fertilization and the embryo quality. Follicular-LF is one of the biological markers guiding the selection of embryos at the time of embryo transfer. A direct effect of follicular-LF on oocyte maturation may be minimal; however, an influence of LF on cumulus cells must be considered. LF receptors on oocytes and cumulus cells suggest a direct involvement of LF in embryo maturation. Thus, the follicular-LF may have an important physiological role in the human reproductive process (Yanaihara et al. 2007).

Amniotic-LF: Amniotic fluid is the first feeding of LF with other critical mucosal immune factors to the fetus. LF exists in both amniotic fluid and cervical mucoids in pregnant women. Detectable levels of LF appear in amniotic fluid after *Week-20* of pregnancy. LF levels are elevated around *Week-30* and remains high until term. Amniotic-LF may play a vital role in the placental iron transfer and host defense during pregnancy. The distribution of iron between the maternal and embryo-placental compartments during the 1st trimester is comparable to that found later stages of gestation (Gulbis et al. 1994).

In cord blood, LF concentration is low. In tissue specimens, the amount of LF is highest in the decidua (9–95 μg/g), moderately present in the amniotic (2–37 μg/g), chorion (2–26 μg/g) membranes and in the trophoblast (5–35 μg/g). In the umbilical cord, the concentration is <1 μg/g. These results suggest a decidual origin of LF (Thaler et al. 1993). Amniotic-LF levels range from 0.5 to 2.5 μg/mL during a normal pregnancy. Levels of amniotic-LF show drastic modulation at a different stage of pregnancy. Accordingly, LF could be detected in 85.4% of amniotic fluid samples, not detectable in all fluid obtained in the mid-trimester, and detectable in all maternal and cord plasma samples. Amniotic-LF content markedly increases with the advancing gestational age. Thus, term parturition is associated with a significant increase in LF levels in the fetal compartment (umbilical cord blood) and a decrease in the amniotic compartment (Niemelä et al. 1989).

LF and immune-redox changes during pregnancy

Physiological levels of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide play important regulatory roles in almost all aspects of catabolic and anabolic metabolism, and increased levels can signal transient adaptive homeostasis as a protective mechanism, or even apoptosis in the case of severe oxidative stress (Davies 2016; Lomeli et al. 2017). During pregnancy, ROS can activate various signaling transduction pathways such as folliculogenesis, oocyte maturation, endometrial cycle, luteolysis, implantation, and embryogenesis (Agarwal et al. 2008). Persistent and elevated generation of ROS could cause a disturbance in redox homeostasis that leads to oxidative stress (Pomatto and Davies 2018). Excessive oxidative stress could be detrimental (e.g. in conditions such as COVID-19); however, moderate levels of oxidants that the reducing systems of the cell can cope up with are beneficial for embryonic and fetal

development. Accordingly, it has been found that increased levels of one ROS species, hydrogen peroxide, modifies key transcription factors that influence gene expression during fetal development, as well as placental and amniotic membrane integrity during pregnancy (Dennery 2004).

Maternal LF is an activator of cell signaling pathways that scavenge free radicals, regulate oxidative stress and various pro-inflammatory cytokines (Legrand et al. 2005). Iron sequestration by LF decreases oxidative stress by lowering the probability of the Fenton reaction, and as such could alter the production of cytokines (Kruzel et al. 2006). These multifunctional activities, combined with redox-based control of oxidative stress, makes LF a potential regulator of innate host defense, including the cytokine release syndrome ('cytokine storm'), acute inflammation-related pathologies such as SARS, MERS, *Systemic Inflammatory Response Syndrome* (SIRS), *Toxic Shock Syndrome* (TSS), etc (Naidu et al. 1986; Naidu et al. 1989; Bharadwaj et al. 2010). Therefore, a fundamental role for LF in the redox biology of COVID-19-*Pregnancy* and COVID-19-*Postpartum* is warranted.

LF in iron homeostasis and oxidative stress

The placenta generates ROS which may contribute to the oxidative stress in normal pregnancy. Elevated oxidative stress in pregnancies may lead to complications such as preeclampsia, intrauterine growth restriction (IUGR) and pregestational diabetes (Myatt 2010). During pregnancy, redox imbalance and oxidative stress are attributed to the intense growth activity of the fetus (Tourville et al. 1969). In human body fluids, the concentration of free available iron must not overcome 10^{-18} M to avoid microbial multiplication and to hinder the precipitation of insoluble ferric hydroxides as well as the formation of free radicals *via* the Fenton reaction. Human-LF, by its iron-binding ability, guarantees that free available iron does not exceed 10^{-18} M (Klebanoff and Waltersdorph 1990; Naidu 2000). In the body, superoxide anions are scavenged by SOD, catalases, and peroxides by redox enzymes such as GSH- and Trx-dependent peroxidases, and peroxiredoxins (Prdx) (Roos and Messens 2011). Any decline in redox enzymes could result in increased free radical levels and subsequently induce lipid peroxidation, protein oxidation, and DNA/RNA oxidative damage. While moderate oxidation triggers apoptosis, severe oxidative stress could lead to tissue necrosis or even cellular death (Davies 1995; Naidu 2013; Sies 2017). Binding of LF to Fe^{3+} ions could block iron-mediated catalysis and oxidative disturbances in the cell membranes. The antioxidative mechanism of LF appears to involve stimulated glycolysis, increased ATP generation and sustaining the ion gradient, membrane potential and morphology of the cell (Maneva et al. 2003). Thus, LF may reduce oxidative stress at the molecular level, and modulate inflammatory responses at the tissue level. Endogenous LF could prevent lipid, protein and nucleic acid oxidation through its iron-binding and metal-sequestration ability (Volden et al. 2012). It turned out that oxidative stress and its related metabolic syndromes are potential risk factors in the pathogenesis of COVID-19 (Ruan et al. 2020). As a regulator of redox homeostasis, maternal LF could play a prominent role in the clinical management of COVID-19-*Pregnancy*.

Several respiratory viruses induce a dysregulated ROS formation, due to increased inflammatory responses at the site of infection. Also, viral infections disrupt antioxidant mechanisms, leading to oxidative stress. The severity of lung injury in SARS-CoV infected patients depends in part on activation of the oxidative stress machinery coupled with innate immunity and activation of transcription factors, such as NF-κB, resulting in an exacerbated proinflammatory host response (Padhan et al. 2008). The major cause of mortality in COVID-19 cases may be due to exacerbated inflammatory response accompanied by uncontrolled oxidative stress as well as severe inflammatory reaction at the lung parenchymal level (Delgado-Roche and Mesta 2020). During COVID-19 infection, any unrestrained inflammatory cell infiltration could mediate lung damage through excessive ROS and secretion of proteases, in addition to direct virus-inflicted damage. This may lead to diffused alveolar damage, including desquamation of alveolar cells, hyaline membrane formation and pulmonary edema (Tian et al. 2020); this could subsequently limit the efficiency of gas exchange in the lung, causing difficulty in breathing and associated hypoxemia (Tay et al. 2020). Intracellular redox changes intertwined with acute-phase inflammatory responses likely represent the main cause of severity and mortality in COVID-19.

Glycan chains in LF structure-function

The molecular basis of LF multi-functionality is attributed to its structural orientation based on glycosylation (Spik et al. 1994; Choi et al. 2008). There are three possible N-linked glycosylation sites in human LF (hLF), one at Asn^{138}, a second site at Asn^{479}, and a third site at Asn^{624}; differential utilization of these sites results in distinct glycosylation variants. hLF glycans are the N-acetyl-lactosaminic type, α1,3-fucosylated on the N-acetyl-glucosamine residue linked to the peptide chain. Unlike the milk-derived LF, the neutrophilic LF form is not fucosylated, and the difference in structure-function activities of these two distinct LF forms is not fully understood. hLF specifically competes with IL-8 for proteoglycan binding sites and may serve as an explanation for the anti-inflammatory effects of LF observed during *in vivo* sepsis models (Elass et al. 2002). Since hLF contains multiple sites of glycosylation, it is recognized by the group of C-type lectin receptors, which includes the mannose receptor and DC-SIGN (specific ICAM-3-grabbing non-integrin). Dendritic cells (DC) pretreated with LF inhibit HIV-1 infection, resulting from LF binding to DC-SIGN blocks its interaction with gp-120 and prevents viral transmission (Groot et al. 2005). Glycosylation is also required for adjuvant activities of LF; increased generation of delayed-type hypersensitive (DTH) response (Kocieba et al. 2002). During an episode of COVID-19-*Pregnancy*, the involvement of specific form(s) of LF glycoproteins in plasma (circulatory), neutrophilic (inflammatory), and placental/amniotic (barrier-defense) portals are currently under investigation. A comparative immuno-functional analysis of these data with LF isolated from breast milk of COVID-19-*Postpartum* mothers may provide critical knowledge of the pathogenic spectrum of SARS-CoV-2 during pregnancy and postpartum and help develop effective clinical strategies to reduce possible vertical 'mother-to-child transmission' (MCTC) of COVID-19 illness.

Polybasic domains in LF structure-function

The SARS-CoV-2 has acquired a unique polybasic cleavage site (*R-R-A-R*) at the junction of S1 and S2, which facilitates an effective cleavage by furin and other proteases (Andersen et al. 2020). This novel virulence trait has significantly enhanced the infectivity, host tropism, and pathobiological spectrum of COVID-19 (Nao et al. 2017). Competitive blocking of SARS-CoV-2 polybasic cleavage site with highly basic innate host proteins or peptides with a stretch of arginine residues may serve as a viral intervention strategy. Milk LF inhibits HIV and the antiviral activity correlates with the negative charge (polybasic arginine residues) on the N-terminal region of LF protein (Swart et al. 1999). Interestingly, LF also demonstrates serine protease activity and cleaves arginine-rich sequences in a variety of microbial virulence proteins, contributing to its long-recognized antimicrobial properties (Hendrixson et al. 2003).

LF is considered the most polybasic protein in host defense against tissue injuries and infections. The highly basic N-terminal domain of LF interacts with various microbial and host targets; thereby elicits antimicrobial effects as well as modulates innate and adaptive immune responses (Kawasaki et al. 2000). The best characterized LF targets are negatively charged molecules, which include proinflammatory microbial factors (e.g. lipopolysaccharide), as well as host cellular components such as DNA, glycosaminoglycan (GAG) chains of proteoglycans, and cell surface receptors (CSRs). These LF-CSR interactions could influence signaling pathways that modulate complex immune machinery and regulate cytokine release (Legrand 2016). A peptide derived from the N-terminus region of *human LF*$^{(1-11)}$ (*GRRRRSVQWCA*) binds and activates monocyte function. The stretch of arginine residues from position 2 to five and the cysteine residue at position 10 are pivotal in the immunomodulatory properties of LF (van der Does et al. 2012). The N-terminal basic stretch of four consecutive arginine residues, R^2-R^3-R^4-R^5, are involved in the binding of human LF with heparin, lipid A, lysozyme, and DNA (van Berkel et al. 1997). Later studies estimated about 80,000 binding sites per Jurkat cell, mainly sulfated molecules, dependent on basic cluster R^2-R^3-R^4, but not on R^5 residue of the N-terminus region (Legrand et al. 1998).

Antiviral activity of LF

Several *in vitro* studies have demonstrated the antiviral activity for LF against both enveloped and naked viruses. Observational and self-report studies have suggested that LF inhibits several viral pathogens that cause infections such as common cold, influenza, gastroenteritis, summer cold, herpes, etc (Wakabayashi et al. 2014). LF appears to reduce viral docking and entry into host cells, indicating a protective effect on the early phase of virus infection. Preincubation of host cells with LF for 5–10 min blocks certain viral infections (e.g. *human cytomegalovirus*, HCMV), even after removing LF from the viral media (Hasegawa et al. 1994). The possible protective effects of LF against *in vitro* and *in vivo* viral infections are attributed to both blocking of the initial viral attachment to host target cells as well as subsequent interference with the cellular entry and replication of the viral pathogen (Waarts et al. 2005). LF may also induce expression of antiviral cytokine mRNA, such as IFN-α and IFN-β that could inhibit viral replication in infected cells (Ishikawa et al. 2013). These inhibitory effects are achieved through

competitive binding of LF to host cell receptors (i.e. HSPG, ACE2, sialic acids, etc.), and/or directly to viral capsid (i.e. S, E, M, N proteins). Antiviral effects of LF are widely studied *in vitro* and several human clinical trials have shed light on possible mechanisms of action, therapeutic efficacy, and safety.

The nuclear localization and endosomal activity of LF in different epithelial human cells suggests that this iron-binding protein exerts its antiviral effect not only in the early phase of viral interaction with the host cell target sites, but also in limiting the intracellular propagation of the viral pathogen through modulation of immune cell cascade. LF protects the host cell by impeding the virus-induced apoptosis. For example, when the *Echovirus* enters a susceptible cell by endocytic pathway, treatment with exogenous LF effectively intercepts the delivery of viral genome into the cytoplasm (Ammendolia, Marchetti et al. 2007). LF binding to viral capsid proteins induce structural alterations and increase viral susceptibility to host defense. Inhibition of *Echovirus* infectivity by LF is dependent on its interaction not only with the cell surface GAG chains but also with the viral structural proteins that facilitate cellular entry process (Ammendolia, Pietrantoni, et al. 2007).

LF effects on viral docking to cell surface receptors (CSR)

LF binds to proteoglycans on cell surfaces and to 'nucleolin' expressed in cell membranes. LF co-localizes with nucleolin and actively endocytosed through vesicles of the recycling/degradation pathway. A small proportion of LF is also translocated into the cell nucleus. Absence of LF endocytosis in proteoglycan-deficient cells despite LF binding, indicates that both nucleolin and proteoglycans are required in the endocytosis of LF (Legrand et al. 2004). Monocytes and peritoneal macrophages bind and internalize the human LF (van Snick et al. 1977). Other cell types such as brain endothelial cells, hepatocytes and placental cytotrophoblasts demonstrate receptor-mediated uptake and internalization of LF (Huang et al. 2007). LF binding to these cellular receptors is mediated by sulfated chains of proteoglycans (Legrand et al. 2006). Both bovine and human LF bind to THP-1 cells, a human monocytic cell line, and this interaction is reduced by blocking sulfonation of the cell surface (Roşeanu et al. 2000; Saidi et al. 2006; Ammendolia, Pietrantoni, et al. 2007).

Glycosaminoglycans (GAGs): LF interacts with endogenous heparin-like molecules and modulates GAG-mediated biological pathways. Five basic residues at the N-terminus region of LF protein: Arg^5, Arg^{25}, Arg^{28}, Lys^{29}, and Arg^{31}, when substituted by alanine, all the LF derivatives showed decreased ability to neutralize GAGs in a dose-dependent manner. The site mutations at Arg^{25} and Arg^{28} demonstrated the most striking decrease in the ability of LF to neutralize various GAGs. Both Arg^{25} and Arg^{28} are identified as the critical basic residues at the N-terminus region of LF for heparin-binding. Other basic residues on the N terminus, Arg^5, Lys^{29}, and Arg^{31}, may serve as additional cationic motifs for heparin-binding by LF (Wu and Church 2003). This GAG neutralizing ability of LF may play a role in blocking the viral adhesion to proteoglycan-rich host cell surfaces.

LF may block viral attachment to cell membranes *via* competitive inhibition of common GAG receptors (Pietrantoni et al. 2015). LF is shown to inhibit viral attachment to

host cells expressing GAGs [i.e. HSPG, chondroitin sulfate (CS), etc.] and may interfere with the early phase of viral pathogenesis. Glycoprotein C (gC) located on the *Herpes Simplex Virus* (HSV) 'capsid glycoprotein C' (gC) binds to GAG and facilitates viral attachment to host cell surface. LF effectively blocks the virus from this critical step of cellular docking. HSV mutants lacking the 'gC-protein' are less inhibited by LF in GAG-expressing cells, suggesting that LF directly binds to the viral capsid and blocks the HSV docking of host cells. LF also binds directly to both HSPG and CS isolated from cell surfaces, as well as to purified preparations of GAG chains. One mechanism for the inhibition of HSV-1 infectivity appears to be dependent on LF interaction with cell surface GAG chains of HSPG and CS (Marchetti et al. 1998; Marchetti et al. 2004).

Sialic acids: Many CoVs use sialic acids, either as receptor determinants or as attachment factors for viral docking to the heavily glycosylated mucus layer (Desmarets et al. 2014). The C-lobe of LF interacts with hemagglutinin (HA) and prevents *Influenza A virus* infection (Superti et al. 2019). The highly conserved peptides of influenza HA are involved in a low-pH-mediated fusion process and plays a critical role in the early steps of viral infection. LF interaction with influenza HA at low pH induces charge alterations and destabilizes HA conformation, subsequently inhibits the fusion peptide activity. LF also appears to attenuate *Dengue virus* (DENV)-2 binding to host cell membrane by interacting with HSPG, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and low-density lipoprotein receptors (LDLR) (Chen et al. 2017). *Hepatitis C virus* (HCV) has two envelope proteins, E1 and E2 that form hetero-oligomers. Both human and bovine LF avidly bind to these HCV envelope proteins and inhibit the HCV genome replication (Yi et al. 1997). This antiviral activity is specific against the HCV ATPase/Helicase NS3 protein and does not affect the HCV RNA-dependent RNA polymerase (NS5B protein). These data suggested a novel antiviral activity of LF against intracellular HCV replication (Picard-Jean et al. 2014).

LF effects on virus-cell membrane fusion

The S-protein of SARS-CoV is a class I viral fusion protein responsible for both receptor binding and membrane fusion during viral entry. Like other class I fusion proteins, the SARS-CoV S-protein undergoes proteolytic priming prior to fusion activation. Several host cell proteases could prime the fusion activation of SARS-CoV, which occurs at the interface of the receptor binding (S1) and fusion (S2) domains (S1/S2), as well as adjacent to a fusion peptide within S2 (S2') (Madu et al. 2009).

CoV S-protein and viral cell entry: Human CoV-229E uses endosomal cathepsin L to activate the S-protein after receptor binding. Clinical isolates of HCoV-229E preferentially utilize the cell surface protease, transmembrane protease serine 2 (TMPRSS2), rather than endosomal cathepsin L (Shirato et al. 2017). The endosome is a main site of Toll-like receptor recognition (TLR), which triggers an innate immune response. Accordingly, HCoV-229E has evolved mechanisms to bypass the endosome by cellular entry *via* TMPRSS2. Thus, the virus uses specific mechanisms to evade the host innate immune system.

Two major mechanisms are responsible for proteolytic activation of viral S-proteins. For many enveloped viruses, cellular proteases (i.e. furin, trypsin, or TMPRSS2) cleave

the glycoprotein during biogenesis, separate the receptor binding with the fusion subunits, and convert the precursor glycoprotein to its fusion-competent state (White et al. 2008). Alternatively, for other viruses, such as SARS-CoV, and MERS-CoV, cleavage of the viral glycoprotein by cell surface or endosomal proteases (i.e. elastase, histone acetyltransferase [HAT] or cathepsin L) induces conformational changes during viral entry following receptor binding (Shulla et al. 2011). After virus/receptor binding, HCoV-229E also utilizes host cellular proteases to trigger viral-membrane-cell membrane fusion. HCoV-229E enters cells at the cell surface in the presence of extracellular serine proteases, such as trypsin, but in their absence, the virus utilizes cathepsin L in the late endosome (Bertram et al. 2013).

LF inhibits viral cell entry: Several charged proteins and peptides are known to inhibit virus entry. Natural milk proteins with high charge or hydrophobicity profile demonstrate potent anti-HIV activity. Bovine milk LF (IC_{50} 0.4 μM) has potent anti-HIV-1 activity. Modest inhibition was also obtained with LFcin, a high positively charged loop domain of LF. LF interferes with HIV-1 receptors CXCR4 and CCR5, thereby blocks the viral entry process (Berkhout et al. 2002). It appears that the antiviral activity of LF may also be related to its positive charge. The addition of positive charges to LF *via* amidation appears to enhance antimicrobial properties in contrast to increasing the negative charges by acylation, which abolished both the antimicrobial and antiviral properties of LF (Pan et al. 2007).

LF exhibits antiviral activity at an early phase of viral infection by interacting with several host CSRs. Human LF and seven hLF-derived synthetic peptides corresponding to the N-terminal domain of the native protein (1–47 amino acids sequence) demonstrated the capacity to prevent *Hepatitis B virus* (HBV) infection and replication (Florian et al. 2013). Four of the peptides showed 40–75% inhibition of HBV infection in HepaRG cells, human $LF^{(1-23)}$ peptide containing the *GRRRR* cationic cluster showed the most potent antiviral activity. This cluster motif is also one of the two GAG binding sites of the native hLF responsible for inhibition of viral replication; however, the mechanism of the $hLF^{(1-23)}$ peptide action was different from that of the full-length protein. The cationic peptide cluster is sufficient to interact with negatively charged residues on the viral envelope to prevent viral attachment to the cells. The *GRRRR* cationic peptide may constitute a nontoxic approach for potential clinical applications in inhibiting viral host cell entry by neutralizing the viral particles (Padhan et al. 2008).

LF effects on cellular internalization of virus

CoVs enter host cells *via* two primary mechanisms: some viruses deliver their genomes into cytosol after their envelopes fuse with the plasma membrane at the cell surface, whereas, others take advantage of the cellular endocytic machinery (Burkard et al. 2014). Although most CoVs use only one of these routes for cellular entry, some viruses use both mechanisms of invasion.

Macro-pinocytosis and viral uptake: Macro-pinocytosis is exploited by many viral pathogens for cell entry. In SARS CoV, S-protein mediates interaction with receptors on adjacent cells, resulting in cell fusion and syncytium formation. Syncytium formation is a cytopathic effect (plasma membrane changes) consistent with macro-pinocytosis

that increases cell-to-cell spreading of the virus (Yamada et al. 2009). Macro-pinocytosis is a type of endocytosis that is morphologically defined by the presence of membranous extensions of outwardly polymerizing actin termed membrane ruffles. Membrane ruffles nonspecific vesicles that surround and internalize fluid cargo into large vesicles or macro-pinosomes (Kerr and Teasdale 2009). An active replicating virus could induce macro-pinocytosis. LF inhibits macro-pinocytosis and impairs viral replication and cell-cell fusion (Freeman et al. 2014).

Endocytosis and viral uptake: SARS-CoV invades the host cell by direct fusion at the plasma membrane (Simmons et al. 2004). Endosomal mode of cellular entry of SARS-CoV involves cathepsin L, an endosomal protease (Yang et al. 2004; Huang et al. 2006). Endosomal conditions such as low pH, high H_2O_2, and proteolytic activity could induce conformational changes in fusion proteins and facilitate viral merger with the host cell membrane (Matsuyama and Taguchi 2009). Endocytic pathway is both clathrin- as well as caveolae-independent, where lipid rafts play an important role (Inoue et al. 2007; Wang et al. 2008). Proteolytic cleavage of S-protein is important for the induction of viral-cell fusion and/or virus entry into host cells. Different cleavage sites have been identified for different CoVs. Some CoV S-proteins are cleaved at the S1/S2 boundary by furin-(like) proteases during transport (Luytjes et al. 1987). Both clathrin-dependent as well as clathrin- and caveolae-independent entry pathways exist in SARS-CoV (Inoue et al. 2007; Wang et al. 2008).

LF effects on viral cell entry: Proteolytic degradation of proteins from both the host and the virus is critical for several physiological processes. Neutrophils secrete LF and serine proteases such as cathepsin G (CatG), neutrophil elastase (NE), and proteinase 3 (PR3) in response to microbial challenge. LF increases the catalytic activity and broadens the substrate selectivity of CatG during inflammatory conditions (acidic pH 5.0). LF also enhances CatG-induced expression of cell surface expression of CD62P and activates platelets. Consequently, LF-mediated enhancement of CatG activity might promote innate immunity during acute inflammation (Eipper et al. 2016). Milk LF and β-casein are potential inhibitors of cysteine proteases. LF is a strong inhibitor of cathepsin L activity. The inhibition kinetics of LF are noncompetitive and heat-sensitive, which suggests that the tertiary structure of LF is critical for the activity (Ohashi et al. 2003).

LF effects on viral replication

LF saturated with ferric (Fe^{3+}), manganese (Mn^{2+}) or zinc (Zn^{2+}) ions inhibits the infection of Vero cells by human *Herpes Simplex virus* type 1 (HSV1) and 2 (HSV2). Intracellular viral replication and plaque formation is effectively inhibited by metal saturated LF in a dose-dependent manner. Inhibitory concentration (IC_{50}) of LF to reduce viral replication ranged from 5.2 to 31 μg/mL. Fe-LF and Mn-LF showed higher IC_{50} values than Zn-LF and apo-LF (Marchetti et al. 1998). Native and conformationally intact LF proteins from serum and milk may thus inhibit the cytopathic effect of HIV-1 and HCMV on MT4 cells and fibroblasts. LF from bovine or human milk, colostrum, or serum completely block HCMV infection (IC_{50}=35–100 μg/mL). Native LF also inhibits the HIV-1-induced cytopathic effect (IC_{50}=40 μg/mL). The specific distribution of positively and negatively charged domains in the LF protein structure is important
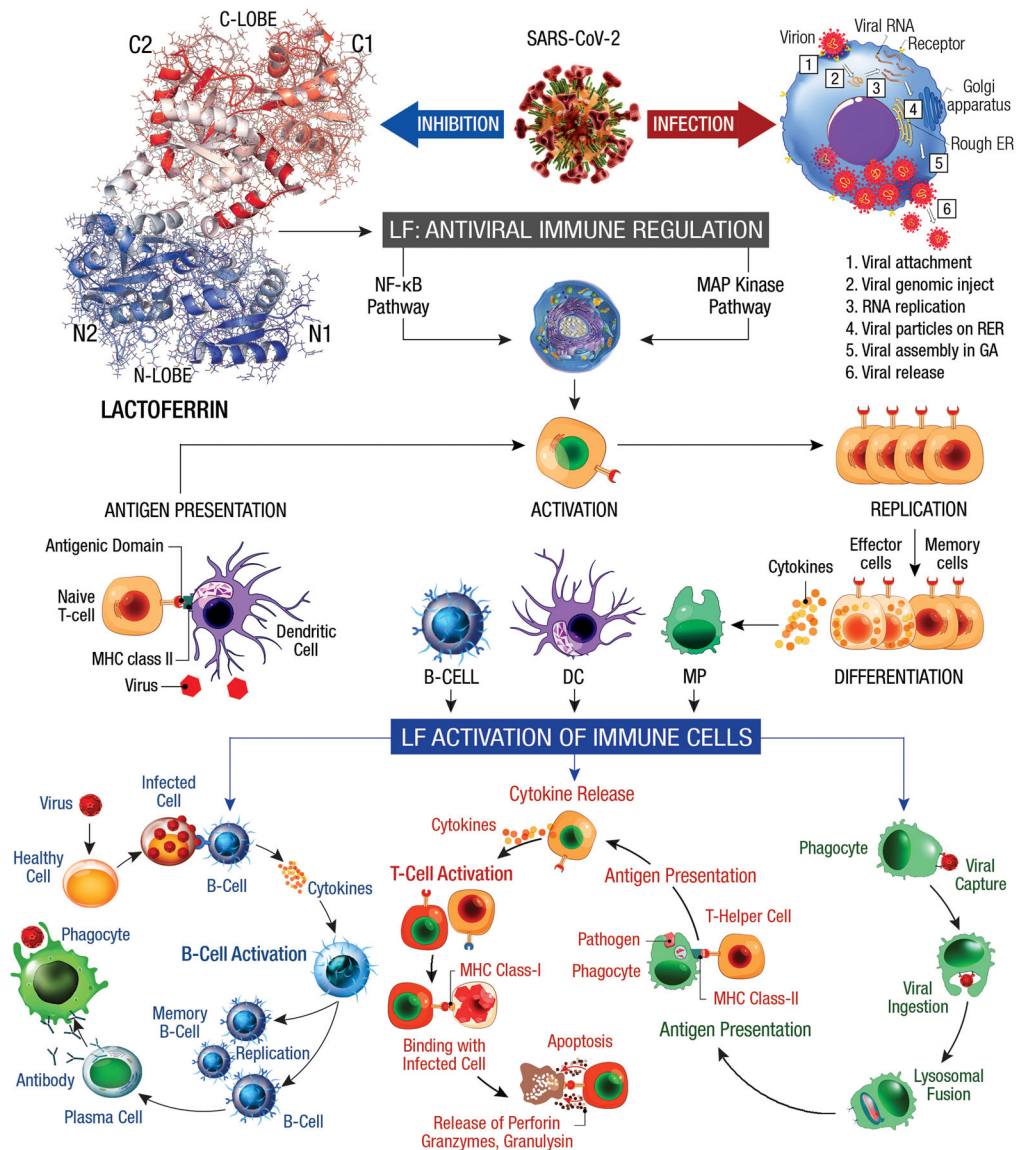
Figure 2. Lactoferrin-regulated antiviral immune responses. Antigen-presenting cells (APCs) mediate antiviral immune responses and act as messengers between the innate and the adaptive immunity. The immune system contains three types of APCs – macrophages (MPs), dendritic cells (DCs), and B lymphocytes. Macrophages are active phagocytic cells that control viral pathogens, either by direct intracellular killing or block viral replication by releasing cytokines. DCs process/present viral particles to T cell surface for antigen recognition. B-cells utilize specific surface receptors to capture foreign antigens and present their associated epitopes to T-cells. Cytotoxic T cells are activated by DCs that express antigen-loaded MH class I molecules. B-cells are activated when antigens bind to their surface receptors. Some activated B-cells turn into plasma cells and secrete antibodies, while others transform into long-lived memory B-cells which are stimulated later to differentiate into plasma cells. At cellular level, LF modulates several pathways of APC biology, including cellular migration and activation; whereas at molecular level, LF affects expression of soluble immune mediators, i.e. cytokines, chemokines and other effector molecules; to regulate inflammatory and immune responses (Actor et al. 2009; Siqueiros-Cendón et al. 2014).

for both anti-HIV and anti-HCMV effects (Harmsen et al. 1995; Swart et al. 1998). Inhibition of intracellular viral replication by N-lobe is 2-fold and 3-fold more effective than that of the C-lobe of LF (Redwan et al. 2014). Importantly, there is *in vitro* evidence that LF may attenuate cytopathic effects of influenza virus, when incubated with the cells after viral adsorption (Pietrantoni et al. 2012).

LF effects on antiviral immune responses

During the COVID-19 infection, the initial damage to lung epithelia triggers a local immune response. Alveolar macrophages and monocytes are the early responders to release cytokines and prime the adaptive immunity (with T and B lymphocytes). Such immune response can resolve the SARS-CoV-2 infection in most cases. However, if the immune reactivity continues, severe local inflammation may ensue, with increased release of pro-inflammatory cytokines and chemokines into the circulatory pool. Patients with severe COVID-19 exhibit higher blood plasma levels of IL-1β, IL-2, IL-7, IL-10, granulocyte colony-stimulating factor (G-CSF), IP-10, MCP1, macrophage inflammatory protein 1α (MIP1α) and TNF-α (Naidu et al. 1989; Yang et al. 2004; Huang et al. 2020). Secretion of these cytokines and chemokines attract immune cells, notably monocytes and T lymphocytes, but not neutrophils, from the blood into the infected site (Xu, Zhao, et al. 2020; Xu, Shi, et al. 2020). Pulmonary recruitment of immune cells from the blood and the infiltered lymphocytes into the airways may lead to lymphopenia and elevate the neutrophil-to-lymphocyte ratio, as observed in 80% of COVID-19 patients (Qin et al. 2020). In addition to local damage, cytokine storm also has ripple effects on the body. Elevated levels of cytokines may lead to septic shock and multi-organ failure resulting in myocardial damage and circulatory failure observed in some COVID-19 patients (Dennery 2004). Earlier studies on SARS-CoV found that the virus may infect other targets in addition to upper respiratory and lung cells. Notably, the virus was found in T-lymphocytes, macrophages, and monocyte-derived dendritic cells (Law et al. 2005; Tseng et al. 2005). Direct virus killing of lymphocytes may cause lymphopenia in patients (Gu et al. 2005).

LF modulates antigen-specific adaptive immunity: Especially in CoVs, viral infection of immune cells such as monocytes and macrophages could result in aberrant cytokine production (Tseng et al. 2005). Therefore, an understanding of both viral as well as innate host factors in the immune responsive pathways of COVID-19 are critical in the development of effective immune-therapeutic protocols. Endogenous or intrinsic LF could play a key role in the immunopathology of many viral infections. LF regulates inflammation (both pro- and anti-inflammatory pathways), as well as the cellular and molecular mechanisms that modulate adaptive immunity (Figure 2). As an integral part of the innate immune defense, LF is recognized as an immunomodulator of leukocyte populations, including neutrophils, peritoneal macrophages, NK cells, T cells, and B cells (Yanaihara et al. 2007; Actor et al. 2009; Siqueiros-Cendón et al. 2014). More importantly, LF as an adjuvant elicits a T cell mediated DTH response against a variety of antigens (Hwang et al. 2016).

LF activates APCs and helps the T-cell-mediated specific antigen recognition (Puddu et al. 2007). There is abundant evidence that LF binds to specific receptors on the

surface of macrophages and increases their phagocytic activity (Birgens et al. 1983; Roșeanu et al. 2000; Wilk et al. 2007). LF also suppresses pro-inflammatory cytokines and type I interferon (IFN α/β) induction; thereby, affecting the ability of phagocytes to present antigens to antigen-specific CD^{4+} T-cells in the adaptive immune system (Suzuki et al. 2005; Latorre et al. 2010). LF could modulate antigen-specific adaptive immune responses (i.e. APC activation, maturation, migration, and antigen presentation) and bridges the functions of both T- and B-cells (Legrand et al. 1997). Structural changes in the N-terminal 'basic' domain of LF facilitates its molecular interactions with B lymphocytes (Padhan et al. 2008). Oral administration of LF could increase in the intestinal secretion of IgA and IgG (Zimecki et al. 1996; Sfeir et al. 2004). LF enables the interaction of antigen presenting B-cells with T cells; thereby, elevates the antibody response. T-helper cell type 1 (T_H1) and type 2 (T_H2) activate macrophages for intracellular killing of microbial pathogens (Hwang et al. 2011). LF promotes T_H1 and inhibits T_H2, which leads to the downregulation of T-cell activity. This lowers the release of cytokines IL-5 and IL-17 with amplification of inflammatory response (Wang et al. 2013). LF accelerates T-cell maturation by inducing the expression of CD4 surface markers (Dhennin-Duthille et al. 2000). LF receptors expressed on all T-cell subsets (Bi et al. 1997; Legrand et al. 1997), bind to T-cell surface receptors, modulate natural killer (NK) cell activity, and restore the humoral immune responses (Artym et al. 2003). LF could reduce T_H1 cytokines and prevent excess inflammatory responses (Kuhara et al. 2000). Oral administration of LF could reduce lung consolidation score and the number of infiltrating leukocytes into bronchoalveolar lavage fluid during viral H1N1 influenza infection. LF increases the expression of IL-12p40, IFN-β, and NOD2 (Shin et al. 2005, 2018). Thus, oral LF appears to augment the transcription of important immune-related genes and such transcriptional activation may promote systemic host immunity. These modulatory effects on APCs suggests a potential role for exogenous LF in the enhancement of adaptive immunity against COVID-19 infections.

LF as adjuvant for immunizations

Adjuvants modulate the immune response to specific types of APCs to enhance the efficacy of a vaccine. Alum and MF59 are common adjuvants used in influenza vaccines, where both elicit migration of neutrophils and monocytes to the site of adjuvant/antigen injection (Calabro et al. 2011). In the case of LF, once on site, neutrophils release the LF from secondary granules and activate both innate and adaptive immune responses by recruiting leukocytes and activating dendritic cells (DC). Thus, LF admixes with immunization may augment the efficacy of vaccines *via* the up-regulation of cytokines synthesis and DTH response (Hwang et al. 2007). Vaccine trials have shown that LF (200 μg) + influenza H1N1 HA antigen (30 μg) could initiate an antibody response comparable to that of alum adjuvant (Sherman et al. 2015). Therefore, injecting LF rather than a traditional adjuvant (perhaps with greater side effects) could eliminate the neutrophil recruitment step and directly facilitate DC recruitment, maturation, and activation (de la Rosa et al. 2008).
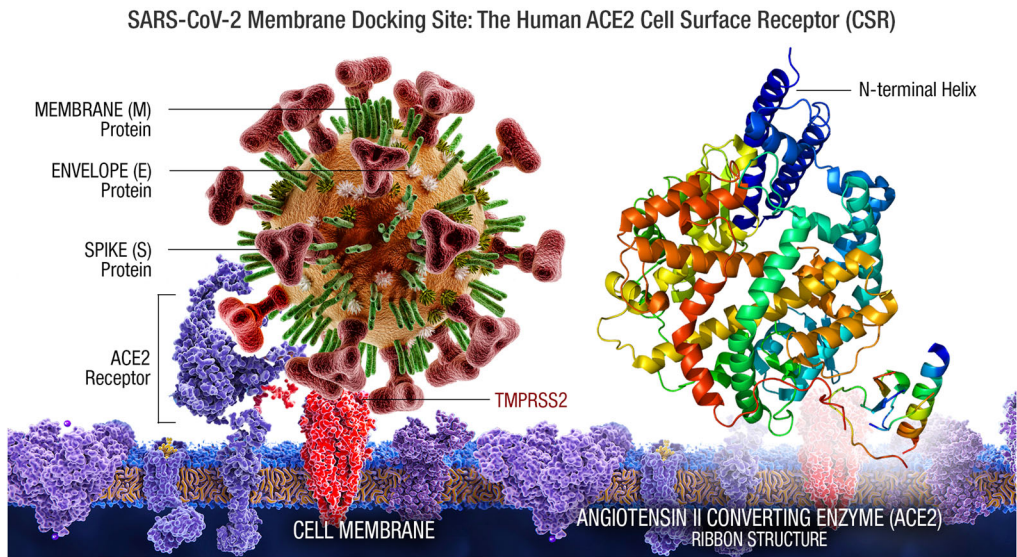
SARS-CoV-2 Membrane Docking Site: The Human ACE2 Cell Surface Receptor (CSR)

Figure 3. The S-protein/ACE2 interface. The S-protein of SARS-CoV-2 facilitates viral docking and entry into host target cells. The S-protein engages ACE2 as the entry receptor and requires the cellular serine protease TMPRSS2 for S protein priming. The efficiency of ACE2 access and utility is a key determinant of COVID-19 infection and transmission. Structure of the ACE2 protein (Right) is based on PyMOL rendering of PDB ID 1R42 (Towler et al. 2004).

The generation of T_H1 immunity against COVID-19 is dependent on APCs such as macrophages, to produce IL-12, a mediator that promotes naïve T-cell development (Naidu et al. 1989). In addition, IL-12 is also a co-stimulator that maximizes the secretion of IFN-γ from T_H1 cells and activates IFN-γ producing cells from memory T-cells (Chen et al. 2016). *In vivo* studies have shown that LF could stimulate APCs and increase TNF-α, IL-6, and IL-12 production (Hwang et al. 2007). Therefore, LF might be studied as an adjuvant to augment subsequent adaptive responses with COVID-19 challenge.

LF and ACE2 expression in COVID-19-*pregnancy*

ACE2 activity in pregnancy

During normal pregnancy, the renin-angiotensin system (RAS) is activated. Estrogen and progesterone upregulate angiotensinogen and renin, which results in the rise of angiotensin (ANG) II levels in the cell surface of lungs, arteries, heart, kidney, and intestines. ACE2 lowers blood pressure by converting the ANG-II into ANG-(1-7), a vasodilator (Figure 3) (Donoghue et al. 2000). In human ovaries, ACE2 is found in primordial, primary/secondary/antral follicles, stroma, and corpora lutea (Reis et al. 2011). ACE2 plays a regulatory role in oocyte maturation, steroidogenesis, ovulation, and atresia (Honorato-Sampaio et al. 2012). ACE2 expression is also upregulated during follicular development and after gonadotrophin stimulation (Pereira et al. 2009). ACE2 may act as a local autocrine/paracrine regulator throughout pregnancy, participating in

the early (angiogenesis, apoptosis, and growth) and late (utero-placental blood flow) events of pregnancy (Neves et al. 2008). During pregnancy, the placenta and the uterus constitute an important source of ACE2 (Levy et al. 2008).

ACE2 receptors in COVID-19-pregnancy

In 2004, ACE2 has been identified as the cellular entry point for the SARS-CoV (Turner et al. 2004). The novel SARS-CoV-2 also uses the analogus ACE2 receptor for cellular entry (Hoffmann et al. 2020). During the 3rd trimester of pregnancy, a systemic vasodilatory condition leads to a lowering of blood pressure and upregulation of ACE2 in the reproductive organs. ACE2 is also over expressed in cells of the maternal-fetal interface such as the stromal and perivascular cells of decidua, as well as cytotrophoblasts and syncytiotrophoblasts in the placenta. ACE2 is also present in specific cell types of human fetal heart, liver, and lung, but not in the kidney (Li, Chen, et al. 2020). Therefore, pregnant women are at risk for COVID-19 infection due to over expression of ACE2 receptors – the prime target sites for SARS-CoV-2 cellular invasion. Mapping of ACE2 expression and its levels in different body sites and fluids could access the vulnerabilities of pregnant women for contracting COVID-19 infections (Zhu et al. 2020). Therefore, both the vertical transmission between mother and neonate; as well as the placental dysfunction/abortion during deliveries of COVID-19-*Pregnancy* demand an in-depth evaluation.

When S-protein binds to the host cell surface, ACE2 is down-regulated and receptor levels remain low for the remainder of the viral infection (Kuba et al. 2005; Dijkman et al. 2012). In the lungs, the ACE2 down-regulation triggers hyperactivation of RAS and causes respiratory failure (Imai et al. 2005). In ovaries, a decrease in ACE2 expression after COVID-19 infection could result in altered ovarian RAS function. Such disturbance in ovarian RAS activity leads to reproductive disorders such as polycystic ovary syndrome (POS), ovarian hyperstimulation syndrome (OHSS), ovarian tumors, and ectopic pregnancy (Yoshimura 1997). However, the clinical impact of COVID-19 induced RAS disturbance on oocyte maturation and ovarian reserve needs further investigation.

LF interactions with ACE receptors

LF is a potential source of anti-hypertensive peptides that affects both the RAS and endothelin systems (Manzanares et al. 2015). LF hydrolysate and its derived peptides are shown to block ACE receptors and inhibit ANG II-induced vasoconstriction (Fernández-Musoles et al. 2014). This inhibition of ACE receptors results in direct relaxation of mesenteric arteries *via* mechanisms involving nitric oxide (NO) release, counteracting modulation by prostanoids, and potassium (K^+) efflux. LF peptides also show indirect vasoactive effects by enhancing the endothelial relaxation (García-Tejedor et al. 2017). An LF-derived peptide (*LRPVAA*) was identified to block ACE receptor activity *in vitro*. A dose-dependent (IC_{50} ∼4.14 μM) reduction of systolic blood pressure by this LF-derived peptide was observed at 60 min after injection and it decreased the blood pressure at a rate of 1 nM/mL/kg. The blood pressure-lowering activity of this LF
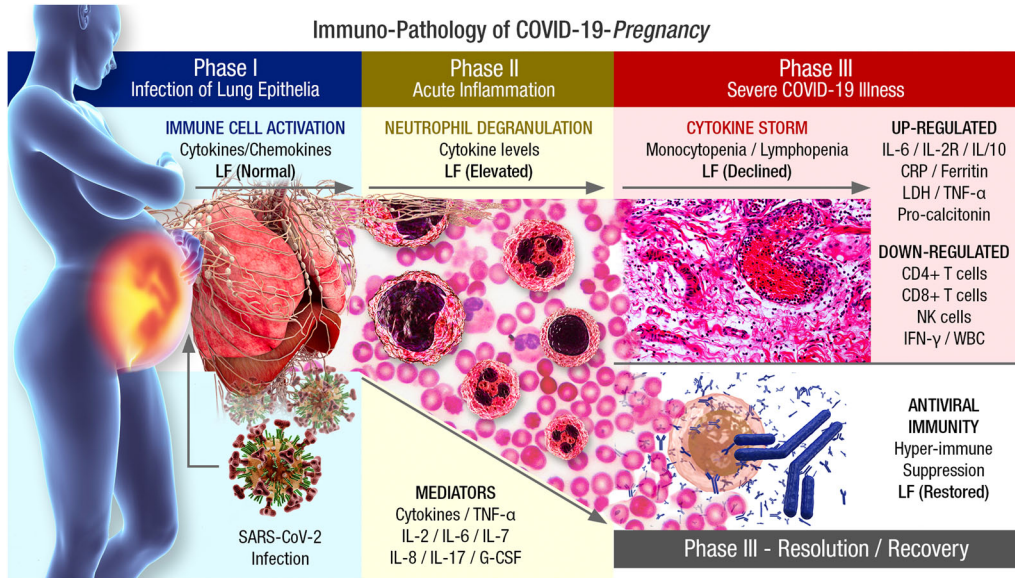
Figure 4. Immuno-pathology of COVID-19-Pregnancy. Clinical outcomes depend on the severity of immune cell activation, inflammatory response, T cell lymphopenia and resulting cytokine storm and *Phase-I*: Infected individuals, based on their immune-competence, either remain asymptomatic or progress to a moderate, pre-symptomatic *Phase-II* exhibiting an increase in IL-6 and a decrease in total T lymphocyte counts, particularly CD4 T cells and CD8 T cells. *Phase-III represent* severe COVID-19 cases with elevated levels of IL-6, IL-2R, IL10, and TNF-α, with a marked decline in total T lymphocytes, particularly CD4 T cells and CD8 T cells, and IFN-γ–expressing CD4 T cells. This extreme immune reactivity leads to pulmonary damage, respiratory distress, and unfavorable outcomes. Based on preexisting data, the immune-modulatory role of LF is extrapolated in the pathobiology of COVID-19-*Pregnancy*.

peptide was about 210% compared to *Captopril* (10 pM/mL/kg) as a positive control (Lee et al. 2006). Taken together, LF levels during pregnancy play a protective role in resolving hypertension *via* downregulation of ACE2; consequently, limiting the membrane receptor access to SARS-CoV-2 for cellular entry.

LF and cytokine release syndrome (CRS) in COVID-19-*pregnancy*

Emerging data suggests that many COVID-19 cases could become fatal due to excessive immune response, characterized by an abnormal release of circulating cytokines, termed 'cytokine release syndrome' (CRS). CRS plays a major role in the symptomatic deterioration of COVID-19 patients, from pneumonia through acute respiratory distress syndrome (ARDS), cumulating in systemic inflammation, and ultimately multi-system organ failure. This phenomenon of cytokine havoc throughout the body is often referred to as 'cytokine storm'. CRS during COVID-19 infection is manifested by acute inflammation with massive oxidative stress. The severity of the CRS is linked to membrane permeability disruption and dysfunction of mitochondria (Exline and Crouser 2008), leading to extensive loss of cellular ATP pool. These clinical conditions lead to a wide range of pathologies during COVID-19-*Pregnancy* such as hypoxia, cytokine storm, and ARDS (Liu, Chen, et al. 2020; Zhu et al. 2020), which may cause to preterm birth, preeclampsia, early pregnancy loss or even death in pregnant women (Figure 4).

COVID-19 is manifested by severe clinical syndromes such as proinflammatory cytokine release, increased expression of adhesion molecules, and massive release of ROS causing widespread oxidative stress (Chen, Huang, et al. 2020). Vascular inflammation ensues rapidly after SARS-CoV-2 infection and coincides with a burst of pro-inflammatory cytokines derived from activated monocytes-macrophages. Clinical data suggest that COVID-19 activates the immune system into a self-perpetuating, generalized state of hyperactivity (Dashraath et al. 2020; Rasmussen et al. 2020; Zaigham and Andersson 2020). LF plays a regulatory role in the clinical management of acute-phase responses and abrogation of cytotoxic damage. Early host defenses during CRS include a rapid rise in LF levels in the plasma (Gutteberg et al. 1989). LF is known to affect leukocytes of the innate immune system by increasing the NK cell activity, promote neutrophil function, enhance phagocytic activity and affect ROS production (Miyauchi et al. 1998; Kawai et al. 2007). LF activates macrophages by increasing cytokine and nitric oxide (NO·) production, thereby, limits intracellular pathogen proliferation (Sorimachi et al. 1997; Wakabayashi et al. 2003; Puddu et al. 2007). Neutrophil degranulation in response to inflammatory signals introduces LF into the cellular milieu populated with innate leukocytes (macrophages, DCs, and NK cells) and adaptive immune cells (T- and B-cells). Several cytokines cause CRS in COVID-19 patients; elevated serum levels of IL-6 seems to correlate with respiratory failure, ARDS, and adverse clinical outcomes (Dennery 2004; Huang et al. 2020). Pro-inflammatory cytokines, TNF-α, IL-6, and IL-1*β*, may be modulated by LF, either to increase (Machnicki et al. 1993; Sorimachi et al. 1997) or decrease (Zimecki et al. 1999; Håversen et al. 2002) cytokine production depending on the type of antigenic stimulus. These complex regulatory effects of LF on inflammatory mediators may play a pivotal role in the development of adjunctive approaches to clinical management of potential cytokine storm during COVID-19-*Pregnancy*.

Maternal-LF in COVID-19-*pregnancy*

Compared to previous SARS and MERS outbreaks, the COVID-19-*Pregnancy* outcomes for the mother appears to be less serious. Pooled data reveals a CFR of 0%, 18%, and 25% for COVID-19, SARS, and MERS, respectively – in the latter two outbreaks, progressive respiratory failure and severe sepsis were the most frequent causes (Wong et al. 2003; Assiri et al. 2016; Rasmussen et al. 2020).

Vertical transmission

To date, the outcomes of 55 pregnant women infected with COVID-19 and 46 neonates reported in the literature, showed no definite evidence of vertical transmission (Li, Zhao, et al. 2020; Zaigham and Andersson 2020). However, there is a theoretical risk of vertical transmission, similar to that observed in SARS, due to ACE2 receptor in the placenta (Levy et al. 2008),, with the common RBD between SARS-CoV-1 and SARS-CoV-2. Two neonates from COVID-19 infected mothers were tested positive for SARS-CoV-2 shortly after delivery, casting concerns about the possibility of vertical transmission (Peng et al. 2020; Woodward 2020; Murphy 2020). However, there are no

confirmed cases of vertical transmission among the 46 other neonates born to COVID-19 infected mothers (Chen, Guo, et al. 2020; Chen, Huang, et al. 2020; Chen, Peng, et al. 2020; Li, Zhao, et al. 2020; Liu, Wang, et al. 2020; Zhang et al. 2020; Zhu et al. 2020). The supporting evidence indicate an absence of SARS-CoV-2 in the amniotic fluid, cord blood, breast milk, and neonatal throat swabs in these patients (Chen, Guo, et al. 2020). It is notable, that most of these women acquired COVID-19 in the 3ʳᵈ trimester. There is no currently available data on perinatal outcomes when the infection is acquired during early pregnancy. Regardless of the risk, COVID-19 appears to manifest as a mild respiratory illness in the pediatric population (Cai et al. 2020; Xu, Li, et al. 2020).

Fetal surveillance

Protracted respiratory compromise increases the risk of FGR due to maternal hypoxia releasing potent vasoconstrictors such as endothelin-1 and hypoxia-inducible factor, causing placental hypoperfusion and reduced oxygen delivery to the fetus (James et al. 2006). Fetal complications include miscarriage (2%), intrauterine growth restriction (IUGR; 10%), and pre-term birth (39%). Fever, with a median temperature of 38.1–39.0 °C, is the prevailing symptom in COVID-19 (Guan et al. 2020).

Maternal LF and fetal defense

Amniotic-LF is an integral part of the repertoire of host defense mechanisms against infections during pregnancy. Intra-amniotic infection is consistently associated with a dramatic rise in the amniotic-LF levels during pre-term labor (3.8 μg/mL), term labor (5.6 μg/mL) and conditions of premature rupture of amniotic membrane (PROM) during pre-term (3.5 μg/mL) compared to the non-infected control group (range: 1.6 to 2.2 μg/mL) (Pacora et al. 2000). The amniotic LF dramatically elevates to 8.8 μg/mL during chorioamnionitis (CAM). It is well documented that amniotic infections induce premature labor and fetal abortion. LF has been shown to inhibit interleukin production induced by endotoxins in cultured amnion cells (Otsuki et al. 1998). The interleukin suppressive mechanism of amniotic-LF has been suggested in possible fetal protection against intra-uterine infections.

LF gene expression can be detected at the 2- and 4-cell stages of embryonic development and throughout the blastocyst stage (prior to implantation). After implantation, LF expression cannot be detected until about halfway through gestation and reappear in neutrophils and epithelial cells of the developing reproductive and digestive systems (Adlerova et al. 2008; Teng 2010). During pregnancy, the plasma levels of LF progressively rise up to Week-29 and remain elevated (Sykes et al. 1982). Several factors contribute to these elevated LF levels, such as pregnancy-associated leukocytosis, the selective increase of LF in neutrophil granules, endometrial tissues, decidua and mammary glands (Levay and Viljoen 1995). LF activates human growth hormone (hGH), and compared to epidermal growth factor (EGF), the effects of LF are more pronounced on small intestine epithelial cells and proliferation of stromal cells in the endometrium (Adlerova et al. 2008).

Maternal-LF in COVID-19-*postpartum*

Newborns are at increased risk of infection due to genetic, epigenetic, and environmental factors. Full-term newborns express a distinct innate immune system biased toward T_H2-/T_H17-polarizing anti-inflammatory cytokine production with relative impairment in T_H1-polarizing cytokine production. This immune condition makes the neonate particularly vulnerable to infection with intracellular pathogens. In addition to such distinct features, preterm newborns also have fragile skin, impaired T_H17-polarizing cytokine production, and deficient expression of complement, antimicrobial proteins, and peptides (APPs) that increase susceptibility to viral infections such as COVID-19. APPs, such as LF could protect the newborn by enhancing immune responses (Cuenca et al. 2013). Maternal-LF in breast milk is known to be a potent antiviral agent to prevent mother-to-child transmission (MTCT) of HIV-1 infection (Zupin et al. 2015).

Breast feeding and COVID-19

Most infants breastfed from their HIV-infected mothers do not acquire HIV-1 despite exposure to the cell-free virus and cell-associated virus in HIV-infected breast milk (Henrick et al. 2017). Paradoxically, exclusive breastfeeding regardless of HIV status of the mother, results in a significant decrease in MTCT of the disease compared to non-exclusive breastfeeding. It is unclear on how the HIV-exposed infants remain uninfected despite repeated and prolonged exposure to the viral pathogen. Prevention of MTCT of HIV-1 is likely due to multiple innate immune factors, including the milk glycoprotein LF. About 4.3×10^{14} human LF binding receptors with an affinity constant of 0.3 μM were estimated per milligram of fetal intestinal brush border membrane protein. The human LF binding is pH-dependent and optimum between pH 6.5 and 7.5 range (Kawakami and Lönnerdal 1991).

Soluble toll-like receptor 2 (sTLR2) inhibits HIV infection, integration, and inflammation. sTLR2 directly binds to selective HIV-1 capsid proteins (p17, gp41, and p24), which leads to reduced NFκB activation, IL-8 production, CCR5 expression, and HIV infection in a dose-dependent manner (Henrick et al. 2017). Human milk-LF helps to protect the neonate against infections by modulating antiviral pathways. Also, it opens the possibilities to develop novel innate immune therapeutics to protect newborns, infants, and children against viral infections such as COVID-19 (Perdijk et al. 2018; Telang 2018).

Previous SARS outbreak revealed that the presence of CoV antibodies in breastmilk depends on the gestation at which maternal infection occurs and any preceding use of high-dose corticosteroids may suppress maternal antibody responses (Woo et al. 2004). Therefore, any corticosteroid prescription to mothers with COVID-19-*Pregnancy* should be exercised with high caution. Based on current published guidelines, breastfeeding is not contraindicated in COVID-19-*Pregnancy*. A retrospective analysis of COVID-19-*Pregnancy* cases indicates that none of the women showed any detectable viral loads of SARS-CoV-2 in breastmilk (Chen, Guo, et al. 2020). Regardless, if a patient prefers to breastfeed, an appropriate face mask should be worn due to the proximity between mother and child to reduce any risk of droplet transmission.

Table 1. LF levels in human breast milk during early stages lactation.

Type of lactation	Lactation days (weeks)	Total samples	Lactoferrin (LF)	
			Level (mg/mL)	% Total Protein
Colostrum	1–5 (<1)	142	58	27
Transitional milk	6–14 (1–2)	106	31	22
Mature milk	15–28 (3–4)	112	20	19
	29–56 (5–8)	34	22	22
	57–84 (9–12)	50	33	30

Human LF in breast milk: The immunological system in human milk undergoes remarkable changes and adapts to the needs of the recipient infant. Human colostrum is an important source of protective, nutritional, and developmental factors (i.e. LF, lysozyme, sIgA) for the newborn. LF levels in colostrum and mature milk vary from 57.5 mg/mL to 50 mg/mL in preterm samples and from 97.1 mg/mL to 29.2 mg/mL in term samples, respectively. High levels of LF in preterm mature milk provides protective benefits for the preterm infant despite small volumes ingested by the neonate (Ronayne de Ferrer et al. 2000). Analysis of 444 breast milk from 64 mothers during the early 12 weeks of lactation showed that the LF levels and the %LF in total milk protein are markedly higher in colostrum compared to transitional or early mature milk. However, in the following weeks, the LF concentration in mature milk gradually increased (Table 1) (Montagne et al. 2001).

An important function of early breastfeeding is its anti-inflammatory effects on the immature gastrointestinal tract of the newborn. Milk LF as well as other components of lacteal secretion such as transforming growth factor (TGF)-β, IL-10, and erythropoietin contribute to the downregulation of inflammatory responses in the neonatal intestine. LF can act individually or in concert with other milk bioactive compounds and may provide nonspecific host defense to the breastfed infant (Walker 2010).

Maternal LF and the development of neonatal immune competence

Maternal LF is an important defense component of colostrum and mature milk that contributes to the protection of the newborn. Specific receptors for LF are located on the intestinal epithelia, playing an important role in iron transport across the mucosal barrier during the early stages of neonatal development (Cox et al. 1979; Iyer and Lönnerdal 1993). Due to low postprandial pH, protein hydrolysis is minimal in infants, LF may have greater bioactive potential in the neonatal gastrointestinal (GI) tract than in adults. LF stimulates the proliferation and differentiation of intestinal epithelial cells in a dose-dependent manner and affects the mass, length, and epithelial digestive enzyme expression of the neonatal GI tract (Nichols et al. 1990; Liao et al. 2012). These intrinsic functional properties make maternal-LF a potent innate defense factor to prevent COVID-19 transmission from mother to newborn.

Conclusions

LF is a multifunctional glycoprotein and an integral part of the placental barrier in the maternal-fetal interface, in the amniotic fluid, in colostrum and breast milk, virtually in

all biological fluids. LF demonstrates a regulatory role in redox homeostasis, inflammatory responses, immune modulation, and antimicrobial activities during pregnancy. The antiviral effects of LF involve blocking the initial viral attachment to host CSRs, as well as subsequent interference with cellular entry and replication of viral pathogen. LF may effectively intercept the delivery of the viral genome into the cytoplasm and reduce the rate of viral replication/propagation. SARS-CoV-2 is a highly adaptable pathogen with extensive virulent traits to infect a variety of host cells. LF binding to viral capsid proteins could induce structural alterations and may increase viral susceptibility to host defense. Spike (S)-protein is a critical virulent factor of COVID-19, responsible for tissue tropism, host range and is one of the main targets for neutralization antibodies. LF may block viral docking sites including putative (ACE2, CD32a) and lectin-type (sialic and GAG) CSRs. Furthermore, ACE2, the prominent CoV receptor for viral docking, is over-expressed in the maternal-fetal interface; therefore, pregnant women are at a potentially greater risk from COVID-19 infection. LF down-regulates ACE2 and thereby may limit CSR access for SARS-CoV-2 entry. The charge neutralizing ability of LF may also play a role in blocking the viral adhesion to the proteoglycan-rich host cell surface. The large spectrum of potentially significant immune functions ascribed to LF include regulation of endogenous inflammation (both pro- and anti-inflammatory pathways), stimulation of neutrophils, peritoneal macrophages, NK cells, T-cells, and B-cells; activation of antigen-presenting cells (APCs); augmentation of T-cell-mediated specific antigen recognition and modulation of adaptive immunity. LF is an innate regulator of acute phase response, which may help abrogate severe cytotoxic outcomes encountered during 'cytokine storm'. Maternal LF in breast milk may be an important antiviral agent and may further contribute to a reduction in MTCT. There is divided and uneven literature that presents *in vitro* and clinical evidence that increasing oral LF intake corresponds to a decreased incidence, severity, and duration of viral infections in humans. Based upon what has been studied and reported, there seems ample justification for designing and conducting rigorous clinical trials of LF supplementation as an adjunctive intervention in reducing the infectious/transmission potential of COVID-19 and also in the management of the associated illness especially in vulnerable periods such as pregnancy and the postpartum phase of life.

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Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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