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Review

The potential for Lactoferrin to reduce SARS-CoV-2 induced cytokine storm

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ABSTRACT

The COVID-19 pandemic is a serious global health threat caused by severe acute respiratory syndrome of coronavirus 2 (SARS-CoV-2). Symptoms of COVID-19 are highly variable with common hyperactivity of immune responses known as a "cytokine storm". In fact, this massive release of inflammatory cytokines into in the pulmonary alveolar structure is a main cause of mortality during COVID-19 infection. Current management of COVID-19 is supportive and there is no common clinical protocol applied to suppress this pathological state. Lactoferrin (LF), an iron binding protein, is a first line defense protein that is present in neutrophils and excretory fluids of all mammals, and is well recognized for its role in maturation and regulation of immune system function. Also, due to its ability to sequester free iron, LF is known to protect against insult-induced oxidative stress and subsequent "cytokine storm" that results in dramatic necrosis within the affected tissue. Review of the literature strongly suggests utility of LF to silence the "cytokine storm", giving credence to both prophylactic and therapeutic approaches towards combating COVID-19 infection.

1. Introduction

Inflammation is the complex biological response of vascularized tissues to environmental harmful stimuli, such as infection due to microbial agents, or mechanisms elicited by trauma. Significant morbidity, and even mortality, results when the inflammatory responses become excessive, leading to the "cytokine storm" and a "paralysis" of cell-mediated immunity. Thus, maintaining control of immune homeostasis is a key factor in protection against any pathogen-induced physiologic imbalance. LF is a front-line, natural immune modulator [1-3] that has ability to counteract excessive immune responses [4,5] and also facilitate the development of adaptive immunity [6].

The cytokine storm is the main cause of mortality and morbidity of patients admitted to intensive care units following septic shock, injury or postsurgical trauma [7,8]. Excessive cytokine release by hyper-reactive individuals is also common in COVID-19 patients [9,10] with interleukin (IL) −6 and IL-10 serving as best markers of the disease prognosis [11]. Other complications resulting from the aberrant response of patients to the viral infection include, among others, increased thrombosis associated with microcoagulation [12] and intestinal microflora dysbiosis [13]. The therapeutic approaches and suggested treatments [14,15] aimed at prevention or amelioration of this violent immune response encompass steroids [16], nonsteroidal drugs [17], low dose radiation [18], convalescent plasma [19], IL-6 inhibition [20], vitamins [21], probiotics [22] and other nutraceuticals [13]. Recently, AlKhazindar commented on the possible benefits of LF to boost natural responses to SARS-CoV-2 [23]. Wang, et al [24] theorized a utility for LF as a tool to control development of pathologies due to coronavirus infection, acting as an anti-inflammatory, anti-infective and immune-regulator. And Chang, et al., suggested that LF may serve to augment intracellular anti-viral mechanisms directly [25]. We agree with this assessment, and further explore mechanisms underlying LF as a therapeutic to function prophylactically or therapeutically to control tissue damage induced during COVID-19 disease states.

Lactoferrin (LF) is a multifunctional, iron binding protein, contained in neutrophils and mammalian excretions. It is long since known that LF has direct anti-microbial functions [26,27], and pertinent to this review, has also demonstrated direct anti-viral capabilities [28-30]. An overall summary of LF immunoregulatory activities is listed in Table 1. Specifically, the importance of lactoferrin in immune cell responses has been detailed in a way to link cellular activation for natural killer and T lymphocytes, mononuclear cells migration, monocytes and neutrophils recruitment to improve overall immune responses to elicit varied aspects of antimicrobial effects [31,32]. LF is deeply involved in many critical physiological functions, namely immune regulatory and the redox homeostasis. By virtue of iron sequestration, LF attenuates the production

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Table 1
Immunoregulatory actions of LF in the context of infections and clinical insults.

Model	LF treatment	Effects	Reference
Mouse and human *in vitro* cell models			
human whole blood cell culture	rhLF in culture	regulation of gene expression involved in oxidative stress	Kruzel, et al. 2013 [55]
PBMC activated by LPS	hLF in culture	regulatory effect on LFA-1 expression mediated by TNF α	Zimecki, et al. 1999 [44]
human macrophages stimulated with EBV	hLF in culture	inhibition of chemokine production via suppression of NFκB	Zheng, et al. 2014 [71]
monocytes from umbilical cord blood activated with LPS	hLF in culture	differentiation of monocytes into macrophages with decreased TLR-4 expression, TLR signaling and proinflammatory cytokine production	Wisgrill, et al. 2018 [121]
hydrogen peroxide induced oxidative damage in epithelial intestinal cells	human apo LF prior to H_2O_2 challenge	increased survival of Caco-2 epithelial cells	Shoji, et al. 2007 [98]
monocyte derived dendritic cells	bLF in culture	generation of dendritic cells with impaired ability to promote Th1 type response	Puddu, et al. 2011 [72]
primary human megakaryocytes	hLF in culture	inhibition of platelet production in matured megakaryocytes	Matsumura-Takeda, et al. 2008 [122]
Viral infections			
human cytomelagovirus (HCMV) and human herpes virus 1 (HSV-1) infection of human embryonic lung cells	bLF in culture	inhibition of virus infection and replication by preventing virus adsorption and cell penetration	Hasegawa, et al. 1994 [123]
rotavirus infection of enterocytic HT-12 cell line	apo and holo bLF in culture	inhibition of replication stronger by apo LF, binding of LF to virus particles	Superoti, et al. 1997 [124]
adenovirus infection of HEP-2 cells	bLF and lactoferricin in culture	inhibition of infection by binding of LF to glycosaminoglucan receptor	Di Base, et al. 2003 [125]
HSV infection of Vero cells	bLF and lactoferricin in culture	bLF inhibits only virus entry to cells and lactoferricin both entry and replication	Andersen, et al. 2004 [126]
rhabdomycosarcoma and neuroblastoma SK-N-SH cells infected with EU71 enterovirus	bLF in culture	inhibition of replication by binding to the target cells and VP1 viral protein	Weng, et al. 2005 [127]
influenza A virus infection *in vitro*	bLF in culture	protection by apoptosis inhibition, block of nuclear transcript	Pietraantoni, et al. 2010 [128]
Egyptian avian lethal influenza A (H5N1) infection of MDCK cell line	bLF in culture	dose-dependent inhibition of replication	Taha, et al. 2010 [129]
influenza virus infection in mice	bLF administered daily by gavage from 1 day before infection to day 6 postinfection	improved histology, lower number of leukocytes infiltrating BALF, lower IL-6 serum level	Shin, et al, 2005 [77]
hepatitis B virus infection of cultured human hepatocytes (PH5CH8)	bLF or hLF in culture	prevention of the infection by preincubation of cells but not the virus by LF	Hara, et al. 2002 [130]
hepatitis C infection of Hep G2 cells	human, camel, bovine or sheep LF in culture	prevention of HCV entry into cells by all LFs via direct interaction with the virus	El-Fakharany, et al. 2013 [131]
echovirus infection of human cells in various assay systems	bLF digested with gastrointestinal enzymes in culture	inhibition of replication with a better effect of digested bLF than intact bLF	Furlund, et al. 2012 [132]
norovirus (MNV) infection of RAW 264.7 cells	bLF in culture	dose-dependent decrease or block of cell damage and virus titres in the medium and cells, increase of IFN α and IFN β production by the cells	Ishikawa, et al. 2013 [133]
Encephalomyocarditis virus infection of splenocytes	rhLF and bLF in culture	both LFs protective to the same degree in young and old mice	Zaczyńska, et al. 2017 [78]
SARS pseudovirus infection of HEK293E/ACE2-Myc cells	bLF in culture	block of the binding of spike protein A to host cells at 4 °C, lack of spike protein /ACE2 integration disruption, role of heparin sulfate block by bLF	Lang, et al. 2011 [33]
SARS-CoV-2 infection in Caco-2 cells	bLF in culture	induction of gene expression for IFNA1, IFNB1, TLR3, TLR7, IRT3, IRF7 and MAV, partial inhibition of virus infection and replication	Salaris, et al. 2021 [134]
Endotoxemia and bacterial infections			
LPS-induced endotoxemia	bLF given i.v. 24 h prior to LPS	significant reduction of TNF α serum level, bLF alone induces serum IL-6	Machnicki, et al. 1993 [73]
LPS-induced endotoxemia	hLF given before, together or after LPS challenge	reduction of TNF α, IL-6, IL-10 and nitric oxide after 1 h pretreatment with hLF, other treatments not effective in reduction of IL-6 and IL-10	Kruzel, et al. 2002 [40]
LPS-induced inflammation	bLF administered i.p. before LPS	protection of gut mucosal integrity, increased survival	Kruzel, et al. 2000 [95]
acute LPS-induced lung injury	i.p. injection of bLF 1 h before or after LPS	both protocols effective in decreasing histological, biochemical and immunological inflammatory parameters	Li, et al. 2012 [135]
Mycobacterium tuberculosis TDM-induced lung pathology	bLF given i.v. together with TDM	reduction in granuloma formation, increased levels of IL-10 and TGF β	Welsh, et al. 2010 [118]
Mycobacterium tuberculosis infection	rhLF in drinking water starting from infection, for 1–3 weeks	lung inflammation and bacteria number significantly reduced, increased total lymphocyte number and $CD4^+$ IFN ɣ and IL-17 producing cells	Welsh, et al. 2011 [116]
Mycobacterial TDM-induced lung pathology	rhLF or rmLF delivered by gavage at day 4 and 6 after TDM	reduced production of IL-12p40, increased IL-6 and CXCL1, increased number of macrophages and diminished granuloma formation	Hwang, et al. 2017 [117]
human monocytes and granulocytes infected with methicillin-resistant *Staphylococcus aureus*	rhLF in culture	increased production of INF ɣ and IL-2, decrease of TNF α,IL-6, IL 1β, IL-12p40, and IL-10	Hwang, et al. 2014 [82]

(*continued on next page*)

of reactive oxygen species (ROS) and subsequent oxidative stress that may lead to wild "cytokine storm". However, at this time, there is little evidence for direct interaction of LF with SARS-CoV-2 and/or the pathologically related Middle East respiratory syndrome coronavirus (MERS-CoV), although, LF may exhibit some inhibition of ACE2 binding via non-specific interaction with surface glycans [33]. It has been shown that LF is able to prevent internalization of some viruses by binding to heparan sulfate proteoglycans (HSPGs) which is present on many cell phenotypes, thus providing generalized protection against a variety of viral infection [34]. The role of LF in controlling fundamental biological processes has been revealed in recent years with emphases on its immune regulatory functions. In this context, LF is theorized as an important component in the toolbox of therapeutics to combat virions due to its maintenance of physiological homeostasis and its link to regulate innate and adaptive immunity [35].

2. Biology of LF in context of multifunctional properties

Lactoferrin is a well conserved, monomeric 80-kDa single polypeptide chain contained in most mammalian exocrine secretions, including milk, tears, saliva, bronchial and intestinal secretions and also in the secondary granules of neutrophils. It is considered a first line defense protein involved in protection against microbial infections [36,37] and subsequent development of systemic inflammatory response syndrome (SIRS) and sepsis [38-40]. LF has been implicated in immunoregulatory functions [35,41-44], as a modulator of vaccine function [45], and containing chemoprotective activity [46]. The primary structure of human LF (hLF) is characterized by a single polypeptide chain containing 692 amino acids organized in two highly homologous lobes, each capable of binding single ferric ion ($Fe3+$). In this regard, LF is considered as an antioxidant because its iron binding ability may prevent the iron-catalyzed formation of ROS namely $H2O2$ and ·OH [47-50]. Lactoferrin is a glycoprotein and in humans the glycans are the N-acetyllactosaminic type, α1,3-fucosylated on the N-acetyl-glucosamine residue linked to the peptide chain. The oligosaccharide component of glycoprotein is often critical for determination of pharmacological properties including in vivo activity, pharmacokinetics, and immunogenicity. Of particular importance is that LF bridges innate and adaptive immune functions by regulating target cell responses, using mechanisms that are highly dependent on the type of carbohydrates attached to the protein.

Bovine lactoferrin (bLF), widely available on nutritional market, shares ~ 70% amino acid sequence homology with human LF (hLF), albeit with a different glycosylation pattern. Although good for nutritional supplementation, bLF cannot be used for parenteral applications in humans due to potential risk of anaphylactic shock. The major concern for LF clinical utility relates not only to amino acid incompatibility but, more importantly, to the glycan structure. Thus, there remains no clinical data on effectiveness of systemically (parenterally) delivered LF in humans. Recombinant human LF (rhLF) has been produced in various expression systems [51], including transgenic plants [52] and animals, but none of the resultant products have been tested and approved for human use for intravenous (systemic) administration. Although there are two rhLFs available for larger studies, one from transgenic rice [53] and the other from *Aspergillus awamori* [54], both present a health threat if administered intravenously due to the antigenic nature inherent within their non-homologous glycosylation patterns. In 2013, Kruzel, et al. reported production of fully compatible, non-antigenic rhLFs obtained in a mammalian expression system using the Chinese Hamster Ovary cell line (CHO), yet no clinical trials with this molecule have been reported [55].

Table 1 (*continued*)

Model	LF treatment	Effects	Reference
Pseudomonas aeruginosa acute and chronic infection in mice	intranasal administration of aerosolized bLF	significant decrease of the neutrophil recruitment and proinflammatory cytokine levels, faster recover and body weight gain	Valenti, et al. 2017 [76]
peritonitis induced by Methicillin-resistant *Staphylococcus aureus*	rhLF given 2 h post-infection	modest increase in survival, reduced IL-6 and IL-17 serum levels	Hwang, et al. 2014 [82]
Normal and manipulated rodents			
surgery in mice	bLF given i.v. or per o.s. before thymectomy or splenectomy	suppression of IL 6 and TNF α after thymectomy, deeper after per o.s. treatment, weaker effects on splenectomy	Zimecki, et al. 1998 [75]
ovalbumin (OVA) induced pleurisy in mice	bLF or hLF given buccally 24 h and 3 h before OVA	decrease of clinical manifestation of pleurisy, decrease of IL5 level in pleural exudates	de la Rosa, et al. 2008 [32]
ovalbumin (OVA) responses in mice	bLF given i.p. before OVA	neutrophil and macrophage recruitment; Th1 polarization	Zimecki, et al. 2012 [81]
hyperoxia in CD-1 mice	bLF in aerosol	reduction in lung edema, cell number in BALF, IL-1 β, IL-6, pulmonary fibrosis and DNA fragmentation, increase of survival from 20% to 66.7%	Chen, et al. 2014 [80]
transcriptome profiling in rat spleen	rhLF per os or i.v.	largely overlapping changes in gene expression by both treatments, associated with upregulation of genes involved in oxidative stress and inflammation	Kruzel, et al. 2020 [69]
Healthy persons and patients			
healthy volunteers	bLF chewable tablets for 14 days	normalization of spontaneous TNF α and IL-6 production by PBMC cultures depending on initial immunological reactivity	Kruzel, et al. 2007 [4]
healthy males	microencapsulated bLF, per os in two four week periods	significant effect for 200 mg daily doses, lowering $CD69^+$ activation marker on $CD4^+$ cells, increase in the volume of most frequent intestinal bacteria species	Dix, et al. 2018 [100]
healthy volunteers, indomethacin-induced enteropathy	ingestion of 5 g rhLF, followed by 2 doses of indomethacin after 9 h and overnight	reduction of the increased by indomethacin small intestine permeability	Troost, et al. 2003 [97]
PMBC from patients with systemic inflammatory response syndrome	bLF in culture	inhibition of both spontaneous and LPS-induced PBMC proliferation, upregulation of IL-6 and TNF α production in septic survivors	Adamik, et al. 1998 [88]
blood from patients diagnosed with severe sepsis or septic shock	rhLF in culture	LPS-induced TNF α production upregulated in patients with sustained anergy, inhibited or without effect in moderately reactive patients	Artym, et al, 2018 [89]
COVID-19 patients	bLF as nutritional supplement, 4–6 daily doses for 10 days	complete and fast recovery within the first 4–5 days, prevention of the disease at lower doses in healthy persons having contacts with the patients	Serrano, et al. 2020 [136]

3. Lactoferrin and immune homeostasis

There are two major metabolic pathways by which LF may support immune homeostasis during environmental insult: a) by controlling the iron-dependent production of ROS, and b) by activating or reducing receptor binding capacity on immune cells. There is convincing evidence of severe oxidative stress in patients with pre-septic conditions. Dysregulation of the immuno-inflammatory response, as seen during the development of sepsis, may culminate in host cell and organ damage. Testing the anti-oxidant properties of LF in human embryonic diploid fibroblasts (MRC5) using 2′,7′-dichlorodihydro-fluorescein diacetate (H2DCF-D), it was demonstrated that LF reduced intracellular ROS levels in a dose-dependent manner. LF also decreases ROS-induced apoptosis [4] and attenuates mitochondrial dysfunction [56].

By acting as a homeostatic agent, LF can work both ends of the immunological spectrum to increase low response or dampen aggressive ones. High affinity of LF to ferric ions (Fe+++) makes it important for the maintenance of iron dependent oxidative stress. Oxidative stress occurs when the production of destructive reactive oxygen species (ROS) surpasses the body's own natural antioxidant defenses, resulting in cellular damage and death. Thus, by virtue of iron sequestration LF protects against tissue injury (Fig. 1), which may be critical during the development of infection such as COVID-19. It has direct capacity to regulate both pro-inflammatory and anti-inflammatory responses [38,57]. The utility of such pleiotropic immune mediator represents a novel therapeutic approach, which is dependent on elicited responses for outcome. Of particular importance is LF's ability to bind to various cell receptors including CD14 [58], Toll-like receptor-2 and TLR-4 [59], as well as possible interaction with receptors involved in coronavirus binding; heparan sulfate proteoglycans (HSPG) [60,61] and angiotensin-converting enzyme 2 (ACE2) [62,63]. The role of LF in correction of the immune status of healthy individuals and patients seems indispensable since the protein can normalize its function in a sophisticated manner by sensing degrees of reactivity of the immune system and act accordingly [4,6]. As a component of the innate immunity, LF exerts diverse immunomodulatory functions. In particular, it can act in an adjuvant capacity. We and others demonstrated that LF (bovine-derived and recombinant human) augments vaccine efficacy by promoting both T and B lymphocytic responses that culminate in subsequent pathological protection against challenge with virulent pathogens [6,64]. Indeed, studies in neonatal mice show that LF had a significant effect to induce influenza neutralizing antibodies when combined with hemagglutinin antigen [65]. This bodes well for theoretical incorporation of LF into existing SARS-CoV-2 vaccines to boost neutralizing antibodies post immunization. In fact, LF secreted into all human mucosal sites is considered a natural adjuvant enhancing immunity to all infectious agents as we encountered during the period of infancy onward [66-68]; it assists in the acquisition and development of protective immunity against infectious-related pathologies during early stages of our ontogeny. Accordingly, exogenous LF given to population during this COVID-19 pandemic may be of great benefit to acquire immunity; this can be accomplished in safe and accelerated matter, especially when considering the safety profile of bovine LF which has been well documented in clinical trials for enterocolitis (discussed below).

Recently we demonstrated that LF given orally to experimental rats has systemic effects as measured by gene expression levels involved in the regulation of physiologic processes. They include changes that may lead to a tempered response to proinflammatory stimuli, such as repression of reactive oxygen species, elevation of anti-inflammatory cytokines (TGF β1 and CSF-1) and downregulation of cell-to-cell interactions [69]. Inhibition of LPS-induced expression of LFA-1 adhesion molecule was also found on human peripheral blood lymphocytes [44]. The effects of LF on expression of adhesion molecules and consequently on cell-to-cell interactions, should limit recruitment of cells to sites of inflammation. LF also affects metabolism of prostanoid family by a moderate induction of cyclooxygenase 1 and more profound inhibition of cyclooxygenase 2 expression in human whole blood cell culture that may account for the anti-inflammatory properties of LF [55]. Such a bidirectional action is rather unusual and may well explain the immunoregulatory nature of LF. Inhibition of immune cell reactivity by LF can also be accomplished by downregulation of toll like receptors, and even PAMPs [6,70]. For example, LF can inhibit Epstein-Barr virus infection not only by blocking CD21 receptor on B cells but also by interference with the activation of TLR-2 and TLR-9 [71]. Monocytes from umbilical cord blood cultured with LF differentiate into macrophages that have decreased TLR-4 expression, TLR signaling and cytokine production; they acquire an anergic state. A similar phenomenon can be observed with monocytes cultured with LF, where they differentiate into poor antigen presenting cells [72].

4. Effects of LF on cytokine production and lung injury in mice

Lactoferrin regulates insult-induced, cytokine-driven inflammation (Fig. 2). Early studies demonstrated that pretreatment of mice with LF

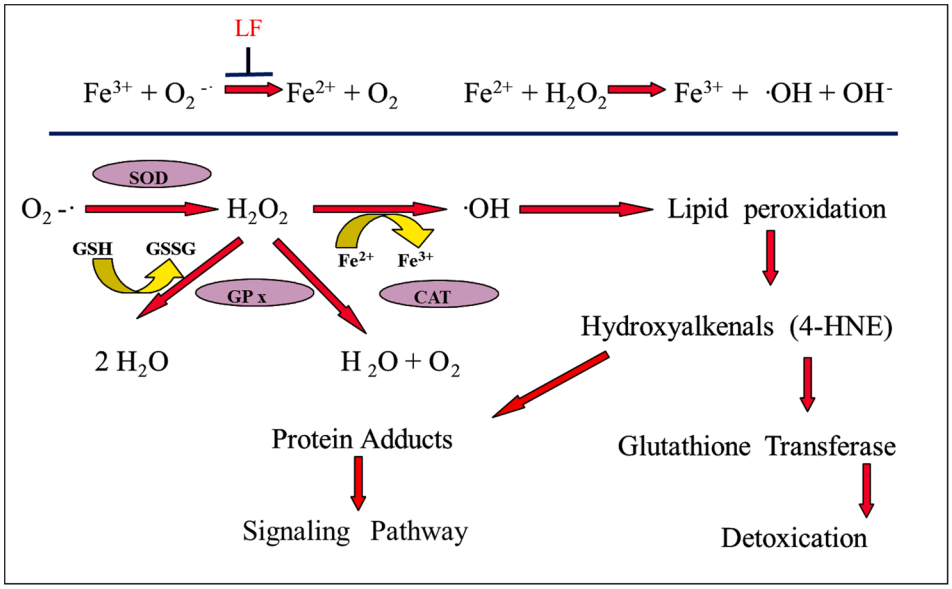

Fig. 1. Lactoferrin to modulate oxidative stress. At normal physiological conditions superoxide will spontaneously dismutate and produce hydrogen peroxide. However, this enzymatic pathway can be circumvented in the presence of ferric ions (top line, right side) so that the hydrogen peroxide is reduced to highly reactive hydroxyl radicals which culminate in toxic protein adducts. By sequestering free ferric ions, LF has the potential to block the production of hydroxyl radicals and their subsequent toxic effects. Adapted from [120].

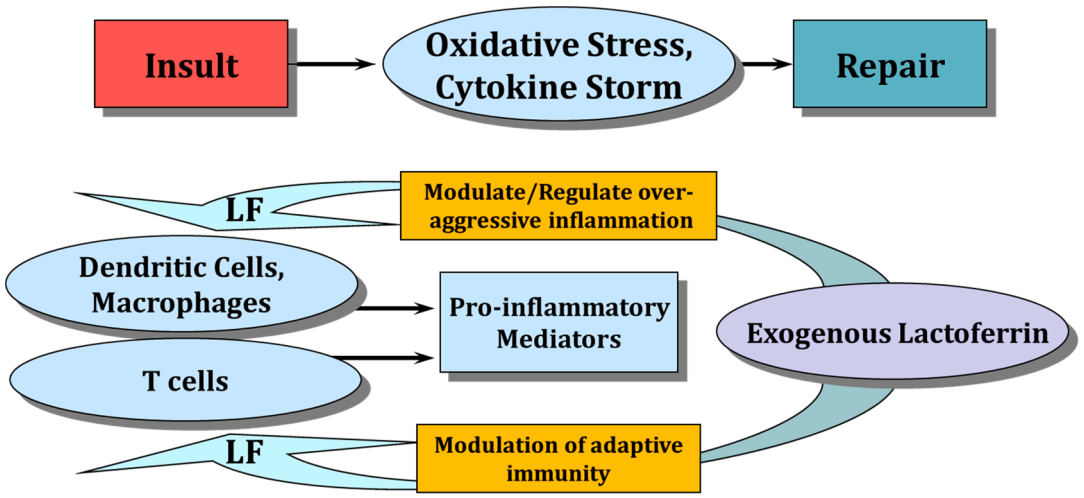

Fig. 2. Potential for LF to regulate insult-induced inflammation. Exogenous LF can modulate insult-induced inflammation in a similar way as endogenous molecule by augmentation of low level responses or modulation of overaggressive cytokine response. Both effects serve to assist with tissue repair after insult. Adapted from [115].

prevented lipopolysaccharide (LPS) –induced high tumor necrosis factor alpha (TNF-α) serum levels [73]. LF alone elicited IL-6 but not TNF-α release into circulation. It also appeared that the protective effect of LF in endotoxemia was associated with induction of IL-10 production [74]. More importantly, LF was effective in lowering serum TNF-α level not only as a preventive but also as a therapeutic agent [40]. TNF-α and IL-6 serum levels induced by splenectomy could be also lowered by pre-treatment of mice with LF [75].

Other studies were crucial in the context of lung inflammation, inherent to coronavirus infection. An intra-tracheal administration of LF in aerosol to mice infected with Pseudomonas aeruginosa prevented acute and chronic lung inflammation by significant reduction of proinflammatory cytokines in the bronchoalveolar fluid (BALF) and neutrophil infiltration [76]. LF, administered per os, extenuated also lung inflammation resulting from intranasally introduced influenza virus, as evaluated by number of cells infiltrating BALF and IL-6 serum levels [77]. Of interest, the antiviral effects of LF with regard to aged mice did not differ in relation to young animals [78]. A possibility also exists that prolonged ventilation of coronavirus patients with oxygen enriched atmosphere may damage lung epithelial cells [79]. Therefore, of a particular importance are results indicating that aerolized LF significantly diminishes lung injury of mice exposed to hyperoxia, taking into account several parameters characteristic to lung inflammation and increase of the survival rate [80]. LF may also protect against lung inflammation in the model of allergic, antigen specific pleurisy [81]. The protein, given prior to the eliciting dose of antigen, significantly lowered indices of inflammation such as edema, alveolar hemorrhage, content of eosinophils and IL-5 concentration in the pleural exudates. In conclusion, lung inflammation caused by various stimuli and mechanisms, could effectively be alleviated by use of LF.

5. Immunoregulatory effects of LF in human volunteers

A specific study to determine clinical relevance for use of LF to treat sepsis used human blood derived monocytes and granulocytes infected with MRSA, and examined cytokine changes when treated with homologous recombinant human LF [82]. The outcome of treatment with LF led to increased production of IFN-γ and IL-2, with a concurrent decrease in IL-6, TNF-α, IL-1β, IL-12p40, and IL-10. The monitoring of the course of COVID-19 infection revealed that high levels of IL-6 and IL-10, but perhaps not always TNF-α, as markers of morbidity and mortality of patients [11]. These reported markers confirm what was identified in earlier reports from septic patients [8]. It also became clear that very low or exceptionally high reactivity of peripheral blood mononuclear cells (PBMC) significantly correlated with mortality rate in septic shock patients, and were accompanied by a low ability to produce IL-6 and TNF-α by septic non-survivors [8]. Such a phenomenon probably resulted from an immune exhaustion following excessive cytokine production and release into circulation. LF may have direct activity to interfere with SARS-CoV-2 infection, perhaps by preventing virion attachment to cell surface ACE2 receptor [63] or binding to a heparin sulfate co-receptor [61]. There is speculation that LF alone may function as a nutritional supplement to ward infection [83]. More likely, LF functions in a manner to regulate/modulate cytokines in the tissue environment.

Since serum levels of IL-6, together with IL-10, represent only the marker of host's response to COVID-19 virus, it is not clear whether these cytokines are important for pathology of the infection or they rather fulfill a protective role. Therefore, it may not be advisable to completely block activity of IL-6. However, the effects of treatment of patients with tocilizumab or sarilumab, IL-6 inhibitors [20,84], seem disputable. Indeed, it may be critical to utilize LF in combination therapies with other agents [85]. For example, hypothiocyanite combined with LF can limit pulmonary damage in models of respiratory infections [86]. In an analogous line of thought, there were no significant effects registered with blocking TNF-α production in septic patients [87]. Our experience showed that LF could regulate LPS-induced IL-6 and TNF-α production by PBMC cultures in septic and trauma patients [88,89], and breaking anergic responsiveness of blood cells by LF was of particular interest. It should also be mentioned that the ability of LF to inhibit platelet aggregation [90] may be of advantage to prevent microcoagulation as a major cause of organ failure [12].

The most intriguing data demonstrating regulatory effects of LF on spontaneous production of IL-6 and TNF-α by PBMC, derives from studies in healthy volunteers [4]. In one trial, volunteers received chewable tablets containing bLF for several days and the production of cytokines by PBMC cultures was measured before, on day 1 and on day 14 after treatment. The subjects were divided into categories of low, moderate and high responders, according to levels of TNF-α production following LF treatment. The study revealed that production of IL-6 successively rose in low responders but diminished in high responders, attaining the same level of reactivity in both categories on day 14. The regulatory action of LF was also evident by elevating the cytokine production in the low responders, transient stimulation in the moderate and a very strong inhibition in the high responder category. In conclusion, it seems that a more reasonable approach to prevent consequences due to

an induced cytokine (septic) shock should be aimed at optimization of immune reactivity of patients with pathogens inducing clinical insults.

6. Gut integrity and microbiota during clinical insult: Preventive and therapeutic utility of LF

We are of the opinion that LF would be most beneficial when applied as a prophylactic nutraceutical supplement to correct the immune status in healthy subjects and improve composition of gut microflora, crucial in maintenance of homeostasis. General health conditions, such as age and nutritional status of individuals, are typically associated with gut microflora composition that can potentially affect the critical course of SARS-CoV-2 infection [91] or trauma patients [92]. Others turn attention to proper function of microbiota-gut-liver-lung axis and provide preliminary evidence that disturbances in this axis may be linked to COVID-19 infection [93]. In fact, human brush border intestinal cells are particularly susceptible to SARS-CoV-2 infection [94]. Preventive and therapeutic actions of LF are directed both to protection of gut mucous layer integrity and composition of microflora. The integrity of gut wall is protected in the model of endotoxemia in mice [95] and rats [96], and is attenuated in healthy volunteers treated with indomethacin [97]. In vitro [98], in turn, LF was shown to protect Caco-2 cells against intracellular oxidative stress. Since gut microbiota dysbiosis may also contribute to pathogenicity of COVID-19 infection [13], the beneficial effects of LF on composition of gut microbiota is of utmost importance [99]. An interesting trial was conducted on volunteers receiving bLF preparations for two 2-week-periods separated by 2-week off term [100]. The authors found beneficial effects of LF on the volume of physiological bacterial species and lower expression of activation CD69 marker on CD4 + cells. Thus, application of nutraceutical preparations containing LF, displaying beneficial effects on human physiology and microbiota would be highly recommended as a preventive measure to optimize the immune system function [101]. Nevertheless, a therapeutic approach by oral or intra-tracheal administration of LF may also be effective considering promising results from animal models and human *in vitro* studies. Indeed, it is well established that oral administration of LF increases antimicrobial related NK cell activity in the small intestines of mice [102].

7. LF in clinical trials for neonates and enterocolitis

Although it is beyond the scope of this review, it should be mentioned that bovine LF has been successfully used in clinical trials of neonates to investigate utility to reduce incidence of enterocolitis. Multiple studies have shown the potential for utility; beginning with landmark studies by Manzoni, et al. [103,104]. Specific studies to note include those reported by Pammi and Shuresh [105], and trials completed by Ochoa, et al. [106] where oral delivered LF prevented sepsis in neonates of very low birth weight (VLBW). Kaur, et al. also demonstrated efficacy of oral bLF to reduce incidence of sepsis in neonates of less than 2000 g [107]. And Akin, et al. reported success in studies on VLBW infants, with an interesting twist showing increased Treg populations in the treated group, albeit a smaller patient was investigated [108]. Finally, use of a recombinant human lactoferrin (talactoferrin) showed some promise in low weight infants [109], although this study had limited enrollment and therefore no statistical difference between groups. Talactoferrin differs in human glycosylation patterns, and may not be fully compatible with human lactoferrin. Overall conclusions comparing all clinical studies to date is limited due to study terms, demographics, LF manufacturer, number of enrolled neonates, and set outcomes for specific pathogens. The ELFIN study represents one of the largest clinical trials, and offers solid hope for general support to use enteral LF to limit preterm infections post birth [110,111]. While promising, questions still remain [112]. Recent *meta*-analyses indicate further need for continued data collection and coordination of trial parameters to determine overall success in this clinical arena [113,114].

8. LF lessens lung pathology during the acute respiratory distress Syndrome (ARDS)

Finally, there may be insights for utility of LF as a therapeutic for SARS-CoV-2 induced pathology garnered from our studies investigating pulmonary ARDS occurring in models of granulomatous inflammation (reviewed in [115]). Specifically, LF was able to diminish or abolish cytokine excess and lung pathology cause by *Mycobacterium tuberculosis* [116,117]. Of course, pathology developed post mycobacterial infections are unique from viral induced tissue damage. However, it should be noted that LF was also able to diminish hyperacute immunopathology developed after administration of isolated surface mycolic acid components [118,119]. Direct reduction of proinflammatory IL-1β, IL-6 and TNF-α were identified post LF treatment, while IL-10 and TGF-β were maintained or even enhanced.

9. Conclusion

Based on the previously collected data from the animal and human studies, we postulate that LF may be of clinical benefit in prevention and amelioration of the cytokine storm and its devastating consequences in lungs and other vital organs. LF can be applied in prophylaxis, as well as a therapeutic agent, by multiple routes of administration (including oral delivery) for individuals at risk of infection, and especially for those demonstrating impairment of innate immune function.

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