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Lactoferrin: a critical mediator of both host immune response and antimicrobial activity in response to *Streptococcal* infections

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Abstract

Streptococcal species are gram positive bacteria responsible for a variety of disease outcomes including pneumonia, meningitis, endocarditis, erysipelas, necrotizing fasciitis, periodontitis, skin and soft tissue infections, chorioamnionitis, premature rupture of membranes, preterm birth, and neonatal sepsis. In response to *streptococcal* infections, the host innate immune system deploys a repertoire of antimicrobial and immune modulating molecules. One important molecule that is produced in response to *streptococcal* infections is lactoferrin. Lactoferrin has antimicrobial properties including the ability to bind iron with high affinity and sequester this important nutrient from an invading pathogen. Additionally, lactoferrin has the capacity to alter the host inflammatory response and contribute to disease outcome. This review presents the most recent published work that studies the interaction between the host innate immune protein lactoferrin and the invading pathogen, *Streptococcus*.

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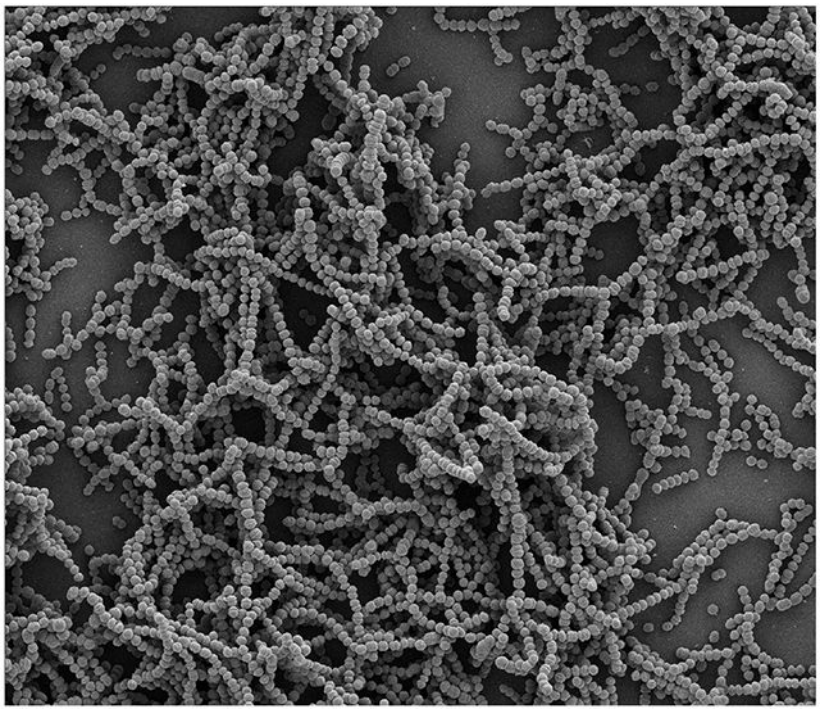
Graphical Abstract



Keywords

lactoferrin; streptococcus; infection; innate immunity; antimicrobial; nutritional immunity; iron; bacteria; pathogenesis; virulence; infectious disease

The requirement for nutrient metals as cofactors in a variety of biological processes is conserved across all kingdoms of life. Invading bacterial pathogens require iron as a cofactor for cellular processes including respiration, DNA replication, electron transport, peroxide reduction, stress response, and cell division. The host exploits this need for nutrient metals such as iron and deploys a variety of factors to bind iron with high affinity, effectively starving the invading pathogen in a process termed, “nutritional immunity”[1]. Nutritional immunity in the vertebrate host includes numerous proteins such as calprotectin, calgranulin C, hemoglobin, ferritin, transferrin, and lactoferrin. Lactoferrin is highly abundant in host tissues infected with bacterial pathogens such as streptococcal species. Interestingly, several of these nutritional immunity proteins also have immunoregulatory properties. This review will focus on the intersection of lactoferrin’s involvement in antimicrobial activity and immune regulation and the pathogenesis of *Streptococcus*.

Streptococcus and human health

The *Streptococcus* genus includes a group of gram-positive cocci bacteria which are some of the leading causal agents of human infectious diseases[2]. The genus is separated into 49 species and eight subspecies, 35 of which are attributed as the source of invasive infections in humans. In particular, four *Streptococcal* species are considered to be the cause of

common human infections. These species include *Streptococcus pyogenes* (Group A *Streptococcus*, or GAS), *Streptococcus agalactiae* (Group B *Streptococcus*, or GBS), *Streptococcus mutans*, and *Streptococcus pneumoniae*. Collectively, these species of bacteria create an immense public health burden[3].

Invasive Group A *streptococcal* infections, caused by *S. pyogenes*, include a number of clinical manifestations locally in the skin, soft tissues, joints, or the lower respiratory tracts, or globally as bacteraemia without a focus of infection[4]. 1.8 million new cases are reported per year with an estimate of 517,00 deaths annual, making GAS one of the top human-infection related cause of death annually[5-6]. In particular, GAS infections can result in fatality in one of two ways: necrotizing fasciitis and sepsis [7-8]. Additionally, invasive GAS infections can be complicated by streptococcal toxic shock syndrome (STSS), which is a condition characterized by hypotension and often accompanied by fever or rash with rapid progression to shock and multi-organ failure[9]. Only 10% of patients develop STSS but early intervention is important as over 25% of these patients will result in death within 24 hours of presentation[10]. GAS is also responsible for non-lethal disease conditions ranging from mild infections, such as pharyngitis and impetigo, to invasive diseases[11]. Serious immune sequelae may be triggered after repeated GAS infections, including acute glomerulonephritis and rheumatic heart disease[12]. Overall, the skin appears to be the most frequent portal of entry as marked by a third of patients have evidence of skin lesions or wounds[13-14]. In particular, injecting drug users at higher risk for acquiring GAS[15].

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is the leading infection-related cause of preterm birth, chorioamnionitis, funisitis, neonatal sepsis, bacteremia, mastitis, and invasive soft tissue infections in diabetics[16-18]. In particular, neonates, newborns, pregnant mothers, and the immunocompromised are susceptible to GBS infection[19]. GBS colonizes 20-30% of healthy adults, including the urogenital tract of females and lower gastrointestinal tract of both males and females[20]. The major reservoir of GBS is the human gastrointestinal tract where it exists as commensal flora[21]. It is estimated that between 20-30% of pregnant women are colonized with GBS, making colonization the leading risk factor for disease[22]. In one longitudinal study in South Africa, Kwatra *et al.* found that up to 50% of women in their study population were colonized with GBS at some point during their pregnancies[23].

GBS is especially a large health burden to mothers and neonates in developing countries. In 2015, it is estimated that 21.7 million pregnant mothers were colonized with GBS. With GBS infection, there is a higher risk of maternal and neonatal death and development[24]. The most recent report of global vaginal GBS colonization estimates a prevalence of 18%, after adjusting for sample collection and methodology, ranged from the lowest regional prevalence in Southern and Eastern Asia (11–13%) to the highest prevalence in the Caribbean (35%)[22]. An estimated four million newborns deaths are reported each year within the first 4 weeks of life globally, and one in four of these deaths stems from severe infection including sepsis or pneumonia. Notably, 99% of neonatal deaths occur in low- and middle-income countries[25]. In developed countries, GBS and *E. coli* are collectively responsible for approximately 70% of early-onset neonatal sepsis of both term and preterm infants[26]. Neonatal colonization occurs in approximately 40–75% of births from GBS colonized mothers, with 1–2% of cases leading to invasive disease[27-30]. The most recent

comprehensive systematic review and meta-analysis estimated the grouped incidence of neonatal morbidity and mortality to be 0.47 per 1000 worldwide, including cases attributed to GBS-associated preterm birth, stillbirth, and neonatal GBS infection[24].

S. mutans is the primary etiologic agent of human dental caries[31], and occasionally infective endocarditis[32]. They can form biofilms on the surface of teeth, referred to as dental plaque biofilm[33]. In fact, *S. mutans* is the prime initiator of plaque and a potent producer of acid, resulting in exacerbation of tooth decay and oral disease. Unlike other pathogens that produce virulence factors that disrupt the host, *S. mutans* has evolved to exist as part of the normal member of oral dental biofilms[34]. As a result, *S. mutans* can easily gain access into the bloodstream during dental surgery, resulting in colonization of injured heart valves and induction of endocarditis[35]. Dental caries is the most common and costly oral disease worldwide, with *S. mutans* as the etiological agent underlying this burden[36].

S. pneumoniae (pneumococcus) causes pneumonia, otitis media, meningitis, and septicemia[37]. The pneumococcus is a commensal in the pharynx and upper respiratory tract of healthy individuals but may cause localized infections, such as otitis media, particularly in children. Invasive pneumococcal infection may become established at a variety of sites including the lung parenchyma and meninges. *S. pneumoniae* is a bacterium that has been widely linked to respiratory infections in immunocompromised populations[38-40]. Additionally, it is the major cause of community acquired pneumonia (CAP) and is an important cause of bacteraemia, particularly in infants and older adults[19].

The populations most susceptible to invasive pneumococcal disease (IPD) are adults above the age of 65, children younger than two years old, and those with certain underlying conditions, such as HIV infection, multiple myeloma and chronic kidney, liver or pulmonary disease[41]. The bacterium is estimated to cause about four million illnesses within the United States and about 450,000 hospitalizations per year[42]. Furthermore, studies indicate that 10% of patients with invasive pneumococcal diseases die of their illnesses[43].

Lactoferrin and the innate immune system

The innate immune system plays an immense role in defense against *Streptococcal* infections. One critical part of the innate immune response is production of a repertoire of antimicrobial proteins to control bacterial growth and viability. One such antimicrobial protein, lactoferrin is a globular glycoprotein which is produced by eosinophils, macrophages and neutrophils in response to infection[44-45]. Lactoferrin is expressed at high levels in secondary granules within granulocytes such as neutrophils[46-47]. Outside of immune cells, lactoferrin is expressed in a variety of tissues and fluids including breast milk, colostrum, saliva, tear fluid, and mucous[46]. In response to encountering a microbial threat, neutrophils release antimicrobial elements by excreting DNA neutrophil extracellular traps (NETs)[47]. NETs are structures decorated with antimicrobial agents, including nuclear chromatin, histones, and other antimicrobial proteins that serve to immobilize and kill or inhibit the growth of invading microbes[47]. Recent work indicates that *S. agalactiae* induces neutrophil extracellular traps that are enriched with lactoferrin[44].

Structure- function relationships of lactoferrin

Lactoferrin is an iron-binding glycoprotein with a size of 80 kDa. The protein consists of a single polypeptide chain containing 692 amino acids, which is folded into two symmetrical lobes (N and C) with a hinge region connecting to two regions (Figure 1) (Anderson et al., 1989). Each lobe is able to bind one Fe^{3+} iron together with one CO_3^{2-} iron within the cleft of the lobe that contains a nonheme iron-binding site[48]. While lactoferrin predominately binds iron, it can also bind Cu2+, Mn2+, and Zn2+ at low affinity[49]. Two regions of lactoferrin have been implicated in the iron-independent activity of the glycoprotein. This first region is referred to as lactoferricin and contains the first 47 residues starting at the N-terminus[50]. The residues from this region come together to form a β-α-β unit at the explore surface of human lactoferrin, isolated from the iron-binding regions. The exposed region includes nine amino acid side chains projecting from its surface. This region is located in a highly charged N-terminal tail (residues 1 to 5; GRRRR) and an amphipathic region near the C terminus of helix A (residues 28 to 31; RKVR) that are in close proximity in the folded human lactoferrin protein[48, 51]. The highly basic region has been implicated in direct bacterial killing[52]. Furthermore, this region of the N-terminus has been shown to bind to the DNA sequences upstream of various genes and act to regulate transcriptional activation[53-54]. Another major binding site is at residues 269-285 in the N-lobe of humans and this region is called lactoferrampin[50]. Pepsin cleaves human lactoferrin to generate these two peptides[55]. Overall, the glycoprotein is very basic, which allows for interaction with negatively charged molecules in solution and on cell surfaces[56].

Polymorphisms in the lactoferrin gene exists in our population with varying effects on microbial clearance. One such SNP is at the 29th position of the N-terminal region. Lactoferrin with lysine at this position exhibited significantly enhanced antimicrobial activity against *S. mutans* and *Streptococcus mitis* compared to the variant with arginine at the 29th position[57]. However, there was no difference observed in antimicrobial activity against gram-negative bacteria. The study also performed genetic analysis and revealed that the K allele was more frequent in their sample of patients with localized juvenile periodontitis, which is a rapid and aggressive form of periodontitis that disproportionally affect African-American adolescents[58]. Interestingly, they found that the K allele was more prevalent in the African-American population, which is correlated with higher incidence of the disease. The authors suggest that the more bactericidal allele (K allele) kill more gram-positive bacteria, which changes the microflora and allows for the gram-negative bacteria *Aggregatibacter actinomycetemcomitans* to colonize[57]. Importantly, *A. actinomycetemcomitans* colonization is linked to localized juvenile periodontitis[58].

In another study, Barber *et al.* used various genetic tools to investigate the evolution of the lactoferrin across the human ancestral timeline. Through the evolutionary history of primates, the lactoferrin gene acquired iron-independent antimicrobial capability through positive selection[59]. They also pinpointed signatures of natural selection, namely pressure from microbial pathogens, acting on lactoferrin in human populations, suggesting that lactoferrin genetic diversity has impacted the evolutionary success of both ancient primates and humans. For instance, the authors point out that there are bacterial receptors that target

transferrin, a related gene, for iron acquisition. The repeated targeting of transferrin may have provided an advantage for antimicrobial activity to arise in the n-lobe of lactoferrin.

Immunoregulatory functions of lactoferrin

While lactoferrin is recognized as an antimicrobial peptide, the glycoprotein also possesses immunoregulatory properties, both dependent and independent of iron chelation. During inflammation, polymorphonuclear neutrophils (PMN) are recruit to the site of infection. Once the PMNs reach the site, they release reactive oxygen species (ROS) to aid in the clearance of the bacterial pathogen. However, reactive oxygen radicals are not only detrimental to bacterial cells, but will also non-discriminately damage the surrounding host tissue. One consequence of cell damage is the release of ferric and ferrous iron. The free iron may then participate in the Haber-Weiss reaction to generate new free radicals, further perpetuating the damage. Lactoferrin plays a crucial role in preventing further damage by chelating iron released by the wounded tissue, resulting in the mitigation of oxidative stress at the site of inflammation[60].

Independent of iron chelation, lactoferrin is able to bind to receptors of immune cells to dampen a pro-inflammatory response[61]. The glycosaminoglycans of membrane proteoglycans on cell surfaces account for 80% of binding by lactoferrin; this interaction is of low affinity (10^{-5}-10^{-6} M)[62]. One consequence of receptor binding is the downregulation of pro-inflammatory cytokines by immune cells. Lactoferrin has been shown to bind to bacterial LPS, the ligand for TLR4, thus mitigating TLR-4 mediated pro-inflammatory cytokine production by macrophages[63]. Another study revealed that lactoferrin can bind to soluble CD14 (sCD14), which normally complexes with LPS to induce production of IL-8, resulting in the inhibition of IL-8 production by epithelial cells[64]. As lactoferrin is known to bind DNA, internalization of the peptide into monocytic cells can inhibit NF-kB binding to the TNF-α promoter and downregulate LPS-induced cytokine production[63]. The modulation of cytokine production by lactoferrin ultimately alters the balance of Th1 and Th2 responses[65].

In addition to altering the cytokine profile of an immune response, binding of lactoferrin to some cell-surface molecules leads to changes in immune cell activation, recruitment, and function. As previously discussed, lactoferrin binds sCD14, preventing the induction of IL-8. This interaction also prevents the expression of E-selectin, and intercellular adhesion molecule 1 (ICAM-1), thus disrupting recruitment of leukocytes to the site of infection[66-67]. Furthermore, the same study demonstrated that lactoferrin is able to compete with IL-8 for proteoglycans and their further presentation to leukocytes. Lactoferrin has also been shown to inhibit hydrogen peroxide production mediated by LPS binding to L-selectin of PMNs[66]. Taken together, lactoferrin is able to target many key processes of the innate immune response and assert its immunoregulatory effects.

Lactoferrin and nutritional immunity

With two binding pockets to bind iron ions at high affinity, lactoferrin is an excellent scavenger for free-flowing iron. Iron is involved in a variety of critical biological processes,

including DNA synthesis, ATP synthesis, and nitrate reduction in the nitrogen cycle. Iron is also required for the formation of heme, as an enzyme co-factor, and in the electron transport system. In bacteria, iron is an essential micronutrient for biofilm formation, cell development, and cell growth[68-69]. As a result, there is an arms-race between the host and the pathogen to acquire imperative nutrients. In order to suppress bacterial invasion, the human immune system produces molecules that bind transitional metals in a strategy coined "nutritional immunity"[1]. Among these iron-chelating proteins are transferrin and lactoferrin[70]. Lactoferrin, however, is able to retain iron at a much lower pH than transferrin (such as those encountered in the stomach or in devitalized tissue), allowing for a more potent iron-sequestration activity[71]. The importance of nutritional immunity is highlighted by the mechanism by which bacteria have evolved to overcome this selective pressure. While only about 10^{-9}–10^{-18} M iron is available within the host, most microbes need 10^{-8} M iron for normal cellular functions[72]. Some bacteria have evolved to bind iron-chelating molecules such as lactoferrin and use these as iron sources. Members of the *Streptococcus* genus have gained iron acquisition mechanisms including siderophores and iron channels to overcome the host pressure[73-74].

Antimicrobial activity of lactoferrin

Lactoferrin has been implicated in defense against a wide range of organisms by a variety of mechanisms. Studies have shown that lactoferrin is able to aid in the control of bacteria, viruses, fungi, and parasites[71]. Among bacterial pathogens, lactoferrin has been shown to exhibit potent antimicrobial activity against *Streptococcus*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Enterobacter* genera[75]. Supplementation with lactoferrin (and other oral enzymes and proteins) in toothpaste, reduced oral bacterial growth, viability, and biofilm formation, a result that was attributed to perturbations in bacterial membrane integrity, specifically in *S. mutans*[76]. Both gram-negative and gram-positive organisms have molecules that are targeted by lactoferrin, lipopolysaccharides and lipoteichoic and teichoic acids, respectively, resulting in the depolymerization of the cell membrane[77]. This leads to the disruption and permeabilization of the bacterial membrane and ultimately cell death. Furthermore, the N-terminus of lactoferrin possesses a serine protease-like activity, which potentially degrades secreted proteins[78].

While lactoferrin is able to defend against bacterial pathogens by direct killing, it is also able to protect by inhibiting bacterial resistance structures known as "biofilms". Biofilms are communities of microorganisms that form highly complex three-dimensional structures that adhere to surfaces and protect against immune assault and nutrient deprivation[79]. The biofilm-altering role of the antimicrobial peptide lactoferrin has been studied intensively in *Streptococcus* species, due to the fact that lactoferrin is abundant in mucosal surfaces such as those found in the oral cavity and in mammalian milk, which are natural reservoirs for *Streptococcus*[80-81]. Allison and colleagues report that lactoferrin significantly inhibits *S. mutans* biofilm at all dilutions, including those within the physiological range found in milk (3 mg/mL), a result that was not seen with other milk components such as lactose and casein[80]. Other *in vitro* studies using lactoferrin alone or in combination with lactoperoxidase and/or lysozyme determined that these antimicrobial molecules inhibit biofilm formation by oral *streptococcal* species, such as *S. mutans,* by preventing bacterial

adhesion[81-89]. One plausible explanation for the mechanism by which lactoferrin inhibits *streptococcal* biofilm formation is via iron sequestration. Indeed, this is supported by results reported by Berlutti *et al.*, which indicates that bovine apo-lactoferrin enhances *S. mutans* aggregation, but holo-lactoferrin (iron-loaded) represses bacterial aggregation[85]. Additionally, the authors discovered that bovine lactoferrin had the propensity to inhibit adhesion of *S. mutans* to abiotic surfaces independent of its capacity to bind iron, demonstrating its iron-independent biofilm-repressing activity. Furthermore, a recent study by Angulo-Zamudio *et al.*, showed that lactoferrin has the capacity to disaggregate pneumococcal biofilms and inhibit the acquisition of antibiotic resistance via DNAse activity[90]. Together, these results underscore the importance of lactoferrin in modulating *streptococcal* aggregation and biofilm formation/maintenance.

In addition to bactericide and disruption of biofilm, lactoferrin is able to engage the innate immune system to aid in bacterial clearance. One study showed that bovine lactoferrin was able to substitute for antibodies in order to efficiently activate the classical pathway of complement and induce opsonization of unencapsulated *S. agalactiae*[91]. Complement is a family of proteins of the innate immune system that activate in a cascade with the final result of pore formation by the membrane attack complex on bacterial membranes or opsonization by phagocytes[92]. However, other studies have shown that lactoferrin from normal human tears can inhibit the complement pathway through preventing the formation of the C3 convertase[93]. As lactoferrin has been shown to have immunomodulatory effects, it is possible that lactoferrin inhibits some but not all of the complement pathways, and that the mechanism by which lactoferrin acts on a surface could be different than interactions with complement components in solution. One study suggests that bovine lactoferrin inhibits the classical pathways but activates the alternative pathways during *Staphylococcus aureus* infection[94]. More studies will need to be conducted to firmly establish the interaction between lactoferrin and the complement system.

Utility as a biomarker

As lactoferrin is an immune derived peptide, it has been implicated in many infectious diseases. As a result, changes in expression levels may allow for lactoferrin to be used as a sensitive biomarker for the diagnosis of many diseases. In the studies of oral health, increased levels of lactoferrin in the saliva are positively correlated with chronic periodontitis[95]. In another study, salivary lactoferrin levels were elevated in periodontal disease in patients who are both HIV positive and negative, suggesting that lactoferrin is a suitable biomarker for periodontal disease[96]. A recent study has demonstrated that lactoferrin can be used as an effective biological recognition element to detect *S. sanguinus*, one of the most prevalent bacterial species in the onset of periodontal diseases[97]. In the realm of maternal and neonatal health, lactoferrin is shown to be elevated in the amniotic fluid of chorioamnionitis patients[98]. Additionally, plasma lactoferrin levels are indicative of newborn preterm infants with sepsis[99]. Taken together, lactoferrin may serve as a powerful biomarker for a variety of human diseases.

Potential use as a chemotherapeutic strategy

Lactoferrin, along with its peptide derivatives (lactoferricin, lactoferrampin, and LF1-11), has been studied as a chemotherapeutic strategy against a range of infectious diseases[100]. *In vivo* studies using lactoferrin alone or in combination with lactoperoxidase and/or lysozyme have revealed that lactoferrin has the capacity to inhibit colonization of oral *streptococcal* species, such as *S. mutans,* in dental caries/lesions by repressing biofilm formation and preventing bacterial adhesion for up to one month post-treatment[82]. Lactoferrin supplementation in toothpaste reduced oral *S. mutans* burden, an important intervention that could be utilized to defend against caries or periodontal disease progression[76]. As lactoferrin has been shown to inhibit the growth of *S. agalactiae*[44], there is merit in studying the use of lactoferrin and its derivatives as a chemotherapeutic strategy.

Conclusions

Lactoferrin is produced by epithelial cells and innate immune cells such as granulocytes and participates in nutritional immunity by binding two molecules of iron[46-47] (Figure 2). Lactoferrin has strong antimicrobial effects against a wide range of microbial infections, including those caused by *Streptococcus spp*[44]. However, the antimicrobial peptide also possesses immunoregulatory properties, making it a very versatile protein with many functions. Lactoferrin interacts with cell surface membranes via proteoglycan receptors as well as pathogen-associated molecular patterns such as LPS to inhibit TLR4 and soluble CD14 receptors to ultimately repress pro-inflammatory signaling and cytokine production[63-64]. Lactoferrin can bind to host DNA and repress NFκB-dependent induction of TNF-α, thereby exerting anti-inflammatory activities[63]. Lactoferrin can also inhibit hydrogen peroxide production by innate immune cells[66]. Streptococcal cells induce neutrophil extracellular trap formation in which these immune cells excrete DNA decorated with antimicrobial molecules, including lactoferrin[44]. Lactoferrin, subsequently inhibits bacterial growth and biofilm formation[68-69]. Lactoferrin also binds to the surface of streptococcal cells to promote complement-binding and opsonization[91]. Furthermore, studies have revealed lactoferrin could be a promising candidate as a biomarker for diagnosis or as a potential treatment to combat microbial infections. As *Streptococcus* infections are a rising global health burden, the investigation of the intersection between lactoferrin immune defense and *Streptococcus* biology will provide crucial scientific knowledge to address the rising concern.

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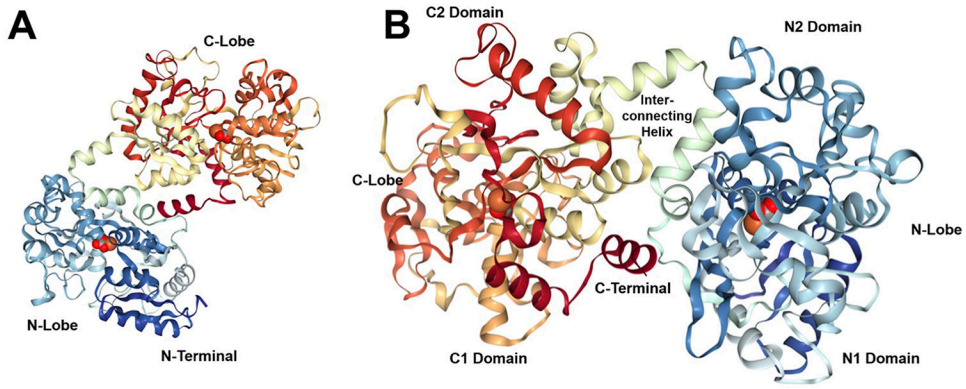

Figure 1.

Structural model of human lactoferrin. Lactoferrin is a glycoprotein consisting of a single polypeptide chain containing 692 amino acids, which is folded into two symmetrical lobes: the N-lobe (in blue) and the C-lobe (in orange and yellow) with a hinge region (in green) connecting to two regions. Each lobe is able to bind one Fe^{3+} iron together with one CO_3^{2-} iron (red spheres) within the cleft of the lobe that contains a nonheme iron-binding site. Each lobe has two domains: the C1 and C2 domain within the C-lobe, and the N1 and N2 domain within the N-lobe. Structure was generated using PyMOL software.

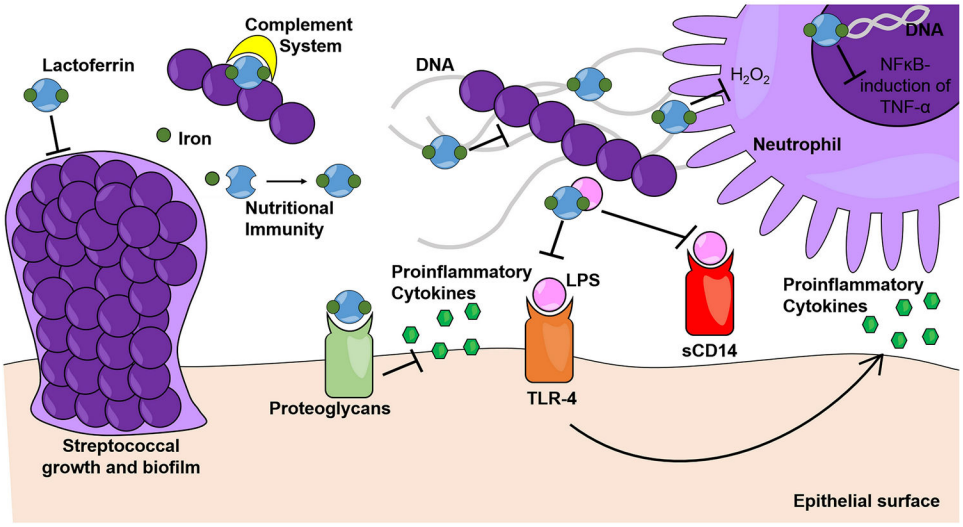

Figure 2.

Conceptual model of the role of lactoferrin in host-pathogen interactions during *Streptococcus* spp. infections. Lactoferrin (also referred to as lactotransferrin) is produced by epithelial cells and innate immune cells such as granulocytes and participates in nutritional immunity by binding two molecules of iron. Lactoferrin interacts with cell surface membranes via proteoglycan receptors as well as pathogen-associated molecular patterns such as LPS to inhibit TLR4 and soluble CD14 receptors to ultimately repress pro-inflammatory signaling and cytokine production. Lactoferrin can bind to host DNA and repress NFκB-dependent induction of TNF-α. Lactoferrin can also inhibit hydrogen peroxide production by innate immune cells. Streptococcal cells induce neutrophil extracellular trap formation. Lactoferrin decorates the neutrophil extracellular trap, which is comprised of DNA, and inhibits bacterial growth and biofilm formation. Lactoferrin also binds to the surface of streptococcal cells to promote complement-binding and opsonization.