The Immunomodulatory Effects of Lactoferrin on the Neutrophil Functions

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Abstract

Lactoferrin is a multifunctional glycoprotein that binds to iron and is found in mammalian milk, other external secretions such as saliva, tears, semen, mucosal surface of the respiratory and intestinal tracts, and the secondary granules of neutrophils. The wide localization of lactoferrin in the body, especially in neutrophils, mostly characterizes its biological functions. The secreted form of lactoferrin is thought to be involved in the host defense against microbial infection at mucosal sites and another neutrophilic form of lactoferrin has notable immunomodulatory function. Neutrophils are the first responder against microbe invasion and a deficiency in their function leads to severe recurrent bacterial and fungal infection and inflammatory lesions. These lesions often affect the respiratory tract, oral mucosa and skin. It was shown that the oral administration of lactoferrin induced an anti-inflammatory effect in the cats with chronic inflammation, by modulating neutrophil functions. In addition, the oral administration of lactoferrin increased the expression of β2 integrin on the surface of neutrophils in canine leukocyte adhesion deficiency (CLAD) dogs, and as a result of this modulating effect, a clinical improvement was observed. Therefore, lactoferrin might be a treatment target for congenital neutrophil dysfunction based on its ability to modulate neutrophil functions. In this review article, we will discuss the modulating effects of lactoferrin on the neutrophil functions, especially in the congenital neutrophil dysfunction.

Keywords: canine leukocyte adhesion deficiency (CLAD); lactoferrin; leukocyte adhesion deficiency (LAD); neutrophil dysfunction

Introduction

Lactoferrin is a multifunctional 80 kD glycoprotein that binds to iron and has been widely studied over the last half century. The multifunctional character of lactoferrin has attracted many researchers to investigate its structure and functions.

Lactoferrin was first identified as a red protein in milk more than a half century ago. It was first discovered in milk at 1937 [1], and was first purified from human and bovine milk in 1960 [2,3,4] Lactoferrin was called lactotransferrin, because of its strong iron-binding activity. Lactoferrin binds to iron similar to transferrin, however, its binding ability is greater than transferrin. Lactoferrin binds to iron under low pH conditions (typically pH 3-4) compared with transferrin (iron dissociates from transferrin at pH 5-6) [5]. Lactoferrin is found not only in mammalian milk, but also in other external secretions such as saliva, tears, semen and mucosal surface of the respiratory and intestinal tracts [6,7]. Interestingly, the following investigation revealed that lactoferrin is found in the secondly granules of neutrophils [8,9]. The wide localization of lactoferrin in the body, especially in neutrophils, mostly characterizes its biological functions. Many following studies have shown the biological effects and antimicrobial activity of lactoferrin on the primary innate-immune defense system [10–16]. The antibacterial properties of lactoferrin were first reported in the 1960s. Previous studies showed two mechanisms related to the anti-microbial effects of lactoferrin: the depletion of iron (iron is essential for microorganisms growth), and direct interaction with pathogens that cause cell lysis[17]. Furthermore, lactoferrin interacts with various cells, including immunocytes such as macrophages, T-lymphocytes, NK-cells and neutrophils. The expression of the lactoferrin receptor on T-lymphocytes was described about three decades ago and indicated that the interaction between lactoferrin and T-lymphocytes induces MAPK activation [18].

The modulating effect of lactoferrin on immunocytes, especially neutrophils, is very important with regard to its protective effects on the primary immune system. The functions of neutrophils, such as chemotaxis, phagocytosis and bacteria-killing ability with superoxide, contribute to the innate host defense system against bacterial infections. Lactoferrin stimulates the release of IL-8, a neutrophil-activating polypeptide, from macrophages [19]. IL-8 increases the number of neutrophils and also primes them. On the other hand, it was shown that the oral administration of lactoferrin induced the beneficial effects in an animal model with chronic inflammation, by modulating neutrophil functions. Our previous study demonstrated the modulating effects of oral lactoferrin-administration on neutrophil function in the cats with chronic inflammation [20]. Subsequent studies revealed that the clinical application of lactoferrin isolated from bovine milk was beneficial in animal models and human cases. These clinical effects of lactoferrin are partly based on the modulating effect of lactoferrin on neutrophil functions as indicated by our previous study [20]. In this review article, we will discuss the modulating effects of lactoferrin on the neutrophil functions,

especially in the congenital neutrophil dysfunction.

Lactoferrin and neutrophil functions

Lactoferrin is found in exocrine secretions, such as saliva, tears urinary-reproductive fluid, the mucosal surface fluid of the respiratory organs in the body, and also detected in the serum. Plasma lactoferrin originates in neutrophils. Lactoferrin is stored in the neutrophilic secondary granules [8,9], and is synthesized during the inflammatory responses. Plasma levels of lactoferrin were significantly elevated following the degranulation of activated neutrophils. Neutrophils contain lactoferrin (2.5-15 μg/10^6 cells) and release it at the sites of infection, which are acidic because of pathogen activity [8.11,21,22]. Plasma concentration of lactoferrin in the patients with sepsis or inflammatory disease were markedly increased, while usual level of lactoferrin in healthy person was very low (0.4-2 μg/ml) [8]. Many studies reported that plasma lactoferrin level varied between 0.1 to 0.5 μg/ml in healthy humans [23-26]. Serum lactoferrin levels are 2.5 times higher than that in plasma [25]. Furthermore, lactoferrin levels in patients with inflammatory disease are higher than those from healthy humans. Patients with pneumonia in the initial phase [27], chronic inflammatory enteritis such as inflammatory bowel disease (IBD), ulcerative colitis and Crohn's disease [28] had high levels of serum lactoferrin. The patients with rheumatoid arthritis also showed elevated levels of plasma lactoferrin [23] . Plasma lactoferrin is thought to reflect the absolute neutrophil count, as well as neutrophil secretory activity .

In veterinary medicine, lactoferrin concentrations in milk are used as a good biomarker of mastitis. Previously, we measured plasma lactoferrin concentrations by ELISA system [29]. In that study, we reported the values of plasma lactoferrin in healthy cows and diseased cows including acute and chronic mastitis. Healthy cows had 0.158 - 0.713 μg/ml of lactoferrin while diseased cows had 0.374 - 11.0 μg/ml of lactoferrin. Therefore, the plasma concentration of lactoferrin might also have diagnostic valuable.

In addition to inflammatory biomarkers, recent studies of lactoferrin have examined it as an immune modulator *in vitro* and *in vivo* models. The relationship with lactoferrin and neutrophils was studied using *in vitro* models at the 1980s. Many reports have demonstrated enhanced reactive oxygen species production by isolated PMN (polymorphonuclear leukocyte: neutrophils) when coincubated with lactoferrin [30,31]. Boxer et al. demonstrated that primed PMN mobilized lactoferrin to their surface, and that lactoferrin had a reduced surface charge causing enhanced "stickiness" and a variety of cell-cell interactions [32]. The antimicrobial activity of lactoferrin seems to be dependent on its cationic properties. In the 1990s, studies revealed two forms of human lactoferrin [33,34]. One form is contained in the exocrine secretions and the other is present in the secondary granules of neutrophils. These two forms are identical in their amino acid sequence but differ in glycan content. While the secreted form is thought to be involved in host defense against microbial infection at mucosal sites, granulocytic/

neutrophilic lactoferrin has an immunomodulatory function [35.36]. These differences are thought to depend on specific glycosylation patterns especially interactions with a specific receptor for sialic acid [36].

In addition to the *in vitro* studies of lactoferrin, the oral administration of lactoferrin was reported to have immunomodulating effects, especially on lymphocyte and neutrophil functions. We previously reported that the oral administration of bovine lactoferrin enhanced phagocytosis and reduced the adherence activity of peripheral blood neutrophil in feline immunodeficiency virus (FIV) infected cats, which showed intractable stomatitis, and improved their chronic inflammation [20]. The oral administration of bovine lactoferrin also enhanced phagocytosis and superoxide production, and decreased adherence activity in healthy volunteers [37]. It was reported that the oral administration of bovine lactoferrin also increased NK cell activity in patients with colorectal polyps [38]. How does the lactoferrin modulate effector cells ? Orally administrated lactoferrin is absorbed into the systemic circulation in neonate calves [39], weaned pigs [40] and adult rats [41]. Takeuchi et al. reported that the intraduodenally administrated bovine lactoferrin was transported into the blood circulation via the thoracic duct lymph fluid in adult rats [41]. It was also reported that lactoferrin receptors are present in the intestine including Peyer's patches, which have a greater binding capability for lactoferrin compared with other tissues in cattle [42]. Iigo et al. [38] reported that serum human lactoferrin level and peripheral blood neutrophil count were increased after ingesting bovine lactoferrin, but that serum bovine lactoferrin was not detected in patients with colorectal polyps. They suggested that ingested bovine lactoferrin primed neutrophils and caused the release of human lactoferrin from neutrophil. In FIV infected cats, orally administered lactoferrin reduced intractable stomatitis. We speculated that orally administrated lactoferrin mediated its clinical effects through interactions with the oral lymph system such as tonsils. In addition, it was reported that bovine lactoferrin administrated by gastric intubation was detected in mesentric fat tissue and decreased the risk of visceral fat accumulation in adult rats [43]. These findings suggest that orally administrated lactoferrin is absorbed via the lymphatic pathway and modulates effector cells, and/or directly acts on the lymphatic glands of the mouth and intestine.

LAD and CLAD

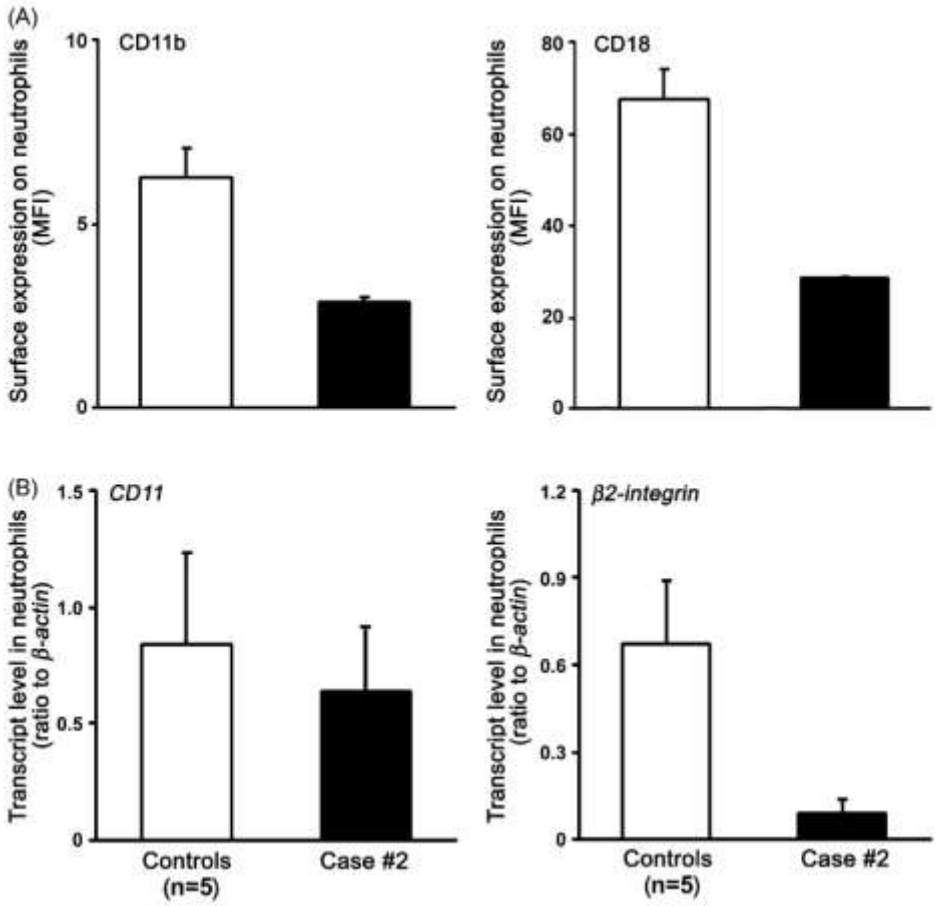
Neutrophils are the first responder against microbe invasion and a deficiency in their function leads to severe recurrent bacterial and fungal infection and inflammatory lesions. These lesions often affect the respiratory tract, oral mucosa and skin. Congenital neutrophil dysfunction accounts for 10–20% of primary immunodeficiencies [44,45]. Congenital neutrophil dysfunction causes the defect in neutrophil adhesion, migration and oxidative killing. Leukocyte adhesion deficiency (LAD) is a congenital neutrophil dysfunction that causes the recurrent bacterial infection, soft tissue abscess and periodontal disease from birth. LAD is divided into three disease types: LAD I, LAD II and LAD III. LAD I is

an autosomal recessive disorder characterized by a defect in CD18 caused by a mutation of the integrin gene *ITGB2*. CD18 is necessary for the stable expression of CD11/CD18. CD18 is a common β chain of four β2 integrins in leukocytes, each containing a different α chain: LFA-1 ($\alpha_1\beta_2$ or CD11a:CD18), Mac-1 ($\alpha_M\beta_2$ or CD11b:CD18, CR3), gp150/95 ($\alpha_X\beta_2$ or CD11c:CD18, CR4), and ADB2 ($\alpha_D\beta_2$ or CD11d:CD18, CR3) [46]. Neutrophil activated by chemoattractants, such as IL8, expresses β2 integrins on their surface that binds to intercellular adhesion molecule-1 (ICAM-1) and -2 (ICAM-2). This process is important for the quick trafficking of neutrophils to the blood vessels and their transmigration into inflammatory tissues. The failure of this process reduces activation of the innate host defense system by neutrophils, because of defective cell adhesion, chemotaxis and phagocytosis. LAD II is caused by the mutations in *SLC35C1* (*FUCTI*) that encodes the membrane transporter of fucose which impairs selectin mediated adhesion. LAD II has a more severe phenotype than LAD Ⅰ because it includes developmental delay and short stature [47]. LAD III is caused by mutations in intracellular protein Kindlin3 (*FERMT3*) which regulates inside-out integrin activation [46]. The expression of β2 integrins is normal but the activation of β2 and β3 is absent in LAD III patients [48]. The mutation analysis of Kindlin3 revealed homozygous mutation in all LAD III patients [49]. LAD III was observed in Turkish child with recurrent bacterial infection and bleeding tendency [50].

Canine leukocyte adhesion deficiency (CLAD) was originally identified by Renshaw et al.[51] in the middle of 1970s in Irish setter dogs. Then, Giger et al. reported the deficiency in the surface expression of the CD11/CD18 complex in dogs [52] snd in Red and White Irish setters [53]. The clinical progress of CLAD dogs closely parallels that of children with LAD. The recurrent bacterial infection takes the form of severe gingivitis, periodontitis, and cutaneous nonhealing wounds are observed in LAD children during the perinatal period. LAD children also show severe leukocytosis ranging from 15,000 to 100,000 cells /μl, but despite marked leukocytosis, their neutrophils do not migrate to the site of infection resulting in an absence of pus at the inflammatory or infectious site [54]. Puppies with CLAD also show similar clinical progress from shortly after birth. Omphalitis is observed after the birth and develops followed by severe gingivitis, lymphoadenopathy, poor wound healing, low body weight, and episodes of infection manifesting as pyrexia and anorexia [55]. Severe leukocytosis was also observed in CLAD puppies. Sequence studies revealed that CLAD dogs have a deficiency in the surface expression of CD11/CD18 complex [52], caused by a single point mutation that results in an amino acid substitution (c365) in highly conserved cysteine residue in the extracellular domain of CD18 [53]. From these findings in CLAD dogs, CLAD is thought to be an animal model for LAD I.

A small number of mixed-breed CLAD dog species have been reported with the exception of Irish setter and their cross bred dogs. We described a mixed-breed canine cases with a defect in neutrophil adhesion ability

caused by the lack of CD11/CD18 and β2 integrin transcript level (Figure 1) [56,57,58].



(C) β2-Integrin cDNA

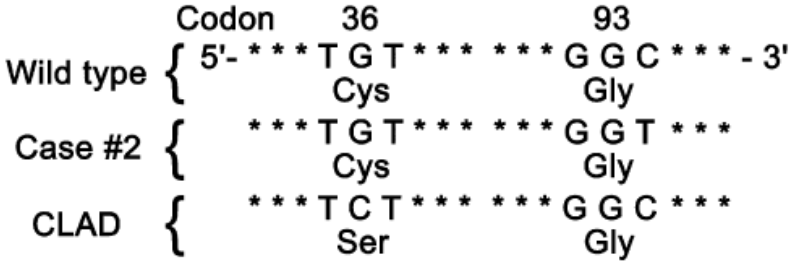


Figure1. Surface expression and transcript level of neutrophil adhesion molecules and sequence of CD18 cDNA in case#2.[56] .

However, the sequence analysis of β2 integrin cDNA in the dog did not show a single nucleotide G-to-C transversion at position 107, which leads to a replacement of cysteine by serine at residue 36 in CLAD dogs. Rather, a single nucleotide transversion at position 279, codon 93 was observed which did not lead to a change in amino acid (Figure 1). From these findings, our dog seemed to be a variant of CLAD. In our canine cases, the dogs showed recurrent bacterial infection such as oculonasal mucopurulent discharge, severe bilateral corneal opacity and upper respiratory bacterial inflammation (Figure 2) [57.58].

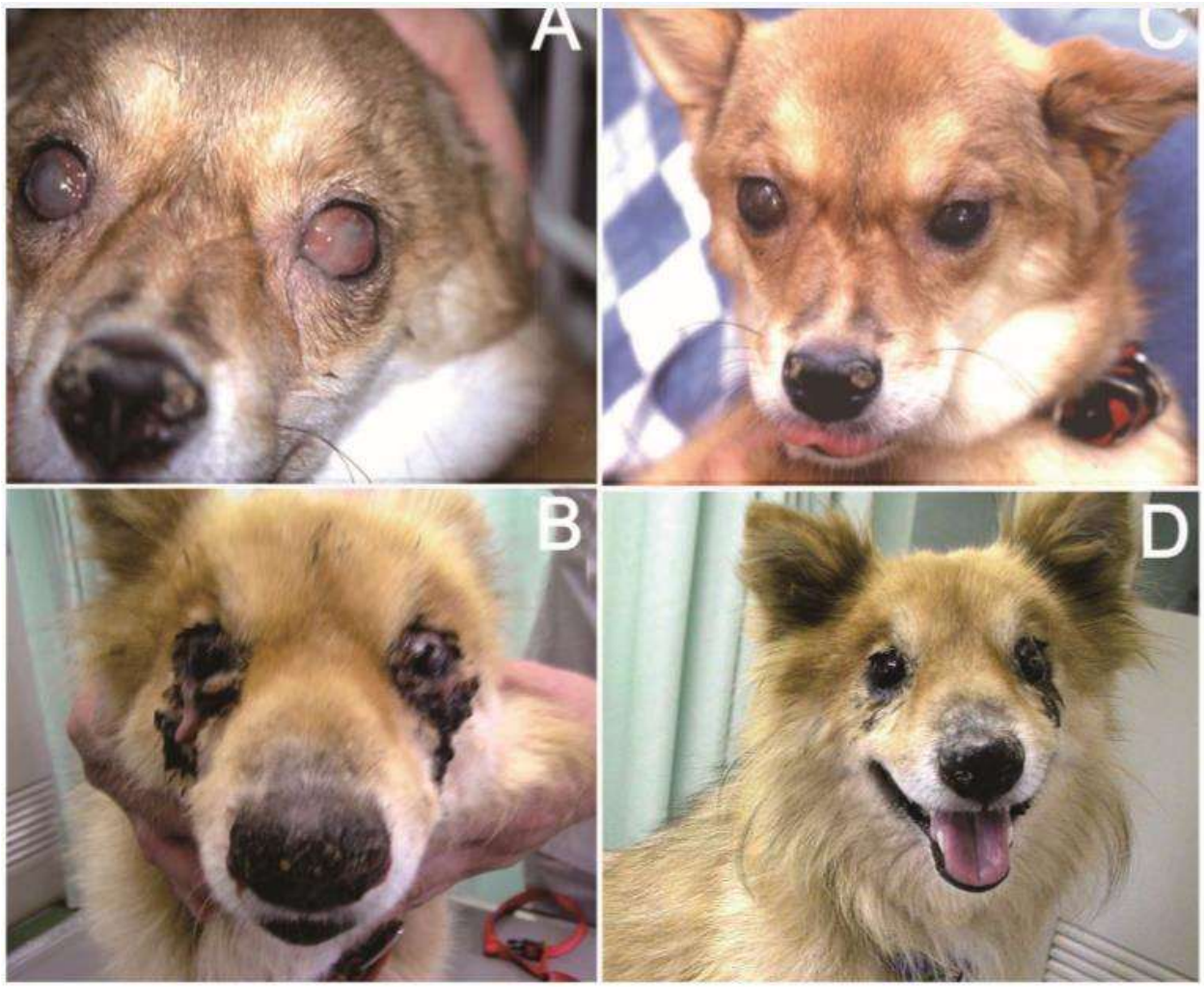


Figure2. Clinical features of dogs with familial neutrophil dysfunction at the first medical examination (A:case#1, B:case#2). Dogs showed the clinical signs of frequent and progressive muco- purulent oculo-nasal inflammation with bacterial overgrowth. The clinical improvement was observed after the beginning of oral lactoferrin- administration (C and D)[58].

Our canine cases showed a severe deficiency of neutrophil functions including adhesion ability and phagocytosis (Figure 3) [56]

How can we explain the findings of our canine cases? The formation of a CD11/CD18 membrane domains requires the participation of specific granules in neutrophils during immune recognition, and these domains are formed by the fusion of lysosome containing CD11b/CD18-bearing specific granules at local site of adhesion. In neutrophil-specific granule deficiency, defect of granular CD11b/CD18 leads to a lack of cluster-formation on the CD11b/CD18-modified cell surface [59,60]. In addition, neutrophils treated with a granule-release stimulator showed increased of CD11b/CD18 clusters on the plasma membrane [60]. Therefore, it is possible that one of reasons which cause the decrease of CD18 in the level of protein without mutations of β2-integrin gene is the disorder of posttranscriptional regulation of specific granules [56]. Recent studies revealed that the functions of integrins are related to cellular activation through inside-out and outside–in signaling, and that integrins clearly depend on a connection to the actin cytoskeleton to perform their functions [61-63]. Integrins do not directly bind to the actin cytoskeleton, rather, this is performed through several F-actin associated proteins such as talin, vinculin and α-actinin [62]. WASP (Wisbott-Aldrich syndrome protein) is another interacting protein related to actin polymerization, and its deficiency leads to WAS (Wisbott-Aldrich syndrome) and a deficiency in neutrophil adhesion and migration. The lack of WASP also causes a deficiency in β2 integrin clustering [64,65]. Furthermore, it was reported that the secretory vesicles in neutrophils carry membrane associated proteins such as β2 integrin component CD11b to the plasma membrane to prime neutrophils for migration [66]. The insufficiency of secretory vesicle function might also affect β2 integrin expression on the neutrophil surface. These findings suggest it is necessary to investigate the proteins associated with the cytoskeleton and secretory vesicle function to help explain the lack of integrin in our mixed-bleed canine cases.

Another type of neutrophil adhesion deficiency was reported as CLADⅢ in German Shepherd dogs and a German Shepherd-mix dog. These dogs demonstrated bacterial infection, mucosal hemorrhages and poor wound healing [67.68]. In these cases, a genetic PCR assay for LADⅢ revealed a homozygous mutation in the gene encoding Kindlin-3 [67]. In both humans and in veterinary medicine, LAD and CLAD are very rare disorders, especially for LAD (CLAD) II and III. CLAD II has not been reported yet.

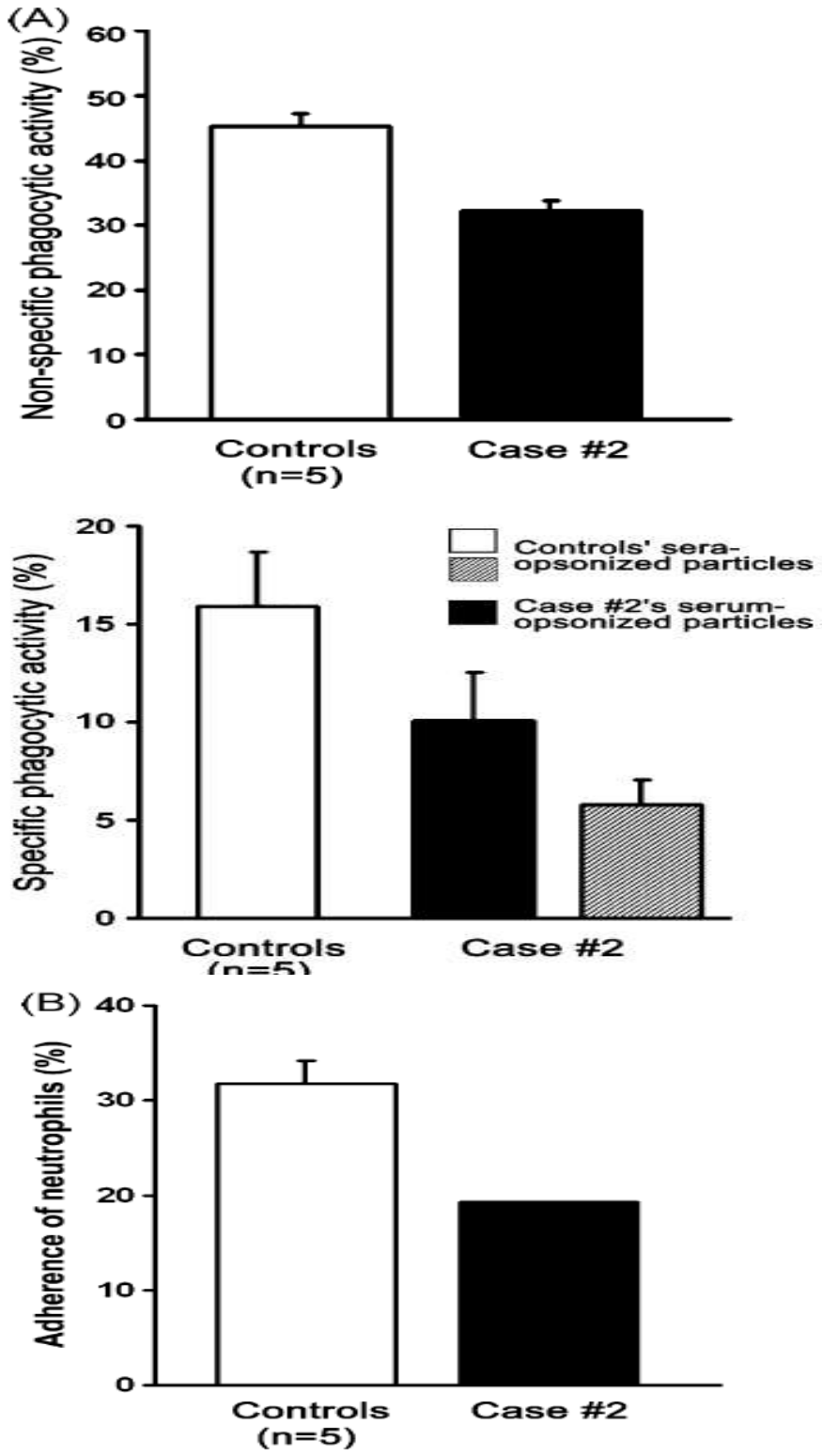


Figure3. Neutrophil phagocytic activity and adherence in case#2. (A) Non-specific or specific phagocytic activity. (B) Neutrophil adherence activity [56].

Lactoferrin and CLAD

As described above, bovine lactoferrin has multiple effects on various types of effector cells both *in vitro* and *in vivo*. In our CLAD case, the oral administration of bovine lactoferrin upregulated of β2 integrin gene expression and normalized β2 integrin-related neutrophil functions, leading to the inhibition of chronic bacterial infection and chronic inflammation (Figure 2) [57,58].

Our CLAD dogs with chronic bacterial infection and chronic inflammation were treated with antibiotics and interferon, and other symptomatic treatments during early hospitalization; however, these treatments failed to reduce the CLAD symptoms. The dogs suffered from recurrent bacterial infection and had chronic inflammation form puppyhood, including upper respiratory bacterial infection with oculo-nasal mucopurulent discharge, pneumonia and severe bilateral corneal opacity. Symptomatic treatments were continued, however, the clinical signs did not improved. Therefore, we administered bovine lactoferrin orally with antibiotics. After the oral administration of bovine lactoferrin, the oculo-nasal mucopurulent discharge and bilateral corneal opacity gradually reduced and coughing frequency caused by pneumonia was reduced. The oral administration of bovine lactoferrin simultaneously increased β2 integrin transcript level and improved the expression of CD18 on the surface of neutrophils (Figure4) [57]. Therefore, the oral administration of bovine lactoferrin modulated the expression of β2 integrin on the surface of neutrophils, and as a result of this modulating effects, a clinical effect was observed in CLAD dogs. Furthermore, interruption of bovine lactoferrin for 14 days (days 126 -140), because of the dog's owner led to a decrease in CD11b and β2 integrin mRNA expression, and worsened the clinical symporns. These results indicate that the oral administration of bovine lactoferrin has clinical effects on CLAD dogs. The mechanisms underlying the increase of β2 integrin mRNA expression by lactoferrin have not been fully defined. As reported by Iigo [38], heterologous lactoferrin modulates leukocyte function and, as a result, neutrophils are primed and release endogenous lactoferrin. It was reported that CD11b/CD18 blockade or Ca^{2+} chelators inhibited both lactoferrin release and superoxide production in human and mouse neutrophils [69,70]. LAD neutrophils were reported to secrete low levels of lactoferrin which reduced superoxide production in response to opsonized zymosan or fMLP [71,72]. These findings indicate that the degranulation of lactoferrin and subsequent superoxide production in response to opsonized zymosan require the sufficient expression of CD18 on neutrophils. Therefore, the expression level of CD18 on the surface of neutrophils is a major requirement for the degranulation of lactoferrin and subsequent superoxide production.Taken together, we speculate that heterologous lactoferrin may activate inside-out and outside–in signaling for β2 integrin and lead the release of endogenous lactoferrin that activates CD11b/CD18 cluster formation. Further studies are required to prove this hypothesis.

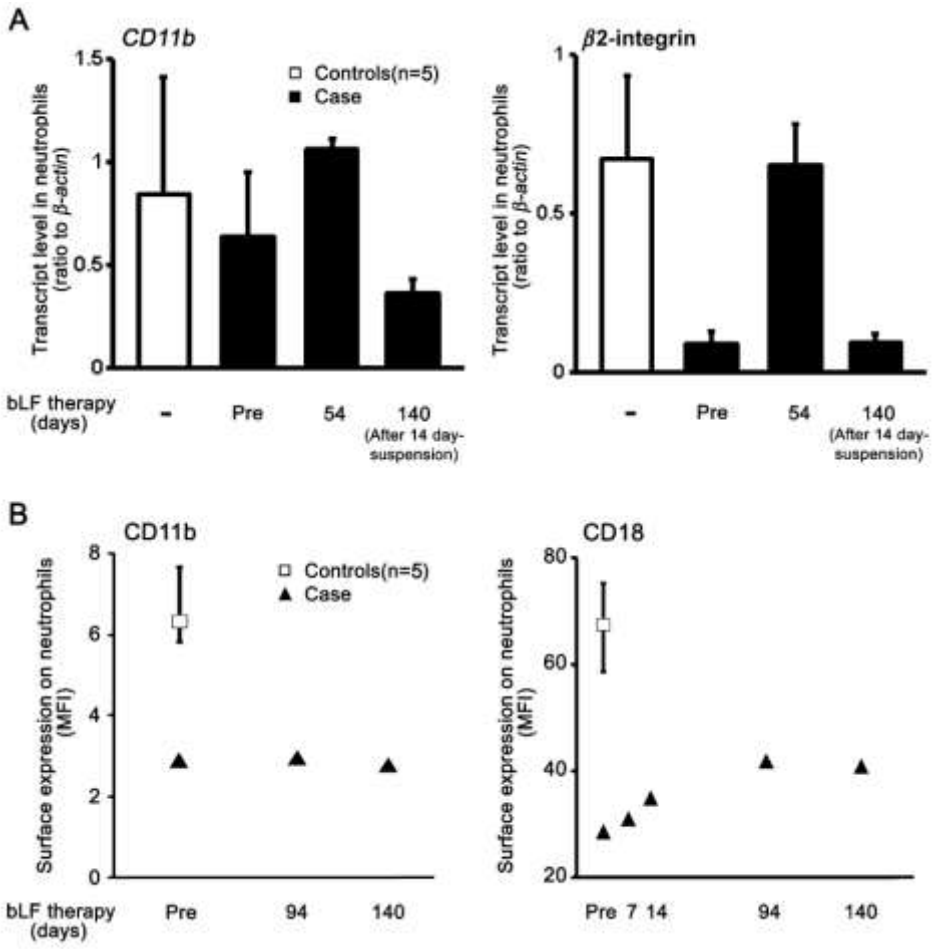


Figure4. Oral administration of bovine lactoferrin led the increaseing of β2 integrin transcript level and improved the express of β2 integrin on the surface of neutrophil. An interruption of bovine lactoferrin for 14 days (on 126 day -140 day), due to dog owner's reason, led the decrease in CD11b and β2 integrin mRNA expression [57].

In our cases of CLAD and FIV-infected cats, the long-term administration of bovine lactoferrin did not induce any type of adverse effects. Therapy for genetic neutrophil dysfunction is very difficult, although gene therapy or hematopoietic stem cell transplantation have been discussed as a definitive therapy. Patients with LAD or CLAD need both therapeutic and prophylactic treatments against the microbial infection, and are usually treated with the long-term administration of antibiotics leading to superinfection or unexpected resistant bacteria. From our previous studies, the oral administration of bovine lactoferrin for a long duration might reduce the dose of antibiotics

required and the administration period, as well as modulating neutrophil functions through enhanced β2 integrin expression. The oral administration of bovine lactoferrin might be suitable for patients with congenital neutrophil dysfunction who require antimicrobial-infection therapy from birth. The oral administration of bovine lactoferrin might be a therapeutic option for patients with congenital neutrophil dysfunction.

Clinical application of lactoferrin and overview

After the accumulation of numerous findings on the amelioratory effects of bovine lactoferrin *in vitro* and *in vivo*, the clinical trials of the oral administration of bovine lactoferrin have been performed globally. Before these clinical trials, the small-scale surveys to examine the clinical application of lactoferrin were performed. We demonstrated the anti-inflammatory effects of bovine lactoferrin on intractable stomatitis in FIV-infected cats [20]. After that, subsequent studies revealed that topical or tablet administration of bovine lactoferrin showed ameliorative results in humans. In addition, the clinical trials of lactoferrin in candidiasis, oral disease and cancer have been performed. In 1995, MacIntosh et al. [73] demonstrated the ingestion of bovine lactoferrin inhibited tumorigenesis. Based on this publication, the relationship between lactoferrin and tumorigenesis has been studied. Tsuda et al. demonstrated the inhibitory effects of ingested bovine lactoferrin on colon and other organ carcinogenesis and metastasis, and the results of human clinical trial showed the inhibitory effects of ingested bovine lactoferrin in patients with adenomatous colon [38,74]. Many studies of lactoferrin are continuing for its potential clinical use.

In this review, we propose the use of the oral administration of bovine lactoferrin as another clinical treatment for congenital neutrophil dysfunction. Lactoferrin might be a therapeutic target for the treatment of congenital neutrophil dysfunction based on its modulating effects on neutrophil functions.

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