Diabetic Lactoferrin Deficient Mice Demonstrates Greater Susceptibility to Experimental Periodontal Disease

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Abstract

The objective of this study is to determine whether alloxan-induced diabetic Lactoferrin knockout (LFKO$^{-/-}$) mice are more susceptible to periodontal disease caused by *Aggregatibacter actinomycetemcomitans* compared to the diabetic wild-type (WT) mice. Diabetes was induced in mice by a single dose of alloxan (60 mg/kg) injected intravenously. Mice were categorized as diabetic when blood glucose levels >250 mg/dL were measured on the 7th day after the injection. Periodontal disease was experimentally induced by *A. actinomycetemcomitans* infection in alloxan induced diabetic WT and LFKO$^{-/-}$ mice. Fasting blood glucose levels and body weight were monitored throughout the study. At the end of the 12th week of infection, mice were sacrificed and bone loss among the groups was estimated by measuring the distance between cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) at 12 sites on the molars. *A. actinomycetemcomitans* infected mice groups developed more alveolar bone loss than sham-infected animals. Diabetic LFKO$^{-/-}$ infected mice exhibited significant bone loss (P<0.01) and a higher mean fasting blood glucose level (P<0.05) when compared to diabetic WT infected mice. No statistically significant difference in fasting blood glucose level was found between the infected and sham-infected groups. Peripheral blood analysis at the end of the 12th week revealed a significant reduction in the platelet counts in LFKO$^{-/-}$ mice when compared to WT mice. Furthermore, diabetic LFKO$^{-/-}$ presented with lower counts than non-diabetic LFKO$^{-/-}$ mice (P<0.01). In conclusion, diabetic lactoferrin deficient mice are at a higher risk of developing periodontal infection induced by *A. actinomycetemcomitans* when compared to diabetic WTI mice.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease associated with a wide range of oral complications such as periodontitis, candidiasis, caries, tooth loss, gingivitis, lichen planus, neurosensory disorders (burning mouth syndrome), xerostomia and impaired taste [1]. The relationship between DM and periodontal disease is often described as a "bidirectional relation"; there is a tremendous body of literature that has studied this interrelation in depth. The results of four meta-analysis studies demonstrate that diabetic subjects are more prone to periodontal disease compared to non-diabetic subjects by a 2:1 ratio [2]. Several studies in children with type-1 DM show that periodontal disease is more severe than in control subjects [3-6]. Increased gingivitis and periodontitis were shown in patients with poor metabolic control compared to well-controlled patients [7,8]. Evidence points to a link between periodontal disease and systemic health. Periodontal infection represents a gateway for oral microorganism-secreted endotoxins and host cytokines to enter the systemic circulation, causing systemic inflammation [9]. These mediators can exacerbate systemic diseases including cardiovascular disease [10], pulmonary disease [11], nephropathy [12], rheumatoid arthritis [13] and DM [14]. *A. actinomycetemcomitans* is a Gram-negative facultative anaerobe linked to localized aggressive periodontitis (LAP). LAP is a form of periodontal disease that causes severe periodontal ligament and alveolar bone loss around the first molars and central incisors [15]. LAP is more prevalent among adolescents of African-American and Hispanic ethnicity [16,17]. The detection of *A. actinomycetemcomitans* was higher in patients with diabetes and periodontitis compared to systemically healthy patients without periodontitis [18,19]. A recent study has also indicated that patients with nephropathy have complications of diabetes and a greater number of *A. actinomycetemcomitans* in their plaque when compared to non-diabetic nephropathy patients [20]. Hyvärinen et al. reported that patients with metabolic syndrome (MetS) have exhibited a higher level of *A. actinomycetemcomitans* serum antibodies and a greater number of missing teeth [21]. Researchers point to possible adverse effects of periodontal disease on glucose tolerance [22] and suggest that treatment of periodontal disease in diabetic subjects is essential for better glycemic control [23]. Periodontal therapy has been observed to result in lower levels of plasma glycated hemoglobin (HbA1c) in diabetic subjects [24].

Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein that possesses antibacterial, antiviral, antifungal, anti-parasitic and immunomodulatory functions. LF exhibits both bacteriostatic and bactericidal activities against a wide range of Gram-negative and Gram-positive bacteria [25]. LF is also suggested to play a role in promoting the general health of subjects with systemic conditions such as DM [26]. Reports indicate that LF is involved in the metabolism of both glucose and lipids. In addition, it increases the cell's sensitivity to insulin [27]. Any decline in LF in cases of obesity could lead to self-perpetuating insulin resistance [28]. Moreno-Navarrete et al. also found abnormally low serum levels of LF associated with DM-2, which they suggest could lead to impaired neutrophil function [29]. Dodds et al. found a substantial increase in the LF concentration in diabetic subject's saliva, despite a decrease in the whole salivary flow [30]. Although LF levels are observed to increase in the diabetic state, most of it is thought to be inactive. According to Li et al. the bactericidal ability of LF is diminished in cases of DM due to LF binding to sugar molecules [31]. The role of LF is supported by a clinical study

where Talactoferrin has shown favorable results in treating diabetic neuropathic foot ulcer with minimal side effects [32].

It has also been demonstrated that LF knockdown in human adipocytes led to a significant decrease in adipogenic, lipogenic and insulin signaling-related gene expression and a significant increase in the gene expression of inflammatory mediators [28]. Our studies demonstrated that Lactoferrin knockout (LFKO$^{-/-}$) mice are more susceptible to *A. actinomycetemcomitans*-induced periodontal disease and bacteremia [33,34], *S. mutans*-induced bacteremia [35], and *C. albicans* induced oral candidiasis [36] when compared to WT or LFKO$^{-/-}$ mice administered human LF.

In our previous study, we observed that LFKO$^{-/-}$ mice infected with *A. actinomycetemcomitans* tended to exhibit more alveolar bone destruction and proinflammatory cytokine secretion when compared to wild type *A. actinomycetemcomitans* infected (WTI) mice [33]. Nonetheless, to the best of our knowledge, there is no study to date that has addressed the impact of LF absence in aggravating the inflammatory processes of periodontitis in diabetic mice. Based on our studies, as well as previous studies, we hypothesize that diabetic LFKO$^{-/-}$ mice are more susceptible to *A. actinomycetemcomitans*-induced periodontitis compared to diabetic WT mice. The results of this study provide a better understanding of the role of LF in modulating the impact of hyperglycemia and controlling progression of periodontal disease in diabetics.

Materials and Methods

Bacterial strains and preparation of inocula

Spontaneous Rifampicin (Rif) resistant variants of a clinical *A. actinomycetemcomitans* isolate, CU1000 nalidixic acid (N) resistant strain were grown in 100 ml of *A. actinomycetemcomitans* growth medium (AAGM) containing 70 μg/ml Rif in tissue culture flasks for 2 days in a 37 ºC incubator containing 10% CO_2/90% air atmosphere. For the adherent clinical isolate, CU1000N Rif, culture flasks were washed three times with phosphate-buffered saline (PBS) and adherent cells were scraped into PBS. The bacterial cells were then further concentrated by centrifugation at 1,000xg, and the total volume was reduced to 1/10 of the original volume [37].

In vivo experimental design and periodontal infection

The experimental groups comprised of 6-8 weeks old male wild type (C57BL/6) and LFKO$^{-/-}$ mice. Mouse colonies were bred and maintained in the transgenic animal facility of Rutgers School of Dental Medicine, Newark, New Jersey. To test our hypothesis, mice were divided into 8 experimental groups, each group comprising at least 6 mice that had free access to water and laboratory food. 1) wild-type control mice (WTC), 2) diabetic wild-type control mice (WTC+DM), 3) *A. actinomycetemcomitans*-infected wild-type mice (WTI), 4) *A. actinomycetemcomitans* infected wild-type diabetic mice (WTI+DM) 5) LFKO$^{-/-}$ control mice (LFKO$^{-/-}$C), 6) diabetic LFKO$^{-/-}$ control mice (LFKO$^{-/-}$+DM) 7) *A. actinomycetemcomitans* infected LFKO$^{-/-}$ mice (LFKO$^{-/-}$I) and 8) *A. actinomycetemcomitans* infected diabetic LFKO$^{-/-}$ mice (LFKO$^{-/-}$I+DM). The protocol was approved by institutional animal care and use committee (IACUC) of Rutgers Biomedical Health Sciences, Newark, New Jersey.

Induction of diabetes

Diabetes was induced in mice by injection of a single dose of 0.1 ml alloxan (ALX; 2, 4, 5, 6-tetraoxypyrimidine) (Sigma Co., St. Louis, USA; 60 mg/kg) intravenously. Other animals were injected with 0.1 ml PBS.

Determination of fasting glucose

The fasting blood glucose levels following an 8 h fast were determined in the blood of all the animals using TRUE track glucose meter (Nipro Diagnostics, Inc., Fort Lauderdale, FL, USA). Glucose levels were measured before the start of the experiment (before Alx or PBS injection), on days 1 and 7 after the injection, and at the end of the experiment. Mice were categorized as diabetic if their fasting blood glucose levels measured above 250 mg/dL on the 7th day after the injection.

Establishment of periodontal infection

The oral cavity of the mice was swabbed once with 0.12% chlorhexidine gluconate (Peridex 3M, ESPE Dental Products, St. Paul, MN) mouth rinse one day before the infection or sham infection. *A. actinomycetemcomitans* in suspension (1x10^8 cells) with 2% carboxymethylcellulose suspension in PBS was placed in the oral cavity of experimental groups using a micropipette. A second dose of bacteria (1x10^9 CFU) in 10 ml PBS was injected into palatal gingival tissue to facilitate the retention of the bacteria, as reported earlier [33]. This procedure was repeated after 48 h and 96 h. In control group mice, oral swabbing was performed with 2% of carboxymethylcellulose in PBS and PBS gingival injection. At the end of 12 weeks, animals were euthanized by CO_2, and heads, blood and organs were collected.

Detection of *A. actinomycetemcomitans* DNA

To detect whether *A. actinomycetemcomitans* was present in the oral cavity, oral cavities of anaesthetized mice was swabbed after two weeks of infection. Genomic DNA was extracted directly from the collected oral samples with a DNeasy Blood & Tissue Kit as described by the manufacturer (Qiagen, Valencia, CA, and USA). The presence of *A. actinomycetemcomitans* DNA was analyzed by PCR using leukotoxin (LtxA) primers (Forward 5-ACCTGTCGCAGGGTTAATTG-3; Reverse 5-CGAGCTGATTCGCGATATGT-3). A negative control without DNA and a positive control with *A. actinomycetemcomitans* DNA were always included. The PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide, and photographed [33].

Determining alveolar bone loss

To determine alveolar bone loss, the maxillae were hemisected, mechanically defleshed and exposed overnight to 3% hydrogen peroxide. They were then treated for 5 min with 1% sodium hypochlorite and then stained with methylene blue dye (Fisher Scientific Company, Fair Lawn, New Jersey) in order to delineate the cemento-enamel junction. The jaws were mounted in utility wax, and the lingual surfaces of the molars were photographed with 10X magnification using an Olympus (SZ61) dissecting microscope (Olympus, Center Valley, PA). The images were digitalized [33] and printed on A4 paper, and bone loss around the three molars was measured at 12 sites by two different examiners. Results were

presented in millimeters, and the significance in bone loss was calculated by comparing bone loss means of the control group with those of the infected diabetic and non-diabetic group of WT and LFKO$^{-/-}$ mice using one-way analysis of variance (ANOVA) [38].

Complete blood count (CBC)

Blood was obtained by retro-orbital phlebotomy under anesthesia, heparinized and complete blood count was determined using an automated H1 Technicon system (Antech Diagnostics, New Hyde park, NY, USA).

Statistics

Statistical analysis was performed for the experiment using one-way ANOVA test to compare intergroup differences. When appropriate, post hoc analysis of significant differences revealed by ANOVA was also performed using an all-pair wise Tukey's HST test with the JMP software SAS 9.1 (SAS Institute, Cary, NC, USA). P values of less than 0.05 were considered statistically significant. Continuous variables were compared by pair wise t test for two independent samples.

Results

Induction of diabetes and blood glucose levels in WT and LFKO$^{-/-}$ mice

Diabetes was induced by a single intravenous injection of alloxan in both WT and LFKO$^{-/-}$ mice. The mice were considered diabetic when their blood glucose level was 250 mg/dL or above. We found that LFKO$^{-/-}$+DM mean blood glucose was significantly higher than WT+DM (P<0.05). No significant difference was found in the fasting blood glucose and food intake levels between the infected and sham-infected in all the experimental groups. We have used the one-way ANOVA to determine the weight gain or weight loss among the groups and within the same group at different time points (0, 3, 6, 9 and 12 weeks). During the experimental period a progressive increase in body weight was observed in all groups of mice. There was no significant difference in the weight gain or loss observed between any of the experimental groups.

Alveolar bone loss due to alloxan-induced diabetes and/or A. actinomycetemcomitans infection

Colonization of oral cavities infected by *A. actinomycetemcomitans* was determined by PCR using LtxA specific primer. In contrast, none of the sham-infected animals were found to harbor *A. actinomycetemcomitans* (Figure 1). Levels of bone loss (distance from the CEJ to the alveolar bone crest) determined 12 weeks after *A. actinomycetemcomitans* or sham infection was significantly less in WTC (0.6 mm±0.35) than in the other experimental groups. Compared to the bone loss levels in these healthy controls, mice that were infected with *A. actinomycetemcomitans*, DM or both tended to have higher amounts of bone loss. These differences were statistically significant except when WTC and LFKO$^{-/-}$ C mice are compared. Generally, hyperglycemic mice exhibited significantly higher bone loss when compared to those with normal glucose levels. Animal who are LFKO$^{-/-}$ tend to have more bone loss than corresponding WT mice. LFKO$^{-/-}$I had significantly greater bone loss (P<0.01) compared to LFKO$^{-/-}$C mice. Diabetic LFKO$^{-/-}$I mice had significantly (P<0.01) higher amounts of bone loss (2.3 mm ±0.63) than animals in any
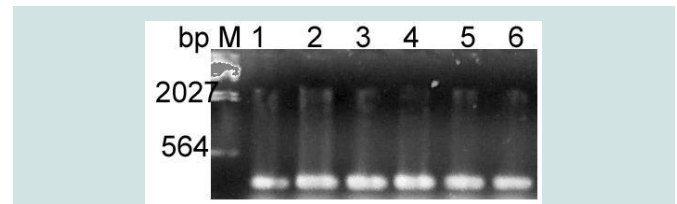
Figure 1: Representative gel picture of detection of *A. actinomycetemcomitans* DNA from the oral cavity of infected diabetic and non-diabetic mice after 12 weeks of post infection. *A. actinomycetemcomitans* DNA was detected by PCR using *A. actinomycetemcomitans LtxA*-specific primers. Genomic DNA extracted from *A. actinomycetemcomitans* was also subjected to PCR as a positive control. 1) Positive control and 2-6) *A. actinomycetemcomitans* DNA from infected mice.

other experimental group. When we compared the bone loss between WTI and WTC+DM, both groups demonstrated similar amounts of bone loss. The same trend was also observed between LFKO$^{-/-}$C+DM and LFKO$^{-/-}$I mice group (Table 1).

Peripheral blood counts

Platelet counts were measured 12 weeks following *A. actinomycetemcomitans* infection or sham infection. Compared to sham-infected non-diabetic mice, infection and diabetes caused a significant increase in platelet counts with the highest counts being in WTI+DM mice. LFKO$^{-/-}$ mice had significantly lower platelet counts than corresponding WT animals with LFKO$^{-/-}$I+DM animals having the lower platelet counts (K/CMM), a value that is significantly lower than counts measured in other groups. Compared to un-infected non-diabetic animals, mice infected with *A. actinomycetemcomitans* and diabetic mice were observed to have increased blood neutrophil counts. All groups of LFKO$^{-/-}$ mice had significantly lower neutrophil counts when compared to WT group. In addition, LFKO$^{-/-}$I+DM mice neutrophil counts (8.5±0.71) were non-significantly lower than other LFKO$^{-/-}$ mice group (Table 2).

Discussion

Table 1: *A. actinomycetemcomitans*-induced alveolar bone loss in WT and LFKO$^{-/-}$ diabetic and non-diabetic mice.

Mice groups	Mean total bone loss/group (mm)
WTC	0.6±0.35
WTI	1.30±0.59*
WTC+DM	1.27±0.53
WTI+DM	1.77±0.50
LFKO$^{-/-}$C	0.93±0.41
LFKO$^{-/-}$I	1.85±0.70$
LFKO$^{-/-}$C+DM	1.69±0.63@
LFKO$^{-/-}$I+DM	2.30±0.63#

Alveolar bone loss was measured at 12 sites on the lingual surface as described in the materials and methods. Asterisks indicate statistical significance between the groups as calculated by one-way ANOVA with post-hoc testing. *P<0.05 significance between WTC vs WTI. #P<0.05 significant difference between WTI+DM vs. LFKO$^{-/-}$I+DM groups, @P<0.05 significant difference between WTC+DM vs. LFKO$^{-/-}$C+DM groups, $P<0.05 significant difference between LFKO$^{-/-}$C vs. LFKO$^{-/-}$I groups.

Table 2: Peripheral blood counts during experimental periodontitis in WT and LFKO$^{-/-}$ diabetic and non-diabetic mice.

Mice groups	Mean Neutrophils	Mean Platelet counts
	Counts (%)	(K/CMM)
WTC	38±1.41	815±21.2
WTI	48±8.49*	921±41.7
WTC+DM	41±1.41	977±10.6*
WTI+DM	43.5±3.54	1379±35.4*
LFKO$^{-/-}$C	19.5±6.43	610±29.9#
LFKO$^{-/-}$I	10.5±0.71#	721±43.1$
LFKO$^{-/-}$C+DM	10±2.81#	310±19.1**
LFKO$^{-/-}$I+DM	8.5±0.71#	394±5.66**

Peripheral leucocytes count during experimental periodontitis. Blood samples were collected after 12 weeks of post-infection with or without diabetics and analyzed for platelet and neutrophil counts. Significance in the levels were analyzed in duplicate experiments using ANOVA and post hoc Tukey' HST test between the groups. The data shown are means ±SEM. Asterisks indicate statistical significance (P<0.05) differences between the groups as calculated by one-way ANOVA. **Platelet counts;** *P<0.05 significant difference between WTC vs. WTC+DM; WTI+DM groups, #P<0.05 significant difference between WTC vs. LFKO$^{-/-}$C groups, $P<0.05 significant difference between WTI vs. LFKO$^{-/-}$I groups, **P<0.05 significant difference between LFKO$^{-/-}$C vs. LFKO$^{-/-}$C+DM; LFKO$^{-/-}$I+DM. **Neutrophil counts;** *P<0.05 significant difference between WTC vs. WTI group, #P<0.05 significant difference between LFKO$^{-/-}$C vs. LFKO$^{-/-}$I; LFKO$^{-/-}$C+DM; LFKO$^{-/-}$I+DM.

Lactoferrin is a part of the innate immune system and possess antibacterial, antiviral and antifungal properties [39]. Knocking out the LF gene, which is responsible for LF protein secretion in LFKO$^{-/-}$ mice, will render their immunity impaired, leaving the host susceptible to invasion by different pathogens. Our current results concur with previous studies that found LFKO$^{-/-}$ mice to be more predisposed to oral bacterial infections compared to WT mice [33].

Our study also demonstrated that diabetic mice are more prone to *A. actinomycetemcomitans*-induced periodontal disease than non-diabetic animals. *A. actinomycetemcomitans* infected diabetic LFKO$^{-/-}$ mice experienced greater bone loss than animals that were either diabetic WT or non-diabetic LFKO$^{-/-}$ mice. Several studies have investigated experimental periodontitis in diabetic animal models and corroborate our findings. Pontes Anderson et al. results are consistent with our results, which showed that Goto-Kakizaki (GK; type-2 DM) rats with ligature-induced periodontitis have greater alveolar bone loss than the non-diabetic rats. Furthermore, when bone levels were examined in groups without induced periodontal disease, diabetic rats demonstrated greater amounts of bone loss than non-diabetic animals [40]. Similarly, Liu et al. reported that Zucker diabetic fatty (ZDF) rats presented with more severe bone resorption when infected with *P. gingivalis*-soaked ligatures around their molars in comparison to controls [22].

A. actinomycetemcomitans oral inoculation stimulates T-cell proliferation, with a high percent of RANKL-expressing, CD4+ T-cells in non-obese diabetic mice (NOD). This explains the greater alveolar bone loss in diabetic NOD mice compared with pre-diabetic and non-diabetic NOD mice [41]. It has been reported that *A. actinomycetemcomitans* infection accelerates alveolar bone loss in diabetic GK rats by increased rates of cell apoptosis in gingival

epithelial and connective tissues [42,43].

After administrating alloxan to WT and LFKO$^{-/-}$ mice the blood glucose level of diabetic groups were measured at different time points. LFKO$^{-/-}$ mice had higher blood glucose levels than their WT counterparts throughout the study, indicating that LF plays a role in regulating blood glucose, reducing hyperglycemia. It has been reported that the LF molecule's C-lobe interacts with different sugar molecules, subsequently lowering blood glucose [44]. Moreno-Navarrete et al. have also reported that LF up-regulates insulin signaling *in vitro* by increasing 473SerAkt phosphorylation in HepG2 and 3T3-L1 cell lines. In addition, they found that LF increases insulin sensitivity *in vivo* [29]. Moreno-Navarrete et al. investigated the effect of LF gene knockdown on human adipocyte and found that it led to reduced expression of adipogenic and insulin-related genes (GLUT4 and IRS1), while the expression of inflammatory genes (IL-6, TNF-α and IL-8) were found to be elevated significantly [28].

We found that neutrophil levels are lower in diabetic LFKO$^{-/-}$ mice compared to their WT counterparts. It has been reported that LF secretion from neutrophils is decreased in subjects with diabetes [29]. Other studies have also pointed to a correlation between low "LF level" and neutropenia [45]. Studies in humans and animals have illustrated defective neutrophil chemotactic, phagocytic and microbicidal actions in diabetics. In addition to impaired neutrophil adhesion to the endothelium and migration to inflamed sites, diabetes affects the production of reactive oxygen species and is associated with a decline in cytokines release and prostaglandin production by neutrophils. In diabetics there is a higher rate of leukocyte apoptosis and decreased lymph node retention capacity [46].

Neutrophils are more sensitive than monocytes to diabetic conditions. Furthermore, it has also been reported that monocyte counts in the blood of type-1 diabetic patients is lower than non-diabetics as side effect of ketosis [47]. Diabetic WT groups presented
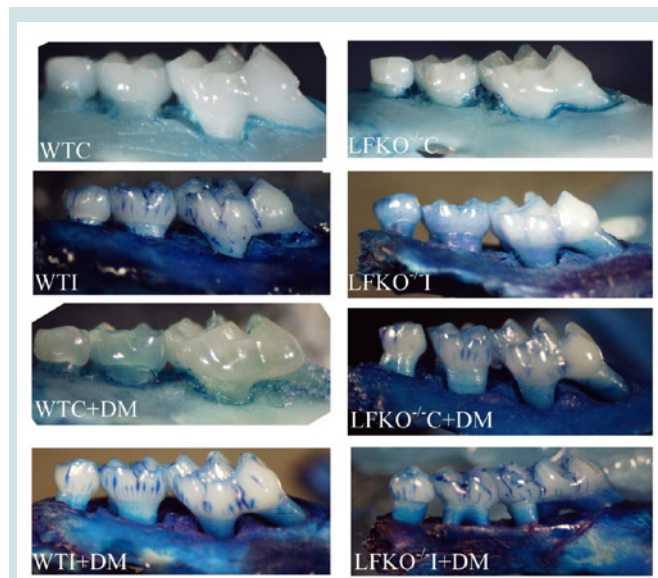
Figure 2: *A. actinomycetemcomitans*-induced alveolar bone loss was evaluated with or without diabetes in WT and LFKO$^{-/-}$ mice 12 weeks after infection or sham infection. Digital images at 10X magnification of defleshed and Methylene blue stained maxillary jaws from all the mice groups.

with higher platelet counts than non-diabetic groups' while the LFKO$^{-/-}$ diabetic groups' platelet counts were lower than those of controls in non-diabetic LFKO$^{-/-}$ mice. These points to possible correlations between LF level and platelet counts. One report has pointed to an elevated LF level in ulcerative colitis and Crohn's disease, and its association with elevated platelet counts [48]. There are contrasting reports regarding platelet counts in diabetic patients. Several reports have found higher platelet counts in diabetic subjects compared to their matched controls [49,50]. Whereas, Hekimsoy et al. have measured the mean platelet volume (MPV) and mean platelet counts in diabetic subjects found that MPV was higher in significant manner, while the mean platelet counts was lower in diabetics compared to non-diabetic healthy subjects [51]. At this point, we don't have any explanation as to why platelet counts are lower in LFKO$^{-/-}$ diabetic mice when compared to WT diabetic mice. However, there are reports, which show that oral administration of LF increased the platelet counts [52]. Furthermore, exogenous LF add-back to LFKO$^{-/-}$ mice studies are needed to elucidate the role of LF on platelet counts in diabetes.

Conclusion

In summary we can conclude from our results that diabetic lactoferrin deficient mice are more susceptible to *A. actinomycetemcomitans*-induced periodontitis compared to diabetic WT mice. LF treatment could be utilized in future studies to explore LF' role in reverting diabetes symptoms and diabetes complications as in periodontitis.

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