Author Manuscript Author Manuscript Author Manuscript Author Manuscript

HHS Public Access

Author manuscript

Mol Oral Microbiol. Author manuscript; available in PMC 2018 June 08.

Published in final edited form as:

Mol Oral Microbiol. 2017 December ; 32(6): 490–499. doi:10.1111/omi.12187.

The in vivo T helper type 17 and regulatory T cell immune responses to *Aggregatibacter actinomycetemcomitans*

S. Mahabady[1], **N. Tjokro**[2], **S. Aharonian**[1], **H.H. Zadeh**[1], **C. Chen**[2], **H. Allayee**[3], and **P.P. Sedghizadeh**[2]

[1]Laboratory for Immunoregulation & Tissue Engineering, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, USA

[2]Division of Periodontology, Diagnostic Sciences and Biomedical Sciences, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, USA

[3]Institute for Genetic Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Summary

The periodontal pathogen *Aggregatibacter actinomycetemcomitans* is known to elicit a systemic immune response in the infected host, and occasionally causes non-oral infections. Detailed information on its immunopathological responses and the involvement of bacterial virulence factors remains to be elucidated. The aim of this study was to assess the systemic immune response to *A. actinomycetemcomitans* oral infection. We used an animal model that simulates systemic dissemination of the bacteria by injecting live wild-type (WT) D7S-1 and a double knockout mutant of leukotoxin and cytolethal distending toxin (*ltx cdt*) *A. actinomycetemcomitans* strains in rat oral mucosa. Draining lymph nodes were examined for regulatory T (Treg) and T helper type 17 (Th17) cell subsets and their associated mediators. An increase in the proportion of Th17 cells and a decrease in Treg cells over the experimental period of 3 weeks were similarly observed for rats challenged with WT and *ltx cdt*. Significant up-regulation and downregulation of proinflammatory cytokines in the Th17 gene pathway was noted, as well as several qualitative differences between WT and *ltx cdt*. Furthermore, we observed differential fold regulation in key genes associated with a proinflammatory response in *ltx cdt*-inoculated rats relative to D7S-1 group. This suggests that although the knockout of these two virulence factors (*ltx cdt*) may suppress certain proinflammatory genes, it causes similar over-expression of other genes compared with D7S-1, indicating a common factor that still remains in the pathogenicity of *A. actinomycetemcomitans*.

Keywords

biofilm; immunity; pathogenesis; periodontal disease; T lymphocytes

---

Correspondence: Parish P. Sedghizadeh, Division of Periodontology, Diagnostic Sciences and Biomedical Sciences, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, USA. sedghiza@usc.edu.

The authors disclose that they have no conflicts of interest related to this work.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

1 | INTRODUCTION

According to the Centers for Disease Control, the prevalence of oral infection in the form of chronic periodontitis is 47% of the US population over the age of 30.[1] Bacteria are believed to be involved in the initiation of the host response, which is ultimately responsible for periodontal tissue destruction.[2,3] Consequently, increased levels of systemic inflammatory markers have been detected in patients with periodontitis.[4] Host inflammatory responses to this oral infection not only affect the periodontal tissues and supporting bone, but can also be associated with other chronic systemic diseases such as cardiovascular disease, diabetes, and more recently pancreatic cancer.[5–9]

Aggregatibacter actinomycetemcomitans is a key pathogen involved in oral infections such as periodontitis and peri-implantitis,[10–12] and the only natural niche for *A. actinomycetemcomitans* is the oral cavity. The presence of *A. actinomycetemcomitans* at other anatomic sites has been reported in disease states such as atherosclerotic plaques, the brains of patients with Alzheimer's disease, and in infective endocarditis, osteomyelitis, or acute pancreatitis.[5–9] With regards to infective endocarditis, *A. actinomycetemcomitans* is part of the HACEK group of Gram-negative bacteria, and extreme cases can cause the infection to be fatal.[13] Moreover, recent prospective large cohort studies have shown significantly increased risk for highly lethal pancreatic cancers in patients with oral salivary carriage of *A. actinomycetemcomitans*.[9] Host immune responses to *A. actinomycetemcomitans* and immunomodulatory effects by *A. actinomycetemcomitans* infection are thought to play a key role in the increased risk for pancreatic cancer as well as other inflammatory conditions associated with *A. actinomycetemcomitans*. *Aggregatibacter actinomycetemcomitans* oral infection, in association with periodontal disease, can also lead to bone loss due to the inflammatory process. Recently, it has been shown that *A. actinomycetemcomitans* infection activates stress pathways in macrophages and upregulates chemokine signaling during inflammation and osteoclastogenesis.[14] Despite recent evidence regarding *A. actinomycetemcomitans*-induced bone loss and proinflammatory mechanisms, there is still limited information on *A. actinomycetemcomitans* invasion and its systemic pathogenesis.

As a result of the known invasive characteristic of *A. actinomycetemcomitans*, we were interested to understand the immunological question of the systemic host response to *A. actinomycetemcomitans* infection. Although *A. actinomycetemcomitans* systemic invasion has been documented in the literature, as described above, there is currently a paucity of information on the role of gene pathways and cytokines involved in the inflammatory process. For this study, we decided to use an injection model of *A. actinomycetemcomitans* bacterial suspension. This method allows us to directly cause local dissemination of the oral pathogen, and thereby analyze its systemic responses and invasion.

The immunological T helper type 17 (Th17) gene pathway is an important pathway in the inflammatory process of many oral infections. This cellular network is derived from the Th1 cytokine network involving cellular immunity (macrophages, CD8, natural killer) and Th2 network involving humoral immunity and allergy (interleukin-4 [IL-4], IL-5, IL-13).[15] The Th17 pathway is based on secretion of the proinflammatory cytokine IL-17, as well as

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

IL-21, IL-22, IL-23, IL-26, and tumor necrosis factor-α; all leading to inflammation and autoimmunity.[15] Regulatory T (Treg) cells, derived from CD4 cells, are involved with immune suppression and counteract these effector Th17 cytokines. Hence, knowledge of the balancing act between Treg and Th17 effector cells is key in understanding the development and pathogenesis of oral infections, including those associated with *A. actinomycetemcomitans*. With the increased interest in this key pathogen and the importance of understanding *A. actinomycetemcomitans* immunological responses in vivo, an animal model is a crucial first step in understanding systemic immune responses and mechanisms of progression.[16] The aim of this research was to evaluate the immunological responses, specifically T-cell regulatory pathways and genes, to oral *A. actinomycetemcomitans* infection in an animal model.

2 | METHODS

2.1 | Microbial culture of *A. actinomycetemcomitans* suspension

Aggregatibacter actinomycetemcomitans wild-type strain D7S-1 serotype A (D7S-1) was originally recovered from a patient with aggressive periodontitis.[17,18] *Aggregatibacter actinomycetemcomitans* double knockout of leukotoxin and cytolethal distending toxin (*ltx* *cdt*) was constructed as previously described.[19,20] Ten colonies each of D7S-1 and *ltx* *cdt* were collected from agar plates with a sterilized loop, dispersed in modified trypticase soy broth (mTSBYE, 3% trypticase soy broth with 0.6% yeast extract) with appropriate antibiotics, and incubated overnight in 5% CO_2 at 37°C. Fresh medium was then added and the optical density at 495 nm (OD_{495}) of the bacterial culture was adjusted to 0.1. Total suspension volume (*A. actinomycetemcomitans* D7S-1, *ltx* *cdt*, or sham control) was 200 μL per injection with 10^9 live bacteria for treatment groups, and sterile media only (mTSBYE) for control.

2.2 | Ethics statement

All animal protocols and procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California (USC), and in accordance with the Panel on Euthanasia of the American Veterinary Medical Association. USC is registered with the United States Department of Agriculture, has a fully approved Letter of Assurance (#A3518-01) on file with the National Institutes of Health, and is accredited by the American Association for the Accreditation of Laboratory Animal Care. The title of our protocol is 'Novel clone-specific virulence determinants of *A. actinomycetemcomitans*' (IACUC Protocol #11838). To ensure quality, reliability, and reproducibility of our animal research, the ARRIVE guidelines[21] were used for accurate reporting of our results.

2.3 | Power analysis

The appropriate number of animals for this study was determined by power analysis using G POWER 3 software.[22] Power calculation indicated an appropriate sample size of six rats per group to detect statistically significant effects between groups. This sample size correlates to an effect size f of 0.40 (F-tests analysis of variance: repeated measures, within–between interaction). For this study, we had three experimental groups (*A. actinomycetemcomitans*

Author Manuscript

D7S-1, *ltx cdt*, and sham control) and two experimental end points (*t*=1 and *t*=3 weeks), and an overall total of 36 animals.

2.4 | Animals

Sprague-Dawley female, virgin rats weighing approximately 200 g each were used for this study. Two to three rats were housed per cage in a vivarium at 22.2°C under a 12-hour light and 12-hour dark cycle, and fed ad libitum with a soft diet (Clear H_2O DietGel 31M, Westbrook, ME, USA). Animals were under full-time supervision by vivarium staff and were evaluated daily by USC veterinarians.

2.5 | In vivo animal experiment

Animals were anesthetized using 4% isoflurane inhalant and given subcutaneous buprenorphine sustained release pain medication (1.2 mg kg^{-1}). Treatment suspensions were injected via 27-gauge syringes into the maxillary buccal vestibule within periosteal and adjacent connective tissues at *t*=0 and *t*=3 days. Animals were clinically evaluated daily during the study, and euthanized at the experimental end point of either *t*=1 week or *t*=3 weeks post-inoculation. Experimental end points were determined to evaluate differences between innate (immediate) immune responses at *t*=1 week compared with adaptive immune responses at *t*=3 weeks, and based on previous experience with our animal model. Animals were evaluated for local clinical inflammation at experimental end points, and draining lymph nodes (ipsilateral to the injection site) were collected for systemic immune response analysis upon euthanasia. Lymph nodes from each rat were divided into two sets, and either processed immediately by flow cytometry, or fixed and frozen for subsequent quantitative polymerase chain reaction (qPCR) analysis.

2.6 | T-cell analysis by flow cytometry

Draining lymph nodes were harvested and washed with RPMI_1640 medium with 10% fetal bovine serum (FBS) immediately upon euthanasia. Single cell suspensions were prepared by pressing the lymphoid tissues through a mesh strainer and centrifugation to remove the RPMI/FBS supernatant for each sample (n=36 samples). Cell surface immunofluorescent staining was performed using CD4-fluorescein isothiocyanate anti-rat antibody (BioLegend, San Diego, CA, USA), and intracellular staining by FOXP3-phycoerythrin anti-mouse/rat/human antibody (BioLegend) or IL-17A-phycoerythrin anti-mouse/rat antibody (eBioscience, San Diego, CA, USA). All staining was carried out on ice with minimal exposure to light. Samples were evaluated using a Facscalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with CELLQUEST software (BD Bioscience, San Jose, CA, USA). As shown in Figure 1, gates were set to read only lymphocyte cell populations, and compensation was adjusted accordingly. Treg cells were specified as $CD4^+$ $FOXP3^+$, and Th17 cells were specified as $CD4^+$ $IL17^+$ so as to compare differences in cell populations between experimental groups (*A. actinomycetemcomitans* D7S-1, *ltx cdt*) and sham control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

2.7 | Gene expression analysis by qPCR

Draining lymph nodes were stored in RNA*later* (Ambion, Thermo Fisher Scientific, Canoga Park, CA, USA) and frozen at −20°C until qPCR analysis. Tissues were thawed and appropriate amounts of each sample were determined (no more than 60 mg tissue). RNA extraction was done with RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA), and resulting sample concentrations and purity were verified using a nanospectrophotometer. Agarose gel electrophoresis was performed to check RNA integrity before proceeding with cDNA synthesis using RT^2 First Strand Kits (Qiagen). RT^2 Profiler Rat Th17 Response PCR arrays (Qiagen) were prepared using RT^2 SYBR Green Mastermix, and processed in a Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iQ5 OPTICAL SYSTEM SOFTWARE (Life Science Research, Hercules, CA, USA). A list of all 84 genes analyzed with the Th17 Response PCR arrays is presented in Table 1. Cycle threshold values were calculated, and melting curve analysis verified qPCR specificity. Data were uploaded online and analyzed using Qiagen's Data Analysis Center.

2.8 | Statistical analysis

Statistical calculations were performed with SPSS 22.0 (IBM, Armonk, NY, USA) and MICROSOFT EXCEL 2016. Quantitative data from experimental results for each group were first analyzed with descriptive statistics to understand the distribution of the data (parametric or non-parametric), and generate the mean, standard error, standard deviation, kurtosis and skewness, and 95% confidence levels. The data were then analyzed using Student's *t*-test or analysis of variance as applicable, and statistical significance was accepted at $P<.05$. Additionally, post-hoc testing using unpaired *t*-tests and Dunnett's test for multiple comparisons were performed for data validation.

3 | RESULTS

3.1 | Clinical findings of oral *A. actinomycetemcomitans* infection

Sham-inoculated rats had only minor and transient localized mucosal edema and erythema. The animals recovered well for the remainder of the experimental period, behaving and eating normally, and draining lymph nodes were unremarkable in size and color upon euthanasia. Rats inoculated with either *A. actinomycetemcomitans* D7S-1 or *ltx cdt* strains exhibited localized mucosal edema and erythema. In addition, the animals exhibited systemic signs such as malaise and ophthalmological discharge during the experimental period. During necropsy, significant lymphadenopathy was noted.

3.2 | T-cell analysis

Flow cytometry results (Figure 2) indicated that, compared with sham-inoculated animals, D7S-1 or *ltx cdt A. actinomycetemcomitans*-inoculated groups exhibited significantly lower proportions of $CD4^+$ $FOXP3^+$ cells at week 1 ($P=.004$), but not week 3. Conversely, higher proportions of $CD4^+$ $IL17^+$ cells were observed in D7S-1 ($P=.023$) or *ltx cdt* ($P=.011$) *A. actinomycetemcomitans*-inoculated groups at week 1. The proportion of $CD4^+$ $IL17^+$ cells remained higher in the *ltx cdt A. actinomycetemcomitans*-inoculated group, compared with the other two groups at week 3.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3.3 | qPCR gene expression analysis

Gene expression analysis by qPCR indicated statistically significant over-expression of IL-4 (*Il4*; *P*=.04, Student's *t*-test), and under-expression of Chemokine (C-C motif) ligand 1 (*Ccl1*; *P*=.01, Student's *t*-test) and IL-23 receptor (*Il23r*; *P*=.02, Student's *t*-test) in the *A. actinomycetemcomitans* D7S-1 inoculated group, compared with the sham-inoculated group at *t*=1 week (Figure 3). By week 3, IL-4 remained significantly over-expressed (*P*=.0004, Student's *t*-test) in the D7S-1-inoculated group. In rats inoculated with *A. actinomycetemcomitans ltx cdt*, colony-stimulating factor 3 was significantly over-expressed (*Csf3*; *P*=.04, Student's *t*-test) and *Ccl1* was under-expressed (*P*=.004, Student's *t*-test) compared with sham-inoculated rats at *t*=1 week (Figure 4). At week 3, rats inoculated with *A. actinomycetemcomitans ltx cdt* exhibited significant under-expression of IL-13 (*Il13*; *P*=.01, Student's *t*-test) and IL-25 (*Il25*; *P*=.01, Student's *t*-test), compared with sham-inoculated rats (Figure 4). Comparison of rats inoculated with *A. actinomycetemcomitans ltx cdt* and D7S-1 indicated significant under-expression of IL-5 (*Il5*; *P*=.01, Student's *t*-test), IL-12 receptor β1 (*Il12rb1*; *P*=.03, Student's *t*-test), *Il13* (*P*=.03, Student's *t*-test), and IL-3 (*Il3*; *P*=.04, Student's *t*-test) in *A. actinomycetemcomitans ltx cdt*-inoculated rats at *t*=3 weeks. Gene expression analysis indicated several other genes with significant fold-regulation values in each group as presented in Figures 3–5. However, these are the genes that had both significant *P*-values (*P*<.05) and significant fold-regulation changes (cut-off 2.0).

Of the fold change values for the remaining genes, it is interesting to note that *Il4* was alternatively under-expressed in rats inoculated with *ltx cdt* (*t*=1 week) compared with D7S-1 group (Figure 5), which had over-expression at both time points (Figure 3). Moreover, over-expression of Chemokine (C-C motif) ligand 20 (*Ccl20*) was observed in all treatment groups at 1 week post-inoculation (D7S-1 relative to sham control, *ltx cdt* relative to sham control, and *ltx cdt* relative to D7S-1). Importantly, the most pronounced gene regulation and fold change was observed for *Ccl20* in the *ltx cdt*-inoculated group relative to the sham control group, as seen in Figure 4. We also noted under-expression of IL17-A in rats inoculated with D7S-1 (Figure 3), and this gene was over-expressed in rats inoculated with *ltx cdt* relative to D7S-1 at *t*=1 week (Figure 5). These genes demonstrated qualitative fold regulation changes, as shown in Figures 3–5, although reference data indicated borderline statistical significance.

4 | DISCUSSION

Aggregatibacter actinomycetemcomitans is one of the key pathogens involved in oral infections, including periodontitis, osteomyelitis, and peri-implantitis. Yet, there is a paucity of information on its systemic effects and immunopathological responses. Up to five-fold more *Aggregatibacter actinomycetemcomitans* has previously been recovered from patients with periodontal disease compared with tissues of healthy patients.[23] In the present study, we investigated the immune response to wild-type *A. actinomycetemcomitans*, as well as mutants generated by deletion of two of its key virulence factors, namely, leukotoxin and cytolethal distending toxin.[24–26]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

We chose to focus on *A. actinomycetemcomitans* as our model pathogen due to its prominent role in oral infection, the availability of mutants with deletion of specific virulence genes generated by our research group, and our significant existing in vitro data on the immune responses to this particular clinical pathogen.[20,27] Previous periodontitis animal models have used oral gavage or ligature placement to study the inflammatory process of surrounding tissues.[28–30] Alternatively, we have also developed a titanium implant animal model to study oral osteolytic biofilm-mediated infection.[31] This *A. actinomycetemcomitans*-inoculated implant model has demonstrated that *A. actinomycetemcomitans* oral infection causes systemic infection, indicated by *A. actinomycetemcomitans*-containing urethral exudate in a subgroup of experimental rats (unpublished results). In order to gain insights into the immune responses to *A. actinomycetemcomitans* wild-type D7S-1 and its deletion mutant strains, we chose to inject live bacteria into oral mucosal tissues. Although this model does not necessarily represent the entire pathogenesis of periodontitis or peri-implantitis, we believe that it is a valid model to investigate the immune response to this pathogen. Similar approaches by injection of live bacteria or their products have been extensively used in the past.[32–37]

In this study, we focused on two important T-cell subsets, namely Treg and Th17 cells, and their associated cytokine and gene expression. The Th17 cellular pathway is thought to play a key role between the immune system and periodontal disease and oral infection. It involves a subset of $CD4^+$ T cells that secretes the proinflammatory cytokine IL-17 (also called IL17-A).[15] Interleukin-17 is a key molecule involved in innate immunity, and is thought to protect against extracellular bacteria and fungal pathogens by recruiting and activating polymorpho-nuclear leukocytes.[38–41] It is also involved in the cross-talk between the immune and skeletal systems through its role in osteoclastogenesis.[39] Previous studies on Th17 pathways associated with periodontal disease in human patients have indicated increased IL-17 expression and the presence of Th17 cells in sites of periodontal inflammation compared with healthy tissues.[42–44] Furthermore, gene expression of IL-17A, as well as IL-12, was demonstrated to be significantly higher in tissues of periodontal disease compared with that of gingivitis.[45] This indicates a strong proinflammatory immune reaction during periodontitis. In our model, we also demonstrated a significant increase in IL-17 cells ($CD4^+$ $IL17^+$) in both *A. actinomycetemcomitans* D7S-1-inoculated and *ltx cdt*-inoculated rats compared with sham-inoculated rats at *t*=1 week (Figure 2). By week 3, IL-17 cells in the *ltx cdt* group were still significantly over-expressed compared with both sham-inoculated and *A. actinomycetemcomitans* D7S-1 groups (Figure 2). This is consistent with previous studies with clinical periodontitis samples indicating an over-expression of IL-17 in periodontal inflammation and immune responses. Moreover, recent studies involving periodontal pathogens have examined the link between *A. actinomycetemcomitans* infection and systemic immune responses. It has been indicated that oral infection of *A. actinomycetemcomitans*, through bacteraemia, can contribute up to 20% of the HACEK bacteria group causing infective endocarditis.[46] It was observed that IL-17 was increased in atherosclerotic mice injected with *A. actinomycetemcomitans*, further demonstrating the link between *A. actinomycetemcomitans* and inflammation associated with Th17 cells.[47]

Other recent studies on the immune response to oral infection with animal models have initiated infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, which are

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

anaerobic bacteria associated with periodontal disease and oral biofilms.[48] In these studies, oral gavage with *P. gingivalis* led to inflammation and periodontal bone loss in experimental mice compared with controls,[34,49] and *P. gingivalis* ligature and injection models caused increased expression of IL-4, IL-6, IL-1b, IL-10, $CD3^+$ $CD4^+$ $FOXP3^+$, and RANKL in *P. gingivalis*-inoculated mice compared with control.[34,50] Using our *A. actinomycetemcomitans* oral inoculation model, we also found an over-expression of IL-4 in D7S-1-inoculated rats compared with sham-inoculated rats, at both *t*=1 week and *t*=3 weeks as seen in Figure 3. *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum* are all major pathogens in periodontitis and inflammation, and expression of IL-4 cytokine may be a key part of humoral immunity and immune responses to the infection.

An inverse relationship between Treg and Th17 cells has been proposed, where Th17 cells have a proinflammatory effect and Treg cells have immunosuppressive properties that downregulate the immune response. Many Treg cell subsets have been identified with varying phenotypic and functional properties, though CD4, CD25, and FOXP3 are common biomarkers to some Treg cells.[51,52] One study on Treg cells and *A. actinomycetemcomitans* oral infection in mice found an influx of Treg cells in experimental periodontitis, which may indicate the protective role of Treg cells during disease state and the attenuation of the inflammatory response and bone loss after infection.[53] Over-expression of FOXP3 cells, as also demonstrated by Marchesan et al.,[50] therefore indicates the influx of Treg cells and an immunoprotective effect during the proinflammatory state and increased immune response caused by *P. gingivalis* oral inoculation. In our study, the data showed a significant decrease in Treg cells ($CD4^+$ $FOXP3^+$) in D7S-1- and *ltx cdt*-inoculated groups compared with the sham-inoculated group at *t*=1 week, and no significant difference by week 3, as seen in Figure 2. However, this may be due to the differences in experimental protocol and bacterial strains. Our study evaluated the proportions of Treg and Th17 cells at 1 and 3 weeks post-inoculation, whereas Marchesan et al.[50] evaluated at 6 weeks. This may reflect the immune response whereby the proinflammatory cytokines are activated immediately after infection and, over time, there is an increase in suppressive immune cells eventually leading to resolution or regulation of the disease state. Furthermore, a recent examination of archival tissues has shown a concomitant presence of Treg (FOXP3) and Th17 (IL-17) cell infiltrate in periodontal disease archival tissues compared with controls,[54] which may also be a result of long-term healing and immune effects. It is interesting to note that expression of IL-4, which is a key proinflammatory cytokine often associated with periodontal infection as described above, was decreased in animals inoculated with *ltx cdt A. actinomycetemcomitans* at *t*=1 week and not expressed by *t*=3 weeks relative to animals inoculated with D7S-1 (Figure 5). This may indicate the difference in virulence of the double-knockout mutant (*ltx cdt*) compared with the wild-type *A. actinomycetemcomitans* strain, which showed statistically significant over-expression of IL-4 at both time points (Figure 3). Alternatively, expression of IL17A, which is another key proinflammatory cytokine in the Th17 pathway as described above, was under-expressed in animals inoculated with D7S-1 (Figure 3) and over-expressed in animals inoculated with *ltx cdt* relative to D7S-1 (Figure 5). This also correlates with our analysis of IL-17A protein expression by flow cytometry (Figure 2), which resulted in an increase in proportion of IL-17A in animals inoculated with D7S-1 and *ltx cdt* relative to sham-inoculated animals.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Aggregatibacter actinomycetemcomitans *ltx* *cdt* indicated a greater increase in the proportion of IL-17 expression by flow cytometry compared with both D7S-1 and control at both time points, as seen in Figure 2. This also suggests the qualitative differences in virulence expression that exist between mutant and wild-type strains of *A. actinomycetemcomitans*. Although the double knockout of two key virulence factors (*ltx* *cdt*) may result in reduced pathogenicity in some aspects of immune response, there is an increase in regulation of other genes in the pathway, as demonstrated here. Furthermore, over-expression of Ccl20, a proinflammatory chemokine in the Th17 pathway, was observed in all three groups, implying that there remains a common virulence factor playing a role in *A. actinomycetemcomitans* pathogenicity. Future studies may explore Ccl20 with various virulence factors to determine its role in immunopathology and mechanisms of disease progression.

The experimental evidence provided in the present study has demonstrated changes in the proportion of phenotypic markers, cytokines, and chemokines associated with T-cell subsets in response to oral inoculation with *A. actinomycetemcomitans*. Although the overall T-cell profiles elicited in rats challenged with D7S-1 and *ltx* *cdt A. actinomycetemcomitans* strains were similar, there were qualitative differences in cytokine expression, as noted above. Further investigation is needed to pinpoint specific virulence factors of *A. actinomycetemcomitans* and their influence on gene regulation. Future studies are also merited to investigate the role of Treg and Th17 T-cell subsets and their associated mediators in the pathogenesis of osteolytic oral biofilm infections such as periodontitis and peri-implantitis.

Acknowledgments

Funding for this research was provided by the National Institutes of Dental and Craniofacial Research grant numbers R01 DE12212 and R01 DE013230.

References

1. Eke PI, Dye BA, Wei L, et al. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. J Periodontol. 2015; 86:611–622. [PubMed: 25688694]
2. Bartold PM, Van Dyke TE. Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. Periodontol 2000. 2013; 62:203–217. [PubMed: 23574467]
3. Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. Periodontol 2000. 2014; 64:57–80. [PubMed: 24320956]
4. Craig RG, Yip JK, So MK, Boylan RJ, Socransky SS, Haffajee AD. Relationship of destructive periodontal disease to the acute-phase response. J Periodontol. 2003; 74:1007–1016. [PubMed: 12931763]
5. Figuero E, Sanchez-Beltran M, Cuesta-Frechoso S, et al. Detection of periodontal bacteria in atheromatous plaque by nested polymerase chain reaction. J Periodontol. 2011; 82:1469–1477. [PubMed: 21453047]
6. Ohki T, Itabashi Y, Kohno T, et al. Detection of periodontal bacteria in thrombi of patients with acute myocardial infarction by polymerase chain reaction. Am Heart J. 2012; 163:164–167. [PubMed: 22305832]
7. Padilla C, Lobos O, Hubert E, et al. Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis. J Periodontal Res. 2006; 41:350–353. [PubMed: 16827731]
8. Sparks Stein P, Steffen MJ, Smith C, et al. Serum antibodies to periodontal pathogens are a risk factor for Alzheimer’s disease. Alzheimers Dement. 2012; 8:196–203. [PubMed: 22546352]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

9. Fan X, Alekseyenko AV, Wu J, et al. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case–control study. Gut. 2016:1–8. Epub ahead of print.
10. Hultin M, Gustafsson A, Hallstrom H, Johansson LA, Ekfeldt A, Klinge B. Microbiological findings and host response in patients with peri-implantitis. Clin Oral Implants Res. 2002; 13:349–358. [PubMed: 12175371]
11. Koyanagi T, Sakamoto M, Takeuchi Y, Maruyama N, Ohkuma M, Izumi Y. Comprehensive microbiological findings in peri-implantitis and periodontitis. J Clin Periodontol. 2013; 40:218–226. [PubMed: 23294017]
12. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC. How we got attached to *Actinobacillus actinomycetemcomitans*: a model for infectious diseases. Periodontol 2000. 2006; 42:114–157. [PubMed: 16930309]
13. Chambers ST, Murdoch D, Morris A, et al. HACEK infective endocarditis: characteristics and outcomes from a large, multinational cohort. PLoS ONE. 2013; 8:e63181. [PubMed: 23690995]
14. Herbert BA, Steinkamp HM, Gaestel M, Kirkwood KL. Mitogen-activated protein kinase 2 signaling shapes macrophage plasticity in *Aggregatibacter actinomycetemcomitans*-induced bone loss. Infect Immun. 2017; 85:e00552–16. [PubMed: 27795356]
15. Gaffen SL, Hajishengallis G. A new inflammatory cytokine on the block: re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. J Dent Res. 2008; 87:817–828. [PubMed: 18719207]
16. Graves DT, Kang J, Andriankaja O, Wada K, Rossa C Jr. Animal models to study host–bacteria interactions involved in periodontitis. Front Oral Biol. 2012; 15:117–132. [PubMed: 22142960]
17. Chen C, Kittichotirat W, Chen W, Downey JS, Si Y, Bumgarner R. Genome sequence of naturally competent *Aggregatibacter actinomycetemcomitans* serotype a strain D7S-1. J Bacteriol. 2010; 192:2643–2644. [PubMed: 20348265]
18. Wang Y, Goodman SD, Redfield RJ, Chen C. Natural transformation and DNA uptake signal sequences in *Actinobacillus actinomycetemcomitans*. J Bacteriol. 2002; 184:3442–3449. [PubMed: 12057937]
19. Cheng YA, Jee J, Hsu G, Huang Y, Chen C, Lin CP. A markerless protocol for genetic analysis of *Aggregatibacter actinomycetemcomitans*. J Formos Med Assoc. 2014; 113:114–123. [PubMed: 24530245]
20. Nalbant A, Chen C, Wang Y, Zadeh HH. Induction of T-cell apoptosis by *Actinobacillus actinomycetemcomitans* mutants with deletion of ltxA and cdtABC genes: possible activity of GroEL-like molecule. Oral Microbiol Immunol. 2003; 18:339–349. [PubMed: 14622339]
21. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. Osteoarthritis Cartilage. 2012; 20:256–260. [PubMed: 22424462]
22. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007; 39:175–191. [PubMed: 17695343]
23. Slots J, Reynolds HS, Genco RJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. Infect Immun. 1980; 29:1013–1020. [PubMed: 6968718]
24. Ando-Suguimoto ES, da Silva MP, Kawamoto D, Chen C, DiRienzo JM, Mayer MP. The cytolethal distending toxin of *Aggregatibacter actinomycetemcomitans* inhibits macrophage phagocytosis and subverts cytokine production. Cytokine. 2014; 66:46–53. [PubMed: 24548424]
25. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of *Actinobacillus actinomycetemcomitans*. Periodontol 2000. 1999; 20:136–167. [PubMed: 10522226]
26. Herbert BA, Novince CM, Kirkwood KL. *Aggregatibacter actinomycetemcomitans*, a potent immunoregulator of the periodontal host defense system and alveolar bone homeostasis. Mol Oral Microbiol. 2016; 31:207–227. [PubMed: 26197893]
27. Zadeh HH, Nalbant A, Park K. Large-scale early in vitro response to *Actinobacillus actinomycetemcomitans* suggests superantigenic activation of T-cells. J Dent Res. 2001; 80:356–362. [PubMed: 11269729]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

28. Lindhe J, Berglundh T, Ericsson I, Liljenberg B, Marinello C. Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog. Clin Oral Implants Res. 1992; 3:9–16. [PubMed: 1420727]
29. Trombone AP, Ferreira SB Jr, Raimundo FM, et al. Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. J Periodontal Res. 2009; 44:443–451. [PubMed: 18973535]
30. Carcuac O, Abrahamsson I, Albouy JP, Linder E, Larsson L, Berglundh T. Experimental periodontitis and peri-implantitis in dogs. Clin Oral Implants Res. 2013; 24:363–371. [PubMed: 23176551]
31. Freire MO, Sedghizadeh PP, Schaudinn C, et al. Development of an animal model for *Aggregatibacter actinomycetemcomitans* biofilm-mediated oral osteolytic infection: a preliminary study. J Periodontol. 2011; 82:778–789. [PubMed: 21222546]
32. Li Y, Lu Z, Zhang X, et al. Metabolic syndrome exacerbates inflammation and bone loss in periodontitis. J Dent Res. 2015; 94:362–370. [PubMed: 25503900]
33. Jin J, Zhang X, Lu Z, et al. Simvastatin inhibits lipopolysaccharide-induced osteoclastogenesis and reduces alveolar bone loss in experimental periodontal disease. J Periodontal Res. 2014; 49:518–526. [PubMed: 24117880]
34. de Molon RS, de Avila ED, Boas Nogueira AV, et al. Evaluation of the host response in various models of induced periodontal disease in mice. J Periodontol. 2014; 85:465–477. [PubMed: 23805811]
35. Gonzalez D, Tzianabos AO, Genco CA, Gibson FC 3rd. Immunization with *Porphyromonas gingivalis* capsular polysaccharide prevents *P. gingivalis*-elicited oral bone loss in a murine model. Infect Immun. 2003; 71:2283–2287. [PubMed: 12654858]
36. Nakajima K, Hamada N, Takahashi Y, et al. Restraint stress enhances alveolar bone loss in an experimental rat model. J Periodontal Res. 2006; 41:527–534. [PubMed: 17076777]
37. Wang L, Guan N, Jin Y, Lin X, Gao H. Subcutaneous vaccination with *Porphyromonas gingivalis* ameliorates periodontitis by modulating Th17/Treg imbalance in a murine model. Int Immunopharmacol. 2015; 25:65–73. [PubMed: 25604387]
38. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology. 2010; 129:311–321. [PubMed: 20409152]
39. Zenobia C, Hajishengallis G. Basic biology and role of interleukin-17 in immunity and inflammation. Periodontol 2000. 2015; 69:142–159. [PubMed: 26252407]
40. Feller L, Khammissa RA, Chandran R, Altini M, Lemmer J. Oral candidosis in relation to oral immunity. J Oral Pathol Med. 2014; 43:563–569. [PubMed: 24118267]
41. Xu S, Cao X. Interleukin-17 and its expanding biological functions. Cell Mol Immunol. 2010; 7:164–174. [PubMed: 20383173]
42. Cardoso CR, Garlet GP, Crippa GE, et al. Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. Oral Microbiol Immunol. 2009; 24:1–6. [PubMed: 19121062]
43. Adibrad M, Deyhimi P, Ganjalikhani Hakemi M, Behfarnia P, Shahabuei M, Rafiee L. Signs of the presence of Th17 cells in chronic periodontal disease. J Periodontal Res. 2012; 47:525–531. [PubMed: 22309127]
44. Behfarnia P, Birang R, Pishva SS, Hakemi MG, Khorasani MM. Expression levels of th-2 and th-17 characteristic genes in healthy tissue versus periodontitis. J Dent (Tehran). 2013; 10:23–31. [PubMed: 23724200]
45. Honda T, Aoki Y, Takahashi N, et al. Elevated expression of IL-17 and IL-12 genes in chronic inflammatory periodontal disease. Clin Chim Acta. 2008; 395:137–141. [PubMed: 18588867]
46. Sharara SL, Tayyar R, Kanafani ZA, Kanj SS. HACEK endocarditis: a review. Expert Rev Anti Infect Ther. 2016; 14:539–545. [PubMed: 27124204]
47. Jia R, Hashizume-Takizawa T, Du Y, Yamamoto M, Kurita-Ochiai T. *Aggregatibacter actinomycetemcomitans* induces Th17 cells in atherosclerotic lesions. Pathog Dis. 2015; 73:1–8.
48. Teles R, Teles F, Frias-Lopez J, Paster B, Haffajee A. Lessons learned and unlearned in periodontal microbiology. Periodontol 2000. 2013; 62:95–162. [PubMed: 23574465]

49. Hajishengallis G, Liang S, Payne MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe. 2011; 10:497–506. [PubMed: 22036469]

50. Marchesan JT, Morelli T, Lundy SK, et al. Divergence of the systemic immune response following oral infection with distinct strains of *Porphyromonas gingivalis*. Mol Oral Microbiol. 2012; 27:483–495. [PubMed: 23134613]

51. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006; 441:235–238. [PubMed: 16648838]

52. Curiel TJ. Tregs and rethinking cancer immunotherapy. J Clin Invest. 2007; 117:1167–1174. [PubMed: 17476346]

53. Garlet GP, Cardoso CR, Mariano FS, et al. Regulatory T cells attenuate experimental periodontitis progression in mice. J Clin Periodontol. 2010; 37:591–600. [PubMed: 20642629]

54. Parachuru VP, Coates DE, Milne TJ, Hussaini HM, Rich AM, Seymour GJ. Forkhead box P3-positive regulatory T-cells and interleukin 17-positive T-helper 17 cells in chronic inflammatory periodontal disease. J Periodontal Res. 2014; 49:817–826. [PubMed: 24506561]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

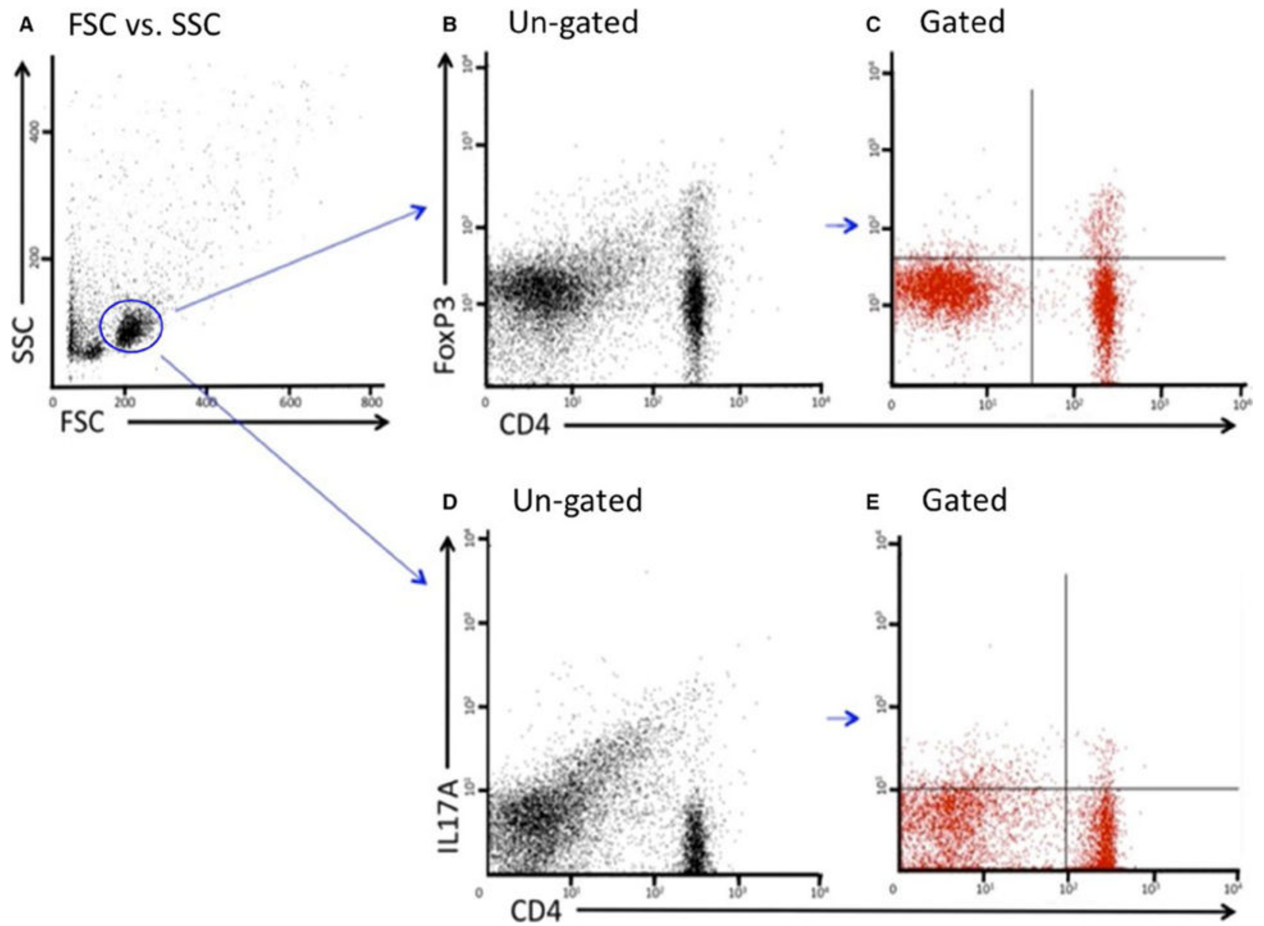

FIGURE 1.

Flow cytometry gating strategy shown for sham-inoculated group at *t*=1 week. Forward scatter versus side scatter of lymphocyte cell population (circled) can be seen in (A). (B, C) CD4$^+$ FOXP3$^+$ cells, (D, E) CD4$^+$ IL17$^+$ cells. Images indicate both before (B, D) and after (C, E) gating strategy was applied. This lymphocyte gating strategy was applied to all samples

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

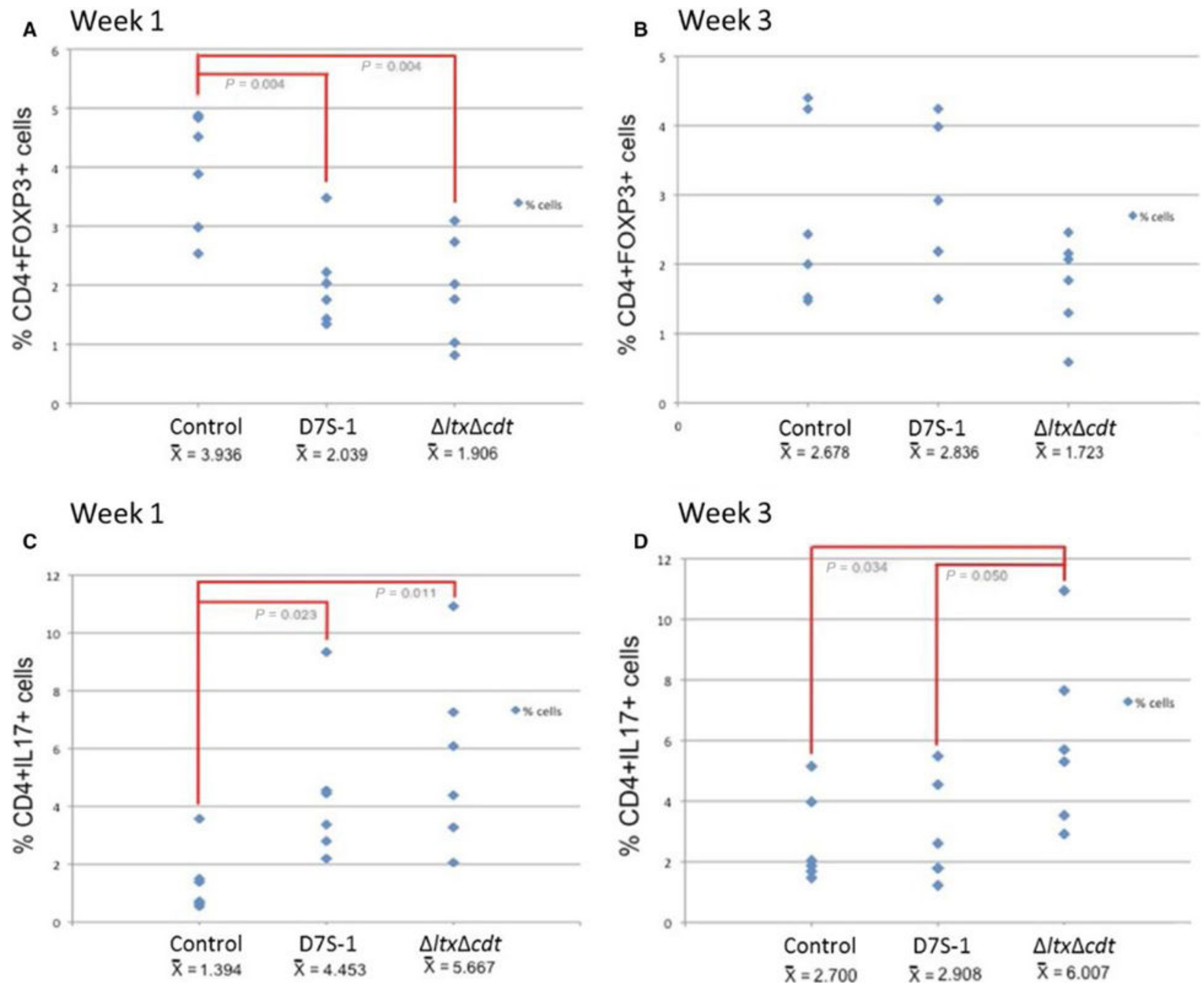

FIGURE 2.

Quantitative results derived from flow cytometric analysis of interleukin-17 (IL-17) and FOXP3 expression among CD4$^+$ T cells. Results of CD4$^+$ FOXP3$^+$ (A, B) and CD4$^+$ IL17$^+$ (A, B) cells at 1 (C, D) and 3 (B, D) weeks are shown

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

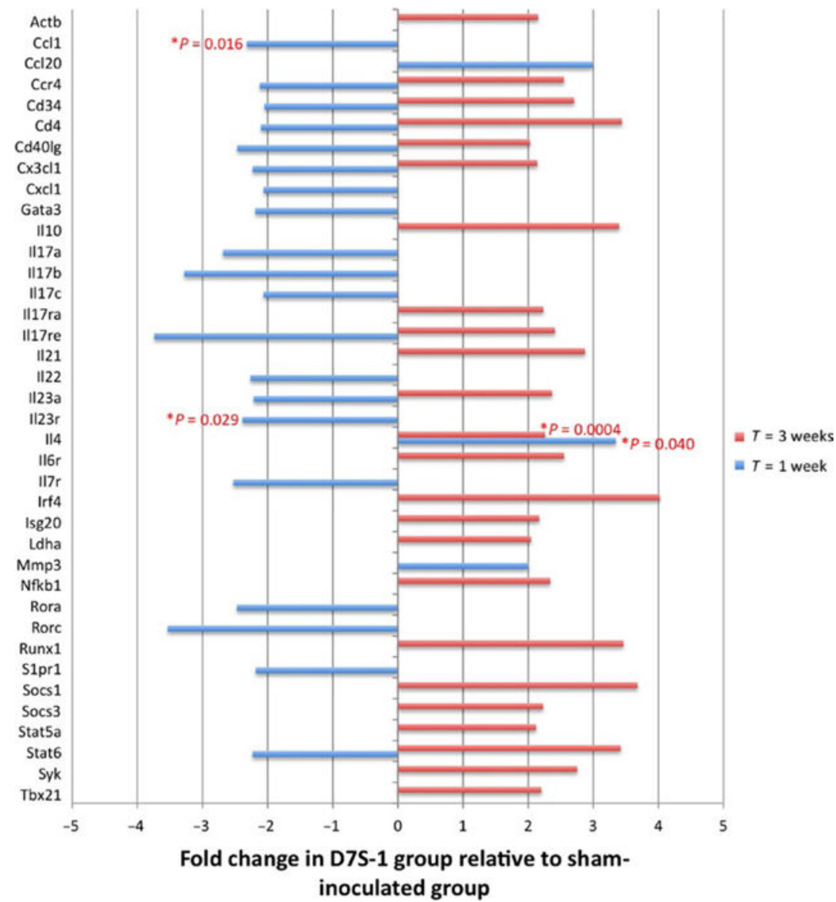

FIGURE 3.

Gene expression analysis of *Aggregatibacter actinomycetemcomitans* wild-type D7S-1 group compared with sham-inoculated group during the experimental period. Results of quantitative polymerase chain reaction analysis indicated statistically significant changes in Chemokine C-C motif ligand-1 (downregulated, t=1 week, P=.016), interleukin-23 receptor (downregulated, t=1 week, P=.029), interleukin 4 (upregulated, t=1 week, P=.040), and interleukin-4 (upregulated, t=3 weeks, P=.0004)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

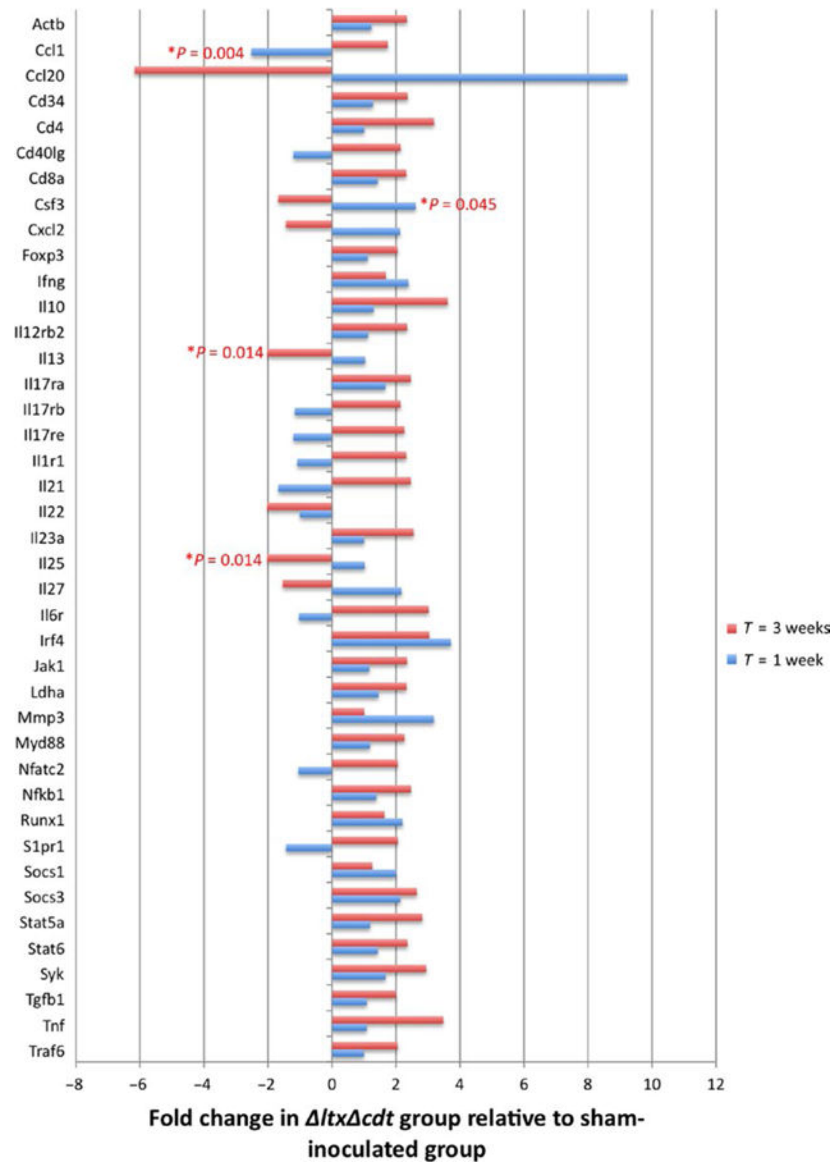

FIGURE 4.

Gene expression analysis of *Aggregatibacter actinomycetemcomitans ltx cdt* group compared with sham-inoculated group during the experimental period. Results of quantitative polymerase chain reaction analysis indicated statistically significant changes in Chemokine C-C motif ligand 1 (downregulated, t=1 week, P=.004), Colony-stimulating factor 3 (upregulated, t=1 week, P=.045), interleukin-13 (downregulated, t=3 weeks, P=.014), and interleukin-25 (downregulated, t=3 weeks, P=.014)

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

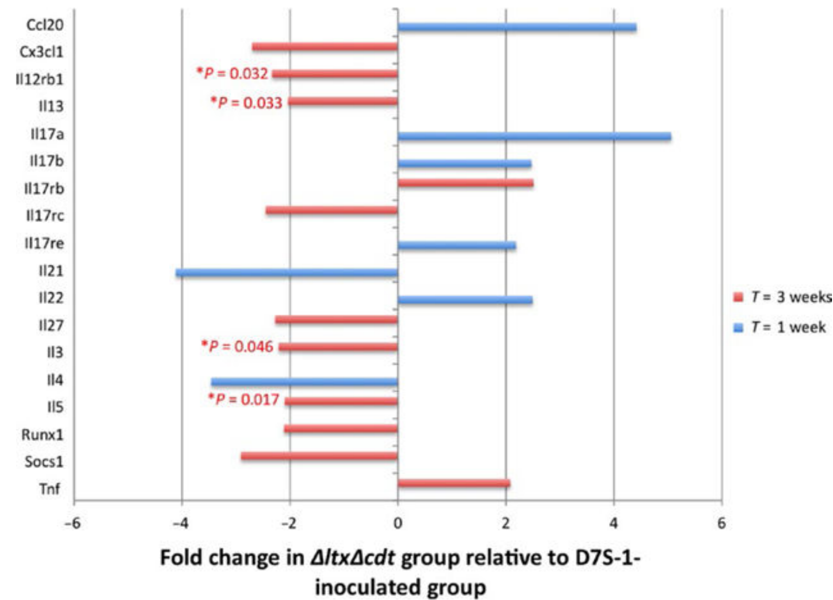

FIGURE 5.

Gene expression analysis of *Aggregatibacter actinomycetemcomitans* *ltx* *cdt* group compared with wild-type D7S-1 group during the experimental period. Results of quantitative polymerase chain reaction analysis indicated statistically significant changes in interleukin-12 receptor β1 (downregulated, t=3 weeks, P=.032), interleukin-13 (downregulated, t=3 weeks, P=.033), interleukin-3 (downregulated, t=3 weeks, P=.046), and interleukin-5 (downregulated, t=3 weeks, P=.017)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

TABLE 1

Gene list for T helper type 17 response RT^2 Profiler PCR array

Cell surface molecules	Cd2, Cd28, Cd34, Cd4, Cd8a, Icam1, Icos, Isg20, Tlr4
Chemokines	Ccl1, Ccl2, Ccl20, Ccl22, Ccl7, Cx3cl1, Cxcl1, Cxcl12, Cxcl2, Cxcl6
Cytokines	Csf2, Csf3, Ifng, Il10, Il12b, Il13, Il15, Il17a, Il17b, Il17c, Il17f, Il18, Il1b, Il2, Il21, Il22, Il23a, Il25, Il27, Il3, Il4, Il5, Il6, Il9, Tgfb1, Tnf
Cytokine receptors	Ccr2, Ccr4, Ccr6, Il1r1, Il12rb1, Il12rb2, Il17ra, Il17rb, Il17rc, Il17re, Il23r, Il6r, Il7r
Signal transduction	Cd40Ig, Cebpb, Clec7a, Foxp3, Gata3, Irf4, Jak1, Jak2, Mmp3, Mmp9, Myd88, Nfatc2, Nfkb1, Rora, Rorc, Runx1, S1pr1, Socs1, Socs3, Stat3, Stat4, Stat5a, Stat6, Syk, Tbx21, Traf6