Gingival Transcriptomics of Follicular T Cell Footprints in Progressing Periodontitis

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Abstract

Follicular helper T cells (Tfh) cells are generally considered critical in secondary lymphoid tissues; however, they are also identified in the circulation and in tertiary lymphoid structures in chronic inflammation. Gingival tissues with periodontitis reflect chronic inflammation so genomic footprints of Tfh cells should occur in these tissues and may differ related to aging effects. Methods: Macaca mulatta monkeys were used in a model of ligature-induced periodontitis [adult group (12-23 years of age); young group (3-7 years)]. Gingival tissue and subgingival microbiome samples were obtained at matched healthy sites, sites during ligature-induced disease, and in samples after clinical resolution. Microarray analysis examined Tfh genes (n=40) and the microbiome samples were examined using 16S MiSeq. Results: An apparent increase in the major transcription factor of Tfh cells, BCL6, was found with disease in both adult and young animals, while the master transcription markers of other T cell subsets were either decreased or showed minimal change. A number of the Tfh related genes, including surface receptors, secreted products and transcription factors were also significantly increased during disease. Unique microbial complexes showed patterns of interactions with Tfh genes that differed in health and disease. Conclusions: An increase in Tfh cell responsiveness occurred later in the progression of periodontitis, affected by age and strongly related to specific microbial complexes. The capacity of gingival Tfh cells to contribute to localized B cell activation and active antibody responses, including affinity maturation may be critical for controlling periodontal lesions and contributing to limiting and/or resolving the lesions.

Introduction

Follicular helper T cells are a subset of $CD4^+$ helper cells that are involved in the regulation and development of antigen-specific B cell immunity. The fundamental function of T helper cells is to provide help to B cells and to regulate their proliferation and immunoglobulin class switching, with antibody affinity maturation. Historically, Tfh cells were considered to be generally localized to the B cell follicle of secondary lymphoid tissues, including tonsils, to interact with germinal center (GC) B cells [1-3]. Functional studies have demonstrated that Tfh cells provide signals that are essential for survival and proliferation of these GC B cells. Thus, they play a critical role in development of B cell protective immunity against pathogens via immunoglobulin switching and recombination to promote increased antibody affinity [2].

Tfh differentiation is a multi-stage process and their functions depend upon the master regulatory transcription factor B-cell lymphoma 6 (Bcl6) [4-6]. Bcl-6 also suppresses the expression of factors that promote the differentiation of other $CD4^+$ T helper cell subsets[7]. Canonical secreted Tfh molecules include CXCL13, IL-21 and IL-4 that are all important for B cell help. IL-21 also regulates $CD4^+$ T cell subsets, including the efficient development of Tfh, as well as B cell differentiation and activation. Tfh cells also

display crucial cell surface markers, including CXCR5, PD1, ICOS, and CD40LG. The early differentiation of Tfh is driven by IL-6, ICOS, and the T cell receptor (TCR). The majority of differentiated Tfh cells are $CXCR5^{hi}PD1^{hi}Bcl6^{hi}Maf^{hi}SAP^{hi}$and $PSGL1^{lo}CD200^{+}BTLA^{hi}CCR7^{lo}$[8]. B cell help signals from Tfh include both cytokines and cell surface receptors [9]. Coupled with CD40LG, both PD1 and ICOS bind to ligands on the B cell surface to regulate the functions of both the B cells and Tfh cells, with direct co-stimulation of B cells via CD40LG and production of IL-21 driving B cell proliferation. The CXCR5 receptor enhances chemoattraction and migration of the Tfh cells into GC B cell zones[10-12].

Of particular interest to this study is that both circulating and tissue resident Tfh cells can be detected that produce cytokines to modulate antibody responses. Moreover, tissues including the periodontium, that contain antigens in a milieu of chronic immune responses are often infiltrated by T cells, macrophages, B cells and plasmacytes [13]. These cellular elements can organize themselves anatomically and functionally in a manner similar to secondary lymphoid organs [13-15]. As the gingival tissues with periodontitis reflect this type of microenvironment we hypothesized that with development and progression of periodontitis, genomic footprints of Tfh cells would occur in the gingival tissues and may differ related to aging effects. It was also predicted that certain bacterial components of the complex oral microbiome would have the capacity to aid in triggering or dampening the various molecules required for functional Tfh cells in the gingival tissues.

Methods

Animals and Diet

Rhesus monkeys (*Macaca mulatta*) (n=36; 17 male, 19 female) housed at the Caribbean Primate Research Center at Sabana Seca, Puerto Rico were examined for periodontal health [16-18]. Adult animals (age: 12-23 years; n=18) and young animals (age: 3-7 years; n=18) were included in the study. The nonhuman primates were fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad, Madison, WI). The diet was supplemented with fruits and vegetables, and water was provided *ad libitum* in an enclosed corral setting.

As we have reported previously the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico and a ligature disease model was utilized [19]. The clinical examination included probing pocket depth (PPD) and bleeding on probing (BOP; 0-5 scale) [20]. Periodontal health was defined by mean Pocket Depth (PD) [?] 3.0 mm and mean Bleeding on Probing (BOP) [?] 1 (0-5 scale) in a full mouth examination excluding 3^{rd}molars and canines [19]. Ligature-induced periodontal disease was initiated as we have previously reported [19] and gingival and subgingival plaque samples taken at 0.5, 1, and 3 months (Initiation/Progression), and 2 months after removal of ligatures and local factors (Resolution). Determination of periodontal disease at the sampled site was documented by assessment of the presence of BOP and probing pocket depth of >4 mm, as we have described previously [17].

Microbiome analysis

Subgingival bacterial samples were obtained from the 36 animals by a curette and analyzed using a MiSeq instrument [21, 22] for the total composition of the microbiome from each sample [23, 24]. Sequences were clustered into phylotypes based on their sequence similarity and these binned phylotypes were assigned to their respective taxonomic classification using the Human Oral Microbiome Database (HOMD V13) (http://www.homd.org/index.php?name=seqDownload &file&type=R) as we have described previously [25]. Raw data were deposited at the NIH NCBI (BioProject ID PRJNA516659). Statistical differences of bacterial OTUs were determined with a *t-test*(p<0.05). Correlations of OTUs within the oral microbiome were determined using a Pearson correlation coefficient analysis (p< 0.05). Correlations between the microbiome components and the gingival gene expression were determined only for matching samples derived from the same tooth in each of the animals. Matching samples with sufficient microbiome signals were compared for 58 samples in adults and 25 samples from the young group obtained at health and throughout the ligature model. As we have reported previously [25], of 396 OTUs identified in the nonhuman primate oral samples the targeted OTU selection for this study was 58 for the adult samples that covered 88% of reads in all samples. Similarly, 49 OTUs were examined in the young samples that covered 91% of reads in all samples.

Gingival tissue sample collection and mRNA analysis

Gingival tissue samples of healthy and disease sites were surgically collected and total RNA extracted for microarray analysis via hybridization to the GeneChip® Rhesus Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) similar to methods we have described previously [17, 26-29] .

Data Analysis

Tfh genes (n=40) (**Table 1**) were targeted in this study. The expression intensities across the samples were estimated using the Robust Multi-array Average (RMA) algorithm with probe-level quintile normalization, as implemented in the Partek Genomics Suite software version 6.6 (Partek, St. Louis, MO). The age groups were initially compared using one way ANOVA. For genes that had significant mean differences, two sample t-tests were used to investigate differences. Statistical significance was considered by a p value [?] 0.05. The data has been uploaded into the ArrayExpress data base (www.ebi.ac.uk) under accession number: E-MTAB-1977.

Results

Tfh gene expression profiles with age and periodontitis

Figure 1 summarizes fold-changes in gene expression for the 40 Tfh genes that were examined in gingival tissues from young (**Fig. 1A**) and adult (**Fig. 1B**) animal gingival tissues compared to baseline healthy tissue samples. The result demonstrated a number of the Tfh related genes, including surface receptors, secreted products and transcription factors were significantly increased during disease. In the young animals, striking increases were observed with BTLA, CXCL13 and IRF4 expression levels, while IFNG, CD40LG and CD200 were all significantly decreased. There was considerable overlap in altered gene expression levels in the young and adult samples. BTLA, CXCL13 and IRF4 were also increased and CD200 decreased with disease in the adult animals. Also of note was the increase in BCL6 in both groups primarily at the 3 month time point late in disease progression. The overall pattern of Tfh gene changes in both groups presented at 1 and 3 months during disease progression.

Figure 2 provides a summary of gene expression changes targeting the master regulatory transcription genes for each of the subsets of T helper cells. In both the adult and young gingival tissues samples the primary increase in transcription factor expression was with BCL6 for Tfh cells, particularly in the latter phases of disease progression, with levels returning to baseline in resolution samples. Also of note was the significant decreases in RORA in both age groups, and RORC (RORγ) in the young animal tissues through disease initiation and progression, and even in resolution samples. Th1 transcription factor, T-bet/TXB21, expression was only increased in the resolution samples.

Gene expression comparison in adult and young samples

Figure 3A-E provide an analysis of the array of gene changes comparing responses in adults and young animals in health (BL), disease (0.5, 1, 3 months), and resolution (5 months). The data was transformed using a z-score to normalize across all adult and young samples and across all data points. **Fig. 3A-B** summarizes the response profiles to Tfh-associated surface receptors. Generally, in the young samples the expression level was lower than adults, and did not show much change through the disease process and resolution samples. Only CD200 appeared elevated in young animals and decreased throughout disease. In contrast, in adults the majority of these receptors showed increases through disease and even in the resolution samples compared to baseline. Only CD10 and IL6RA demonstrated an increased with disease initiation (0.5 months) that tended to decrease with disease progression and resolution, and CD57 showed limited changes overtime.

Fig. 3C-D presents a similar analysis for cytokine/chemokine expression. In adults, IL10 and IL17F decreased from baseline, and IL21 and CXCL13 each increased with the disease process. In comparison with the young samples IFNG decreased with disease and CXCL13 and IL4 were increased in disease samples.

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The expression of crucial transcription factors and adaptor molecules for Tfh functions is shown in **Figure 3E.** Both IRF4 and SH2D1A/SAP showed substantial increases as disease progressed in both adults and young samples with dominant expression in the adult samples. BCL6 increased in adult samples at disease initiation, while this crucial transcription factor decreased in the young samples with disease progression. STAT3 demonstrated an increase at disease initiation and ASCL2 at disease resolution, in the young animal samples.

Microbiome and host Tfh gene expression in adult and young animals samples

Table 2 provides a summary of the microbiome relationships to gingival tissue transcriptome in samples from the two groups of animals. The data presents complexes of bacterial OTUs that were significantly correlated with individual genes related to Tfh phenotype and functions. An initial comparison of the Tfh transcriptome genes and the microbiome across all adult and young samples demonstrated few significant correlations within the potential 2320 comparisons, with only 2% showing a significant correlation (data not shown). However, the complexes and relationships were substantially different in health, disease and resolution in adult or young animals samples. Additionally, the same complexes were significantly related to multiple Tfh genes with both positive and negative correlations (see examples Tfh-H3A, Tfh-R4A), while during disease the complexes were only positively correlated with gene expression levels. Similarities on the component members of the complexes showed some overlap in adults (see Tfh-H1A/Tfh-D2A; Tfh-H4A/Tfh-D3A), although these similarities did not appear in the microbiome complexes in the young animals. Moreover, minimal overlap was noted in the complexes between adult and young animals. In adult animals, the complexes were associated with multiple genes in health, disease and resolution; however, in the young samples, generally, only with the disease associated microbiome were multiple gene correlations observed. Finally, IL6RA, CD84 and CD10 were the Tfh related genes showing the most frequent correlations with the microbial complexes. IL6RA and CD84 were predominantly associated with the microbiome in adults, while CD10 related to microbiome complexes in both adult and young samples.

In addition to exploring the relationship between bacterial complexes and profiles of host Tfh genes with related abundance or expression levels, we identified the individual bacterial OTUs that predominated among these correlations (**Table 3**). The results showed that multiple bacteria historically associated with changes in the disease microbiota, including *Fretibacterium* species, *Treponema*species, *Porphyromonas* species, and *Prevotella* species demonstrated a high frequency of correlations with Tfh genes. Also of note, *Haemophilus, Leptotrichia,* and *G. morbillorum*generally considered as commensals also demonstrated an elevated prevalence of correlations with the Tfh gene patterns. A number of the bacteria also appeared to primarily correlate with Tfh gene expression in adult samples compared to young samples. Finally, the was a predominance of individual bacterial-host correlations in resolution samples compared to health or disease in the young animals.

Profiles of microbiome and host Tfh gene expression

Although we were able to identify complexes that were significantly correlated with host Tfh genes, this approach did not provide a full picture of the potential host-bacterial interactions that occur in the adult and young samples. **Figure 4A-C** provides a heatmap of the overall bacterial-gene correlations, and highlight specific bacterial complexes and associated targeted gene expression profiles. In young healthy samples there existed correlations with multiple bacterial complexes and numerous host genes showing both positive and negative relationships for the same bacterial complex. A similar pattern was noted in the resolution samples suggesting a close interaction of host responses with the oral microbiome in these types of clinical conditions in younger animals. However, during disease, the bacterial components making up the complexes were more limited, and fewer Tfh genes appeared to be responding to these complexes, with both positive and negative correlation patterns. The adult samples (**Figure 5A-C**) generally showed fewer organized bacterial complexes related to Tfh gene expression patterns in health, disease and resolution. In healthy adult samples 4 complexes of bacteria showed patterns of correlations with 3 of the complexes demonstrating these interactions with a small repertoire of the Tfh genes. These host-bacterial interactions were more limited in adult disease samples with 3 complexes all generally correlated with an overlapping set of genes. The adult

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resolution samples showed 5 identified complexes. Three of these complexes demonstrated a relationship to an overlapping set of genes. Of interest was that complex 4 exhibited a very broad negative correlation pattern to 20/40 Tfh genes, while complex 5 showed a strong negative correlation to only CD57/B3GAT1.

Figure 6 provides a schematic of a protein interaction network related to the Tfh genes examined that significantly correlated with bacterial complexes. This includes interactions of transcription factors, cell surface receptors, and soluble products produced by Tfh cells. We highlight the genes expression profiles that were altered in adults and young animal samples stratified into health, disease, and resolution samples. These findings showed that cell receptors CD40LG, CD10/MME, IL6RA, CD84, and the composite of CD3D/E/G were particularly enriched for these correlations. Additionally, of the cytokines/chemokines IL12 and IL17A showed an enrichment for microbiome correlations. Also of interest was that these Tfh gene-bacterial relationships were represented in both adult and young samples.

Discussion

The CD4$^+$ T helper cell population in humans is composed of multiple subsets with clear phenotypic and functional differences, including Th1, Th2, Th17, Th9, Treg, and Tfh. The development of each of the Th cell subsets is driven by lineage-specific transcription factors: T-bet/TXB21 for Th1, GATA3 for Th2, RORα/γ for Th17, FOXO1 for Th9, and FOXP3 for Treg cells. The critical transcription factor for Tfh cells is Bcl6[1, 4, 6, 30]. These various cell types respond to different stimuli including antigenic challenge from pathogens and support development and maturation of both B and T cell effector functions [31]. Cell subsets with similar features have been identified in most mammals, particularly related to rodent and nonhuman primate disease models. Using a nonhuman primate model of ligature-induced periodontitis, we have shown aging effects on the genomic footprints of various T cell subsets in gingival tissues with health, progressing disease lesions, and clinical resolution [19]. Additionally, we have identified major gingival responses in the model, particularly later in disease progression, that reflected adaptive immune system triggering with substantial expression of rearranged Ig genes [26, 27, 32]. Of particular note for this study is that chronically inflamed tissues commonly accumulate lymphoid aggregates that would facilitate localized T-B cell interactions. These lymphocytes that migrate into chronically inflamed tissues can form ectopic lymphoid structures with functional germinal centers and have been termed tertiary lymphoid structures (TLS) [14] that demonstrate prototypical T follicular helper (Tfh) cells with elevated expression of Bcl6, CXCR5, PD1, and ICOS[1]. Understanding the range of T cell populations that can provide help to B cells within these chronically inflamed tissues is essential to a mechanistic clarification of the biology of these inflammatory conditions. More specifically within the gingival tissues of periodontitis lesions, Tfh cells may be important for effective responses to pathogens, as well as commensal bacteria, as they are required for antibody response quality.

This study targeted gene expression of an array of Tfh genes including various transcription factors (BCL6, STAT3, ASCL2, BATF, c-MAF, IRF4)[4, 33-37]. BCL6 was increased in gingival tissues from both age groups, particularly later in disease progression and the pattern differed from other T helper cell regulatory transcription factors. A major role for high Bcl6 levels is directing the positioning of Tfh cells to B cells through upregulation of CXCR5 and downregulation of CCR7, PSGL1, and EBI2 [13]. Generally low levels of these 3 genes were also noted with disease in tissues from both age groups. IRF4 and BATF are both essential transcription factors for early Tfh differentiation[10, 35, 36]. BATF showed minimal change with disease; however, IRF4 increased significantly in both age groups, particularly at the disease progression sampling points. STAT3 is also crucial for Tfh cells role for inducing GC B-cells. Both STAT3 and IRF4 are also critical transcription factors for production of IL-21 that impacts Tfh cell development[34, 38, 39]. STAT3 was increased at disease initiation and progression, and decreased to baseline levels with resolution.

IL-21, IL-12, IL-23, and TGFß are all required for development of Tfh cells and IL-21 plays a critical role in regulating Ig production and GC formation. It also acts in an autocrine fashion for Tfh cell development [40-42]. While IL-21 is induced by IL-6 through the STAT3 transcription factor, the mRNA for this cytokine showed minimal change with disease in young animals but was significantly increased later in disease in the adult animals. IL-12 induces generation of Tfh cells from naïve CD4$^+$ T cells [43, 44], while IL-23 has been

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suggested to contribute to the induction of autoantibody formation in various inflammatory diseases [45-47]. Neither of these factors were altered at the mRNA level in this chronic inflammatory disease model.

Tfh cells show elevated expression of various cytokine (IL6R, IL21R) and chemokine (CXCR4, CXCR5) receptors[7, 10]. Tfh cells co-localize with B cells by Bcl6 up-regulating CXCR5 and down-regulating CCR7, with CXCR5 enhancing migration of Tfh to B cell regions via interaction with its ligand CXCL13 [1, 9, 15, 31]. We identified significant increases in CXCR5 in both adult and young tissues that increased later in disease and actually remained elevated in the resolution samples, while no change in expression of CCR7 was seen. CXCR4 was also elevated, but appeared earlier in the disease process and then dropped to baseline in resolution samples. ICOS is expressed on activated T cells and its ligand, ICOS-L (B7h) is broadly expressed on APCs, non-lymphoid tissues and is constitutively expressed on B cells[48, 49]. The interaction of ICOS/ICOS-L is critical for increases in antigen-specific CXCR5$^+$PD1$^+$ Tfh cells and GC-B cells and has been shown to be dependent on IL-21, IL-6 and STAT3[12]. ICOS is also important for IL-21 and IL-4 production under control of SLAM molecules [39]. Importantly, ICOS engagement induces c-Maf expression that supports Tfh formation and maintenance of these cells through production of IL-21[37]. ICOS mRNA is increased later in disease in both adult and young animals, although levels of c-Maf mRNA were generally unaffected.

CD40LG expressed on Tfh cells binds to the CD40 receptor on B cells as a major co-stimulatory molecule [50, 51]. This interaction induces expression of CD25 on the B cells that helps control B cell receptor expression, and represents a phenotype of memory B-cell population with improved proliferative and antigen-presenting capacity. We noted that increases in message for CD40LG was a response change with disease primarily in adult animals. IL-6 production is generally felt to arise from follicular dendritic cells for Tfh efficient cell responses[5, 52], albeit dendritic cell numbers and functions have also been reported within periodontitis lesions [53-55]. Furthermore, T cell-specific deletion of the IL-6 receptor α chain (IL6RA) results in impaired T cell responses *in vivo* , including a defect in Tfh functions [56]. The periodontitis model showed increases in IL6RA only in adult disease, thus representing one of the varied differences in gene expression related to Tfh functions based upon age. PD1 on Tfh cells binds to PD1L on B cells and engages APCs via TCR signaling that provides transient help to B cells, but not Tfh proliferation[57]. It has been found that elevated PD1 actually limits Tfh proliferation through dampening TCR signaling. However, no changes in the mRNA for this important Tfh receptor was observed in this disease model.

CD84/SLAM5 and CD150/SLAM molecules have been identified on GC Tfh cells (Calpe 2008). Through the activity of signaling lymphocyte activation molecule (SLAM)-associated protein (SAP/SH2D1A) adaptor molecule enables these receptors to have a critical role in B-cell help and antibody class switching related to Tfh development [58-60]. While the Tfh interactions with DCs appear primarily via integrin and TCR receptors, the sustained phase of Tfh interaction with B cells requires SAP. The SLAM receptors on Tfh cells are engaged in prolonged T-B cell contact and optimal Tfh functions [61, 62]. In samples from both adult and young animals, both CD84 and CD150 genes were up-regulated later in the disease process, consistent with an increased role of Tfh cells and adaptive immune responses at this stage of the periodontal lesions.

Sphingosine-1-phosphate (S1P) is a biologically active metabolite of plasma-membrane sphingolipids that is essential for immune-cell trafficking. Its receptor, S1PR1, signaling is a crucial factor in regulating naive Tfh-cells egress from draining lymph nodes [63]. The gene expression for this receptor was significantly increased in gingival tissues of both adults and young animals primarily at early time points in the disease process and then returned to baseline levels in resolution samples. This is consistent with a role in localization of Tfh cell help within the developing periodontal lesion that could contribute to enhancing control over the host-bacterial interactions during disease. Additionally, CD10/MME/neprilysin is a marker of immature T and B cells and GC B cells and is virtually absent on circulating mature T cells [64]. However, it is not only expressed on Tfh cells, but was substantially increased early in the disease process, again potentially related to the localization and communication of Tfh cells in this inflammatory environment.

Finally, a number of biomolecules associated with Tfh cells specifically down-regulate functions associated with T-B cell interactions. CD200 is a glycoprotein surface molecule that is a member of the immunoglobulin

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superfamily and is expressed on B cells and a subset of T cells, including Tfh particularly in reactive lymphoid tissues [65, 66]. It binds to CD200R1, which is an inhibitory receptor for inflammatory mediator production from lymphoid cells. Of interest is the significant decrease in this molecule across all disease time points, thus potentially contributing to less control over the developing chronic inflammatory lesion. Similarly, B- and T-lymphocyte attenuator (BTLA, eg. CD272) is a co-inhibitory receptor also belonging to the immunoglobulin superfamily. It is induced during T cell activation except on Th2 cells [67]. BTLA interacts with the B7H4 co-stimulatory molecule and inhibits T cell effector functions. In the nonhuman primate gingival tissues BTLA mRNA levels were significantly increased during disease, which may contribute to overall regulation of the immune responses occurring through Tfh help in the gingival tissues. SELPLG encodes for P-selectin glycoprotein ligand-1 (PSGL-1) that is an adhesion and regulatory molecule involved in immune cell trafficking. However, PSGL-1 has emerged as an important checkpoint in negatively regulating Tfh cell function during adaptive immune responses[5, 9]. As with BTLA, this gene was significantly up-regulated only during disease that might be predicted to be an important portion of the T cell regulatory environment in the gingival tissues. It has been reported that IL-2 signaling is another potent inhibitor of Tfh cell differentiation [68]. While IL2RA mRNA levels increased significantly with disease in both adults and young samples, generally, IL-2 gene levels were generally low in the gingival tissues. STAT5 is the downstream target of IL-2 engagement of IL-2RA. Thus, although STAT5 would also represses Tfh differentiation there was no change in STAT5 mRNA with disease in either age group.

As we noted these substantive changes in the footprint of genes related to Tfh functions, it must be recognized that these changes result from alterations in the quality and quantity of the microbiome at disease sites and the resulting clinical changes reflecting the disease process. The current paradigm in periodontitis is the transition from a healthy symbiotic microbiome to a dysbiotic microbiome at disease sites [69-73]. This dysbiosis reflects altered biology of both pathogens and commensals in the disease ecology, the summation of which drives host response changes, dysregulates these responses, and triggers tissue changes of periodontitis. However, a more detailed profile of the bacteria and/or bacterial complexes related to specific changes in host responses *in situ* remains to be elucidated. Studies of human gingival transcriptomes and associated targeted bacteria have identified relationships of changes in the expression of specific genes with altered levels of some microbes as indicative of disease versus health; however, these are "point-in-time" samples with minimal knowledge regarding the true dynamics of the lesion development [74-78]. Thus, using this nonhuman primate model of progressing disease, we examined the interrelationship of the microbiome with the pattern of gene expression to discern the potential that certain bacteria or complexes of bacteria afforded a high level of "control" over the gene expression profiles. Multiple outcomes of this analysis were detected. First, there were a finite number of complexes of bacteria that strongly correlated with the Tfh gene expression. The complexes differed in health, disease and resolution sites, as well as showing unique differences in the adult and young microbiomes. Moreover, these complexes were not simply composed of only bacteria historically considered as periodontopathogens versus oral commensal bacteria, but generally were mixtures of these types of microorganisms. Secondly, certain of these complexes were significantly correlated with multiple genes that generally were either positively or negatively correlated with the panel of Tfh genes (eg. Tfh-H1A, Tfh-R2A, Tfh-R4A, Tfh-D1Y). Finally, while we could estimate statistical significance of specific correlations of complexes of bacteria with specific Tfh genes, a systems approach to the relationship of bacterial abundance to gene expression levels provided a visualization of broad correlations between specific bacterial complexes and panels of the Tfh genes. Of note, the same bacterial complex demonstrated positive and negative correlation patterns with different groups of genes within the Tfh footprint.

Thus, Tfh cell functions are mediated through the production of cytokines that also promote B-cell survival and antibody production, and through the engagement of T-APC and T-B cell co-stimulatory molecules (eg. TCR, ICOS, CD40LG, PD1,) and other receptors (eg. SLAM, CXCR5, etc.), which favor these strong interactions leading to successful and mature B-cell immunoglobulin responses. Coupled with existing data in humans [79-85] and nonhuman primates [20, 86, 87] detailing local and systemic antibody production that can be enhanced by immunization, supports that localized Tfh functions in gingival tissues may provide an important adaptive immune regulatory mechanism for controlling the progression and magnitude of disease.

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Author Contributions

JLE and OAG were responsible for the design, conduct of the experiment, clinical data and sample collection, interpretation of the data, and preparation of the manuscript. SSK was responsible for preparation of the gingival tissue mRNA and bacterial DNA for analysis. LO contributed to the clinical measures, and JGM contributed to the animal husbandry and conduct of the experiment.

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Table 1: T follicular helper cell gene expression targets

Gene ID	Fxn	Gene ID
ASCL2	Achaete-Scute Family BHLH Transcription Factor 2	IFNG
BATF	Basic Leucine Zipper ATF-Like Transcription Factor	IL2RA
BCL6	BCL6 Transcription Repressor; master transcription factor Tfh	IL4
BTLA	B And T Lymphocyte Associated (CD272) attenuator; Inhibitory receptor on lymphocytes	IL6RA
CCR7	C-C Motif Chemokine Receptor 7	IL7R
CD3D	TCR component; binds MHCII on APCs	IL10
CD3E	TCR component; binds MHCII on APCs	IL12
CD3G	TCR component; binds MHCII on APCs	IL17A
CD4	Essential role in immune responses; subset of T cells	IL17F
CD10/MME	Neprilysin; membrane metallopeptidase	IL21
CD40LG	T cell surface ligand; binds CD40 on B cells	IL21R
CD57/B3GAT1	Biosynthesis of L2/HNK-1 carbohydrate epitope on glycoproteins	IL23
CD84/SLAMF5	Signaling lymphocyte activation molecule (SLAM) family	IRF4
CD150/SLAMF1	Signaling lymphocyte activation molecule (SLAM) family	c-maf/MA
CD200	OX-2; Costimulates T-cell proliferation	PD1/PDC
CXCL13	C-X-C Motif Chemokine Ligand 13	RORA
CXCR4	C-X-C Motif Chemokine Receptor 4	RORC
CXCR5	C-X-C Motif Chemokine Receptor 5	S1PR1
EBI2/GPR183	G Protein-Coupled Receptor 183	SELPLG/
FOXP3	Forkhead Box P3; master transcription factor Treg	SH2D1A/S
FOXO1	Forkhead Box O1; master transcription factor Th9	STAT3
GATA3	GATA Binding Protein 3; master transcription factor Th2	TGFB1

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Gene ID	Fxn	Gene ID
ICOS	Inducible T Cell Costimulator; binds ICOSL on B cells	T-bet/TX

Table 2: Significant correlations of microbiome OTU complexes with genes related to Tfh phenotype and functions. Blue denotes significant positive correlation and red denotes a significant negative correlation.

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD15	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Health	Tfh-H1A	Fretibacterium sp. 361 Chloroflexi [G-1] sp. 439 Fretibacterium fastidiosum 363 Treponema sp. 246 Peptostreptococcaceae [XIII] unclassified Porphyromonas gingivalis 619 A. actinomycetemcomitans 531 Filifactor alocis 539	+																		

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-H2A		Selenomonas_unclassified	-				+	+	+											
		Leptotrichia_unclassified																		
		Veillonella unclassified																		
		Prevotella unclassified																		
		Prevotella enoeca 600																		
		Aggregatibacter unclassified																		
		Neisseria oralis 014																		

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-H3A		Bacteria_ un- clas- si- fied Tre- ponema un- clas- si- fied Pyra- mi- dobac- ter pis- colens 357 Se- lenomonas sputi- gena 151 Veil- lonel- laceae_ [G- 1] sp. 155 Pre- votella in- ter- me- dia 643 Pre- votella sp. 820 Tre- ponema mal- tophilum 664						+	+	+	+									

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-H4A		Eubacterium infirmum 105 Streptococcus Prevotella sp. 526 Gemella morbillorum 046 Porphyromonas sp. 279 Moraxella catarrhalis 833 Haemophilus sp. 035	+														+	+			

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Disease	Sch-D1A	Leptotrichia_-unclassified Veillonella unclassified Selenomonas sputigena 151 Fretibacterium unclassified Treponema maltophilum 664 Fusobacterium sp. 203 Veillonellaceae_-[G-1] sp. 155 Prevotella sp. 317 Selenomonas unclassified	+					+					+	+						

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-D2A		Fretibacterium fastidiosum 363 Peptostreptococcaceae_-[XIII]_-unclassified Treponema unclassified Fretibacterium sp. 361 Treponema denticola 584 Prevotella enoeca 600	+				+					+			+					

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-D3A		Haemophilus sp. 035 Streptococcus sp. 058 Porphyromonas sp. 279 Moraxella catarrhalis 833 Gemella morbillorum 046 Porphyromonadaceae															+			
Tfh-D4A		Porphyromonas endodontalis 273 Porphyromonas gingivalis 619 Treponema sp. 246 Fusobacterium unclassified	+													+				

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Resolution Tfh R1A		Streptococcus_unclassified Veillonella parvula 161 Haemophilus sp. 035 Prevotella sp. 311 Megasphaera micronuciformis 122 Prevotella denticola 291 Prevotella unclassified Veillonella unclassified Veillonella dispar 160										+		+						

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R2A		Streptococcus sp. 058 Pre-votella sp. 317 Catonella morbi 165 Lep-totrichia_-un-clas-si-fied Se-lenomonas_-un-clas-si-fied Tre-ponema socran-skii 769 Se-lenomonas unclassified			+											+	+	+	+	

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R3A		Fretibacterium unclassified Gemella morbillorum 046 Capnocytophaga_unclassified Porphyromonas sp. 279 Fusobacterium unclassified																	+	

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R4A		Fusobacterium sp. 203 Chloroflexi_[G-1] sp. 439 Prevotella sp. 526 Eubacterium infirmum 105 A. actinomycetemcomitans 531 Treponema unclassified Treponema denticola 584	+						+	+									+	

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R5A		Filifactor alocis 539 Peptostreptococcaceae_-[XIII]_-unclassified Fretibacterium sp. 361 Porphyromonas gingivalis 619 Porphyromonas endodontalis 273 Fretibacterium fastidiosum 363 Desulfobulbus sp. 041										+								

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Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Health	Tfh-H1Y	Gemella morbillorum 046 Fusobacterium_unclassified Fretibacterium_unclassified Fretibacterium sp. 361 A. actinomycetemcomitans 531 Fretibacterium fastidiosum 363										+								

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Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-H2Y		Prevotella intermedia 643 Treponema denticola 584 Prevotella enoeca 600 Treponema sp. 246 Treponema_unclassified Treponema maltophilum 664 Prevotella sp. 313	+																	

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
	Tfh-H3Y	Bacteria_-un-clas-si-fied Veil-lonella parvula 161 Pre-votella sp. 526 Chlo-roflexi_-[G-1]sp. 439 Pre-votella sp. 820																		
Disease	Tfh-D1Y	Bacteroidetes_-un-clas-si-fied Fretibac-terium_-un-clas-si-fied Pep-tostrep-to-coc-caceae_-[XIII]_-un-clas-si-fied Tre-ponema sp. 246						+	+			+			+	+				

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-D2Y		Bacteroidetes_-un-clas-si-fied		+																
		Fretibac-terium_-un-clas-si-fied																		
		Pre-votella fusca 782																		
		Tre-ponema_-un-clas-si-fied																		
		Catonella morbi 165																		
		Tre-ponema den-ti-cola 584																		
		Pre-votella sp. 304																		
		Por-phy-romonas gin-gi-valis 619																		

Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-D3Y		Haemophilus sp. 035 SR1_-[G-1]sp. 345 Pre-votella sp. 317 Cap-no-cy-tophaga_-un-clas-si-fied Por-phy-romonas sp. 279 Pre-votella sp. 526					+													

Category	ID	Compl	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-D4Y		Bacteroidetes_-unclassified																		
		Fretibacterium_-unclassified																		
		Prevotella fusca 782																		
		Treponema_-unclassified																		
		Treponema denticola 584																		
		Prevotella sp. 304																		
		Porphyromonas gingivalis 619																		
		Fretibacterium fastidiosum 363																		
		Chloroflexi_-[G-1]sp. 439																		
		Bacteria_-unclassified																		
		Veil-																		

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Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD15	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R5Y		Gemella morbillorum 046 Pasteurellaceae_unclassified Streptococcus sp. 058 Fretibacterium sp. 361 Filifactor alocis 539																		

Category	ID	Compl	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD15	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Resolution	R1Y	Prevotella sp. 304 Fretibacterium_unclassified Filifactor alocis 539 Fretibacterium fastidiosum 363 Chloroflexi_-[G-1]sp. 439 Fretibacterium sp. 361 Leptotrichia sp. 223 Veillonellaceae_-[G-1]_-unclassified Eubacterium_-[XI][G-1]infirmum 105 Porphyromonas gingivalis 619 Pre-																			

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Category	ID	Complex	CD3G	IL6RA	CD40L	CD3D	IL12	IL21R	CD84	CD150	CD4	CD57	CD10	IL17A	CXCR5	BTLA	c-maf	IL21	IFNG	PD1
Tfh-R2Y		Prevotella_-unclassified															+			
		Moraxella catarrhalis 833																		
		Prevotella sp. 317																		
		Fusobacterium_-unclassified																		
		Prevotella enoeca 600																		
Tfh-R3Y		Bacteroidetes_-unclassified														+				
		Treponema sp. 246																		
		Prevotella sp. 313																		
		Veillonella parvula 161																		
		Bacteria_-unclassified																		

Table 3: Significant correlations of individual bacterial OTUs with Tfh genes in samples from both age groups and in health, disease, and resolution sites. + denotes a significant correlation between the OTU and gene expression levels, – no significant correlations, and NA signifies the OTU was not represented in the

microbiome members that were evaluated.

Bacteria	ADULT Disease	ADULT Health	ADULT Resolution	YOUNG Disease	YOUNG Health	YOUNG Resolution	
Fretibacterium sp. 361	+	+	+	+	+	+	6
Fretibacterium fastidiosum 363	+	+	+	+	+	+	6
Haemophilus sp. 035	+	+	−	+	+	+	5
Treponema unclassified	+	+	+	−	+	+	5
Fretibacterium unclassified	+	−	+	+	+	+	5
Prevotella sp. 526	−	+	+	+	+	+	5
Porphyromonas gingivalis 619	+	+	+	+	−	+	5
Chloroflexi_[G-1] sp. 439	−	+	+	+	+	+	5
Fusobacterium unclassified	+	−	+	+	+	−	4
Leptotrichia_unclassified	+	+	+	−	−	+	4
Treponema denticola 584	+	−	+	−	+	+	4
Porphyromonas sp. 279	+	+	+	−	−	+	4
Prevotella enoeca 600	+	+	−	+	+	−	4
Gemella morbillorum 046	+	+	+	−	−	+	4
Treponema sp. 246	+	+	+	−	−	+	4
Bacteria_unclassified	−	+	−	−	+	+	3
Veillonella unclassified	+	+	+	−	−	−	3
Prevotella unclassified	−	+	+	+	−	−	3
A. actinomycetemcomitans 531	−	+	+	−	+	−	3
Streptococcus sp. 058	+	−	+	−	−	+	3
Treponema maltophilum 664	+	+	−	−	+	−	3
Porphyromonas endodontalis 273	+	−	+	−	−	+	3
Eubacterium infirmum 105	−	+	+	+	−	−	3
Prevotella sp. 820	−	+	−	+	+	−	3
Prevotella sp. 304	−	−	+	+	−	+	3
Prevotella intermedia 643	−	+	+	−	+	−	3
Leptotrichia sp. 223	+	−	+	+	−	−	3
Prevotella sp. 317	+	−	−	+	−	+	3
Filifactor alocis 539	−	+	−	+	−	+	3
Peptostreptococcaceae_[XIII]_unclassified	+	+	−	−	−	+	3
Moraxella catarrhalis 833	+	+	+	−	−	−	3
Selenomonas_unclassified	−	+	+	−	−	−	2
Streptococcus_unclassified	−	+	+	−	−	−	2
Veillonella parvula 161	−	−	+	−	+	−	2
Selenomonas sputigena 151	+	+	−	−	−	−	2
Catonella morbi 165	−	−	+	−	−	+	2
Capnocytophaga_unclassified	−	−	+	−	−	+	2
Veillonellaceae_[G-1]	−	−	−	+	−	+	2
Fusobacterium sp._oral_taxon_203	+	−	+	−	−	−	2
Veillonellaceae_[G-1] sp. 155	+	−	−	−	−	+	2
Prevotella fusca 782	−	−	+	−	−	+	2
Pyramidobacter piscolens 357	−	+	−	−	−	−	1
Prevotella sp. 313	−	−	−	−	+	−	1
Streptococcus parasanguinis II 411	−	−	+	−	−	−	1
Aggregatibacter unclassified	−	+	−	NA	NA	NA	1
Pasteurellaceae_unclassified	−	−	−	−	−	+	1
Bacteroidetes_unclassified	−	−	−	−	−	+	1

	ADULT	ADULT	ADULT	YOUNG	YOUNG	YOUNG	
SR1_[G-1] sp. 345	–	–	–	–	–	+	1
Prevotella sp. 311	–	–	+	NA	NA	NA	1
Neisseria oralis 014	–	+	–	–	–	–	1
Desulfobulbus sp. 041	+	–	–	NA	NA	NA	1
Megasphaera micronuciformis 122	–	–	–	NA	NA	NA	0
Porphyromonadaceae	–	–	–	–	–	–	0
Peptostreptococcaceae_[XI]_unclassified	–	–	–	NA	NA	NA	0
Veillonella dispar 160	–	–	–	NA	NA	NA	0
Treponema socranskii 769	–	–	–	NA	NA	NA	0
Prevotella sp. 443	–	–	–	NA	NA	NA	0
Prevotella denticola 291	–	–	–	NA	NA	NA	0
TOTAL	25	29	32	17	17	28	1

Figure Legends

Figure 1: Expression levels of Tfh genes in gingival tissues from young (**A**) and adult (**B**) animals. The data are expressed as fold-change in expression with disease (0.5, 1, 3 months) and resolution (5 months) compared to baseline healthy samples for each animal. The bars denote group means. The asterisk and horizontal bracket signifies significant difference from baseline at $p<0.01$.

Figure 2: Expression of master transcription factor genes for various subsets of $CD4^+$ T help cells [T-bet/TXB21 - Th1, GATA3 - Th2, RORα/γ - Th17, FOXO1 - Th9, FOXP3 – Treg, BCL6 - Tfh cells]. Points denote group means for young and adult animals at each time point expressed as fold-change from baseline. Asterisk denotes significantly different from baseline at $p<0.01$.

Figure 3: Z-score values for Tfh surface receptors (**A & B**), cytokines/chemokines (**C & D**), and transcription factors (**E**) in adult and young animal gingival samples obtained in health (BL), during disease (0.5, 1, 3 months) and clinical resolution (5 months). Points denote normalized group means comparing z-scores for expression of a specific gene across all animals and timepoints.

Figure 4: Heatmap of correlations between bacterial OTUs relative abundance and individual gene expression young healthy (**A**), diseased (**B**), and resolution (**C**) samples. Boxes provide estimate of gene and assigned bacterial complex interactions.

Figure 5: Heatmap of correlations between bacterial OTUs relative abundance and individual gene expression adult healthy (**A**), diseased (**B**), and resolution (**C**) samples. Boxes provide estimate of gene and assigned bacterial complex interactions.

Figure 6: Schematic of potential protein interactome among Tfh associated genes. Blue nodes are surface receptors, green nodes are transcription factors, and orange nodes are secreted products. The symbols identify significant positive or negative correlations of the gene with bacterial complexes in healthy (H), diseased (D), and resolution (R) samples from adult and young animal gingival tissues. Both denotes that individual bacteria within the complex demonstrated both significant positive and negative correlations the particular gene.

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