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Immune System Transcriptome in Gingival Tissues of Young Nonhuman Primates

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Abstract

Young/adolescent humans demonstrate many microorganisms associated with periodontal disease in adults and substantial gingival inflammatory responses. However, younger individuals do not demonstrate the soft and hard tissue destruction that hallmark periodontitis. This study evaluated responses to the oral microbial ecology in gingival tissues from clinically healthy young *Macaca mulatta* (<3 years old) compared to older animals (5-23 years old). Global transcriptional profiling of four age groups revealed a subset of 159 genes that were differentially expressed at least across one of the age comparisons. Correlation metrics generated a relevance network abstraction of these genes. Partitioning of the relevance network revealed seven distinct communities comprising functionally related genes associated with host inflammatory and immune responses. A group of genes were identified that were selectively increased/decreased or positively/negatively correlated with gingival profiles in the animals. A Principal Components Analysis created metagenes of expression profiles for classifying the 23 animals. The results provide novel system-level insights into gene expression differences in healthy young tissues weighted towards host responses that were associated with anti-inflammatory biomolecules or those linked with T cell regulation of responses. The combination of the regulated microenvironment may help to explain the apparent "resistance" of younger individuals to developing periodontal disease.

Keywords

Keywords: nonhuman primates; periodontitis; inflammation; aging

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Introduction

The nonhuman primate has been documented as a model of periodontitis that demonstrates extensive similarities in clinical, microbiological and immunological features of human periodontitis (1-7). The human subgingival ecology has been shown to exhibit over 700 species of bacteria (8) and differs both qualitatively and quantitatively in health, gingivitis, and periodontitis (9). Recent studies have demonstrated a very similar microbiota inhabiting the oral cavity of rhesus monkeys (*Macaca mulatta*) (B. Paster, AADR 2014, abst. #1588, personal communication).

It is clear that the oral microbiome is acquired early in life and varies among individuals including the types of commensal bacteria, as well as varies in the quality and quantity of proposed opportunistic pathogens that trigger periodontitis later in life (10-13). There is minimal evidence that these pathogens are acquired exogenously in adults that develop periodontitis, thus, research has attempted to focus identification of risk by examining local inciting or environmental factors that would help trigger the bacterial changes in the disease and identifying genetic polymorphisms that could contribute to dysfunctional responses in the periodontium to the microbial challenge.

However, opportunistic pathogens can emerge in the ecology leading to chronic immunoinflammatory lesions and tissue destructive events. It has been recognized that an individual's oral microbiome is acquired early in life, evolves and matures over some time interval, but clearly becomes an intra-individual autochthonous ecology. In a subset of the human population, and our data support in nonhuman primates as well, this ecological changes either trigger a local disease process in the periodontium, or reflect changes in the oral environment that select for more pathogenic biofilms. Routinely when this process occurs it is as an adult or aged individual. However, we have negligible information regarding the ontogeny of the various innate immune, inflammatory, and adaptive immune response pathways in gingival tissues of young individuals, nor is there any data available that describes how variations in the evolving oral microbiome “drive”, not only the maturation of these pathways at the mucosal sites, but also how these microbial variations can result in dissimilarities in the maturation of host response capabilities in the tissues.

We have been using the nonhuman primate model of periodontitis to explore functional genomics that would be involved in creating a local environment in the gingival milieu related to health, or disease, or increased risk for disease. As such we have been targeting specific molecular pathways to determine the transcriptome in gingival tissues, as a representative mucosal tissue, obtained from animals representing young individuals (approximately 10 year old humans) to aged individuals (approximately 70-80 year old humans) (1, 14-16). These studies have shown significant differences in apoptosis pathway gene expression profiles associated with aging, even in healthy gingival tissues (15, 16). Differences were also noted in inflammasome gene pathways, including both receptors critical for signaling, and downstream effector functions (17), and in antigen processing and presentation pathways (18), all focused on changes with aging that could presage disease risk and account for the increased incidence and severity of disease in aged individuals.

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Based upon the existing literature that supports that young individuals and adolescents harbor many of the oral microorganisms considered to contribute to periodontitis in adults (12, 19, 20), and generally demonstrate a high prevalence of gingivitis, it is very infrequent that they develop destructive periodontitis (20). This report posits that the lack of progression of chronic inflammation in young individuals to a tissue destructive process that is the hallmark of periodontitis, will be reflected by differential expression of genes in response to the bacterial biofilm challenge that are more tissue protective and help maintain the integrity of the tissues even in the presence of persistent inflammation.

Methods

Nonhuman primate model and Oral Clinical Evaluation

Rhesus monkeys (*Macaca mulatta*) (n=34; 14 females and 20 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Healthy animals (5-7/group) were distributed by age into four groups: 3 years (young), 3-7 years (adolescent), 12-16 years (adult) and 18-23 years (aged). A protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, enabled anesthetized animals to be examined for clinical measures of periodontal health including probing pocket depth (PPD), and bleeding on probing (BOP) as we have described previously (21). Health was defined as mean mouth values of PPD <3mm and BOP <1.

The nonhuman primates were typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet was supplemented with fruits and vegetables, and water was provided *ad libitum* in an enclosed corral setting.

Tissue sampling and gene expression microarray analysis

A buccal gingival sample from healthy sites from the premolar/molar maxillary region of each animal was taken using a standard gingivectomy technique, and maintained frozen in RNAlater solution. Total RNA was isolated from each gingival tissue using a standard procedure as we have described and tissue RNA samples submitted to the microarray core to assess RNA quality analyze the transcriptome using the GeneChip® Rhesus Macaque Genome Array (Affymetrix) (16, 22). Individual samples were used for gene expression analyses.

Based upon the microarray outcomes we selected 5 genes and performed a qPCR analysis using a standard technique in our laboratory employing a Roche 480 LightCycler (23). qPCR primers were designed using software PrimerQuest at Integrated DNA Technologies website (www.idtdna.com) and were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Primers were prepared for PSMB8 (forward - GGCGCTGTCATCGATTTCTT; reverse – ATGGCTTTGTAGACGCCTTTC; amplicon 103 bp), IL1A (forward – CTGAAGAAGAGACGGTTGAGTT; reverse – CGACCTGGGCTTGATGATT; amplicon 99 bp), IL22 (forward – GAGCGCTGCTAT CTGATGAA; reverse – GCACCACCTCCTGCATATAA; amplicon 100 bp), IL17F

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(forward - ATCTCCATGAATTCCGTTCCC; reverse – AACAGTCACCAGCACCTTC; amplicon 105 bp), TNFSRSF17 (forward – GGCAGGACTGGTGATGAAA; reverse – GTGGAAAGCAATGGTCAGAATC; amplicon 118 bp) and GAPDH (forward – GGTGTGAACCATGAGAAGTATGA; reverse – GAGTCCTTCCACGATACCAAAG; amplicon 123 bp) genes. The level of message was determined according to our previously published methods (23) and those levels compared across the RNA samples prepared from each of the healthy groups.

Data analysis

Normalization and background subtraction was accomplished using the RMA approach (24). Parametric t-test ($\alpha = 0.01$) was subsequently to determine genes that changed significantly across a given pair of groups. A fold change cut-off (2-fold) was subsequently imposed to eliminate noisy expression profiles. Differentially expressed genes that satisfied the fold-change cut-off at least across one of the six pair-wise comparisons was chosen for subsequent analysis. JMP (version 10.0, SAS Inc., Cary, NC) was used to create metagenes independently of group classification using principal components based on the correlation matrix. The plots are of the first two PCA scores across the healthy tissues. The variability is explained by each of the scores indicated on the plots. The data has been uploaded into the ArrayExpress data base (www.ebi.ac.uk) under accession number: E-MTAB-1977.

Results

Differential gene expression analysis using parametric t-test (p-value < 0.01, fold change 2) across the 4 groups resulted in 159 genes. Relevance network (25) abstraction of the 159 genes was subsequently by connecting the highly correlated genes (Pearson-Correlation, p < 0.01) by an undirected edge. Duplicate genes and those transcripts that were not annotated were dropped from the relevance network abstraction. Yifan-Hu visualization of the relevance network is shown in Figure 1. The giant component of the network comprised of 85 nodes and 235 edges where each node is connected to the other directly or indirectly was subsequently partitioned into distinct communities using the Louvain method for community structure detection (26) implemented in Gephi 8.1 (27). Seven communities with varying connectivity and number of genes were observed (Table 1, Figure 1) in the giant component. Community 1 consisted of 17 genes including PIM1, IGJ and NAP1L3 with degree centralities of 14, 14 and 13, respectively. Interestingly, the IGJ gene codes for the immunoglobulin J polypeptide, which is the linker protein for IgA and IgM polypeptides. It also contributes to binding these immunoglobulins to secretory component at mucosal surfaces. PIM1 oncogene belongs to the serine/threonine protein kinase family and is expressed primarily in B-lymphoid cells and contributes to both cell proliferation and survival (28). NAP1L3 (nucleosome assembly protein 1-like 3) has been suggested to contribute to the RIG-I-like receptor signaling pathway and may have some role in the mucosal immune network (http://immunet-dev.princeton.edu/genes/detail/homo-sapien/NAP1L3/). The average degree centrality of Community 1 was the largest among all the communities (~10) indicating a densely connected community and dominant players in the network. The average expression profile of the genes in Community 1 is shown in Figure 2 and exhibits an increasing trend as a function of age. Community 2 consists of 20 genes with

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average degree centrality (~6). Genes with a high degree of centrality in Community 2 include (SOSTDC1, CD179B, SLC1A1) with degree centralities of 11, 11 and 9, respectively. SOSTDC1 (sclerostin domain containing 1) is a member of the sclerostin family and functions as a bone morphogenetic protein (BMP) antagonist, as well as enhancing Wnt signaling and inhibiting TGFβ signaling (29). CD179b (IGLL1; immunoglobulin lambda-like polypeptide 1) is a receptor found on the surface of pro-B and pre-B cells. It transduces signals for cellular proliferation, differentiation, and allelic exclusion at the Ig heavy chain gene locus, was well as promoting Ig light chain gene rearrangements (30). The SLC1A1 [solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1] gene encodes a member of the high-affinity glutamate and aspartate transporters. The SLC1A1 protein provides cysteine uptake for GI epithelial, neuronal, and immune cells, and its activity is decreased during oxidative stress and, thus, it has been implicated in the intestinal immune network for IgA production (31). The average expression profile of the members in Community 2 is similar to that of Community 1 with a general decrease with adolescence and increasing levels of expression in adult and aged gingival tissues (Figure 2). Of the 17 genes in Community 1, 10 are associated with host immune responses, and Community 2 contains 12 genes with 5 related to host responses and inflammation within this network. The average expression profile across Community 7 also exhibited an increasing trend somewhat similar to that of Communities 1 and 2. In contrast, Communities 3, 4, 5, and 6 exhibit an increased expression in adolescence, and then show a decreasing trend in the average expression profile through adult and aged tissues to levels comparable to young animals (Figure 2). Within these 4 communities, only Community 5 showed 8/12 genes that were related to host immune responses. Finally, a group of 3 genes (IL1A, MUC4, DEFB4A) was also identified as an isolated community that was connected to the giant component. The average expression profile was similar to that of Community 2. Interestingly all three of these genes are intimately associated with host responses in the oral cavity, and were also identified as altered in exploring the immunology array of genes.

Based upon the features of the immune system network of differentially expressed genes in Communities 1, 2, and 5, we explored an array of 511 genes reflecting host innate immune, inflammatory, and adaptive immune responses (target set derived from Human Immunology Kit, NanoString Technologies; http://www.nanostring.com/media/pdf/PDS_nCounter_Human_Immunology.pdf). The results of this targeted gene identification in Figure 3 are displayed in a Volcano Plot that identifies the immune system genes that were differentially expressed in gingival tissues from young animals compared to the other age groups.

Table 2 is a summary of the differentially expressed and aging correlated immune system genes in the healthy gingival tissues. From this analysis we identified 97 genes that were lower in the young [under-expressed and/or significantly positively ($p<0.05$) correlated] and 26 genes that were higher in the young gingival tissues [over-expressed and/or negatively correlated]. Some striking observations can be discerned from this catalogues of changes. First, evaluation of the cytokine/chemokine differences support a more anti-inflammatory milieu in the gingival tissues of the young animals. This is evidenced by elevated expression of anti-inflammatory cytokines/receptors such as IL22, IL17F, IL5, and TGFB1, with

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decreased expression of a range of pro-inflammatory cytokines/receptors (*ie.* IL18, IL1A, IL6, TNFSF13B, CCR5,). Chemokines related to inflammatory and more tissue destructive potential (*eg.* CXCL11, CCL5, CXCL13, CCL19) were all decreased in expression in the young tissues. Decreased transcription factor gene expression was identified for AIRE, CIITA, NFATC1, NFKB1, SKI, SOCS1, and TP53, in the young tissues.

NOD2, a pattern recognition receptor for intracellular infections and linked to proteasome function, levels were increased in young gingival tissues. Proteasome molecules, PSMB5 (proteasome subunit, beta type, 5), a catalytic subunit that is not present in the immunoproteasome and is replaced by catalytic subunit PSMB8 was also increased in the young healthy tissues. Generally genes related to intracellular signaling molecules were decreased in the young tissues, except SMAD5 (SMAD family member 5), involved in TGFβ signaling pathway and TYK 2 (component of both the type I and type III interferon signaling pathways) were elevated in young tissues. Similarly cell communication molecules were generally decreased in young tissues with only NCAM1 and PDGFB decreasing from young to aged tissues. Gene expression of molecules associated with cell development were decreased in young tissues except for EEF1G (eukaryotic translation elongation factor 1 gamma), which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. All of the complement components that were differentially expressed in the healthy tissues were increased with aging. Changes in apoptosis related genes demonstrated that both PDCD2 (programmed cell death 2), encoding a nuclear protein that responds to BCL6 in regulating apoptosis, TNFSF10 (tumor necrosis factor ligand superfamily, member 10, TRAIL) that preferentially induces apoptosis in transformed and tumor cells, and TNFSF15 that acts as an autocrine factor to promote activation of caspases inducing apoptosis (particularly in endothelial cells) were elevated in tissues from the young animals. Finally, a range of antimicrobial peptides were altered in the gingival tissues, with both DEFB4A (human beta defensin 2) and IFIT2 (interferon-induced protein with tetratricopeptide repeats 2) were increased in young gingival tissues compared to the other age groups.

Table 3 provides an evaluation comparing the differences in gene expression using the microarray to those obtained from a set of genes analyzed using qPCR. The results demonstrate that the expression profiles exhibited identical changes indirection of expression with some variation in the absolute magnitude of difference in the young gingival tissues using these two independent assessments.

Figure 4 provides the results of a Principal Components Analysis of the immune system genes in comparing the patterns of gene expression in healthy gingival tissues from the young animals versus the other age groups. The graph suggests a grouping of the young animals based upon this composite metagene; however, the animals from the other age groups tended to be spread across the various quadrants of the plot, suggesting many similarities in gene expression. This is evidenced in that only 29% of the variation in expression is related to the age distribution for expression in healthy gingival tissues. The crucial gene profile determinants of the PC1 and PC2 were evaluated and are displayed in Table 4. The loading values of 69/319 genes (PC1) showed an elevated correlation in distributing the young animals compared to other age groups. Similarly, 41/319 genes

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primarily contributed to the PC2 variation. Of the genes in PC1 59/69 were highly positively correlated with substantial representation of cyotkines/chemokines, transcription factors, receptors, and cell communication molecules. In contrast, PC2 genes were primarily negatively correlated (23/41) displaying a different set of cytokines/chemokines and receptors.

Discussion

Periodontal disease manifests as a persistent inflammatory response of the local tissues that has been suggested to reflect changes in the characteristics of the subgingival microbial ecology at diseased sites (32-34). Additional findings in studies of periodontitis report the increased frequency and severity of disease with aging (35-37), leading to the consideration that periodontitis is a disease of aging related to altered immune functions that occur with increasing prevalence coincident with decades of life in the general population (38), or potentially a reflection of changing oral environments that select for a microbial ecology with greater pathogenic potential.

Of additional interest is the other side of the aging pendulum in which it has been described that gingivitis is nearly universal in children and adolescents, and generally responds well to improved oral hygiene and periodic professional care (20). Gingivitis is the most common and prevalent disease form of the periodontium among children and adolescents with the incidence and severity increasing from childhood to adolescence, reaching a peak prevalence of 80% at 11– 13 years of age (39). However, beyond the small percentage (*eg.* about 0.5-1%) of children/adolescents that express a rather unique form of periodontitis that has been termed localized/generalized juvenile periodontitis, early onset periodontitis, or localized/generalized aggressive periodontitis (40, 41), the destructive form of this chronic inflammation of the gingiva does not generally occur in young individuals. This observation contrasts with the age dependent inflammatory reaction of the gingival tissues that has been related to changes in the qualitative and quantitative microbiome of the dental biofilms, the characteristics of immune responses, hormonal changes, and morphological differences in the periodontium that have been shown to increase the frequency of transition from the reversible inflammation of gingivitis to the irreversible tissue destruction of periodontitis in adults. Of particular interest is that beyond the clinical features of inflammation in the gingiva of children, available data demonstrate the existence of oral bacterial species identified as critical to pathogenic biofilms for periodontitis in the supra- and subgingival plaque of many children (42, 43). Thus, the microbial stimuli for triggering periodontitis are in the ecology, and the individuals respond to accumulation of these bacteria with gingival inflammation, but uniformly do not progress to periodontitis. However, 3 decades later, a large percentage of these children/adolescents will develop periodontitis based upon current epidemiologic evidence (44, 45), and apparently in the absence of extrinsic acquisition of new oral pathogens (46, 47). One interpretation of these observations and the hypothesis to be tested in this study is that the localized response of the gingival tissues in children to the microbial challenge is molecularly different than those responses in adults and results in a non-destructive management of the bacterial population.

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The results of this study demonstrated a range of genes related to innate immunity, inflammation, and adaptive immunity were expressed in gingival tissues of the young nonhuman primates. Using a network analysis strategy on a set of 159 genes from the total microarray analysis, we identified distinctive patterns of communities of networked genes that were differentially expressed in young gingival tissues. These communities demonstrated a high representation of components of immunologic pathways that were expressed in healthy young gingival tissues compared to healthy tissues from other age groups. We then targeted, more specifically a framework of a set of about 511 gene probes that are linked to innate immune, inflammatory, and adaptive immune responses. From this we identified an array of approximately 123 that demonstrated differential expression in young healthy tissues and/or showed a significant correlation related to healthy gingival tissues across the lifespan.

Generally, these gene profiles were identified for cytokines/chemokines, transcription factors, receptors, signaling molecules, cell communication factors, cell development molecules, complement components, apoptosis pathway molecules and antimicrobial peptides. Of the 123 genes, 79% were decreased in young tissues compared with healthy gingiva from other age groups. A high frequency of expression of immune related genes was related to transcription factors where ~35% of the genes that were differentially expressed were increased in the young tissues. These included AIRE (role in immunity by regulating the expression of autoantigens and negative selection of autoreactive T-cells), CIITA (essential for transcriptional activity of the HLA class II promoter), NFATC1 (plays a role in the inducible expression of cytokine genes in T-cells, especially in the induction of the IL-2 or IL-4 gene transcription), NFKB1 (pleiotropic transcription factor present in almost all cell types responds to a vast array of stimuli for many biological processes, including inflammation, immunity, and apoptosis), SKI (a repressor of TGFβ signaling), SOCS1 (part of a classical negative feedback system that regulates cytokine signal transduction), and TP53 (regulates expression of target genes related to cell cycle arrest, apoptosis, and senescence). While the exact relationship among this array of transcription factors is not obvious, it does appear that they relate to T cell regulation of responses, control of anti- and pro-inflammatory responses, and can contribute to increased apoptosis, which we have noted previously in young tissues (16, 48). This was also observed with multiple pro-apoptotic genes that were elevated in the young tissues (PDCD2, TNFSF10, TNFSF15).

A limited array of cytokines/chemokines were at elevated levels in the young gingival tissues. IL17F is expressed by activated T cells, and stimulates the production of cytokines, including IL-6, IL-8, and GM-CSF. IL-5 is anti-inflammatory cytokine synthesized by Th2 immune cells and acts as a growth and differentiation factor for B cells and eosinophils. IL-22 is a member of the IL-10 family of anti-inflammatory cytokines. It plays a role in coordinating adaptive and innate immune responses and primary targets are non-hematopoietic cells including epithelial cells. TGFB1 (transforming growth factor beta 1) is a member of the TGF-beta family of cytokines that regulate proliferation, differentiation, adhesion, and migration of many cell types. It is a potent stimulator of osteoblast functions and considered an anti-inflammatory cytokine. Thus, it appears that some of these unique features of the responses in young gingival tissues are also related to controlling T cell functions and creating a more prominent anti-inflammatory regulatory microenvironment.

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These findings suggest that novel gene patterns could provide some guidance regarding the apparent "resistance" of the periodontium in the young in response to a microbial challenge eliciting an inflammatory response but lacking progression to destructive periodontitis, even in the presence of this clinical/molecular gingival inflammation. However, we still have little understanding of how the acquisition of the oral microbiome contributes to the development and maturation of the immune response repertoire in gingival tissues (49). Knowledge of this process will potentially help to clarify the early tissue alterations that could translate into longer-term risk for disease, as well as focusing efforts on approaches to effectively modulate the microbial acquisition by children to improve long term oral health.

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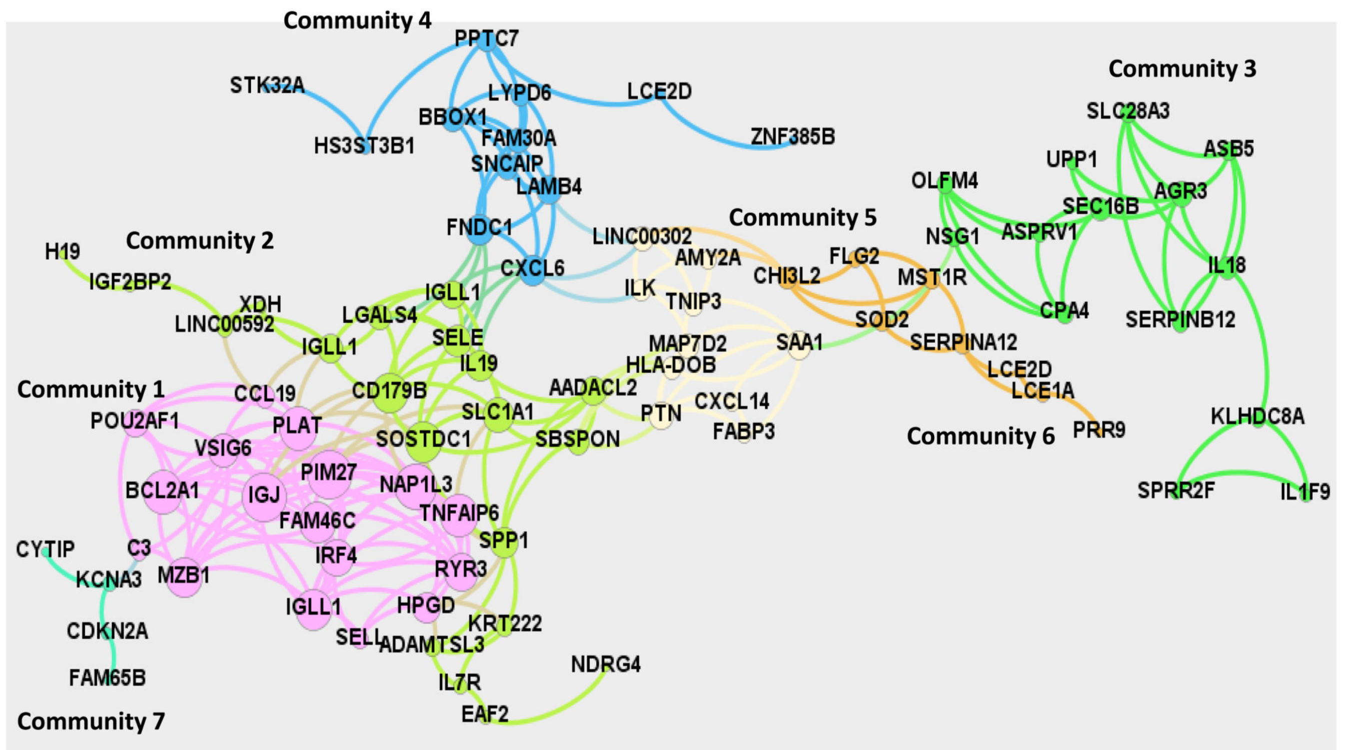

Figure 1.

Yifan-Hu visualization of the relevance network stratified into eight distinct communities. Each point denotes a gene and the number of lines signify the strength of the association of expression across all 4 age groups. Genes within each of the communities are represented by the same color.

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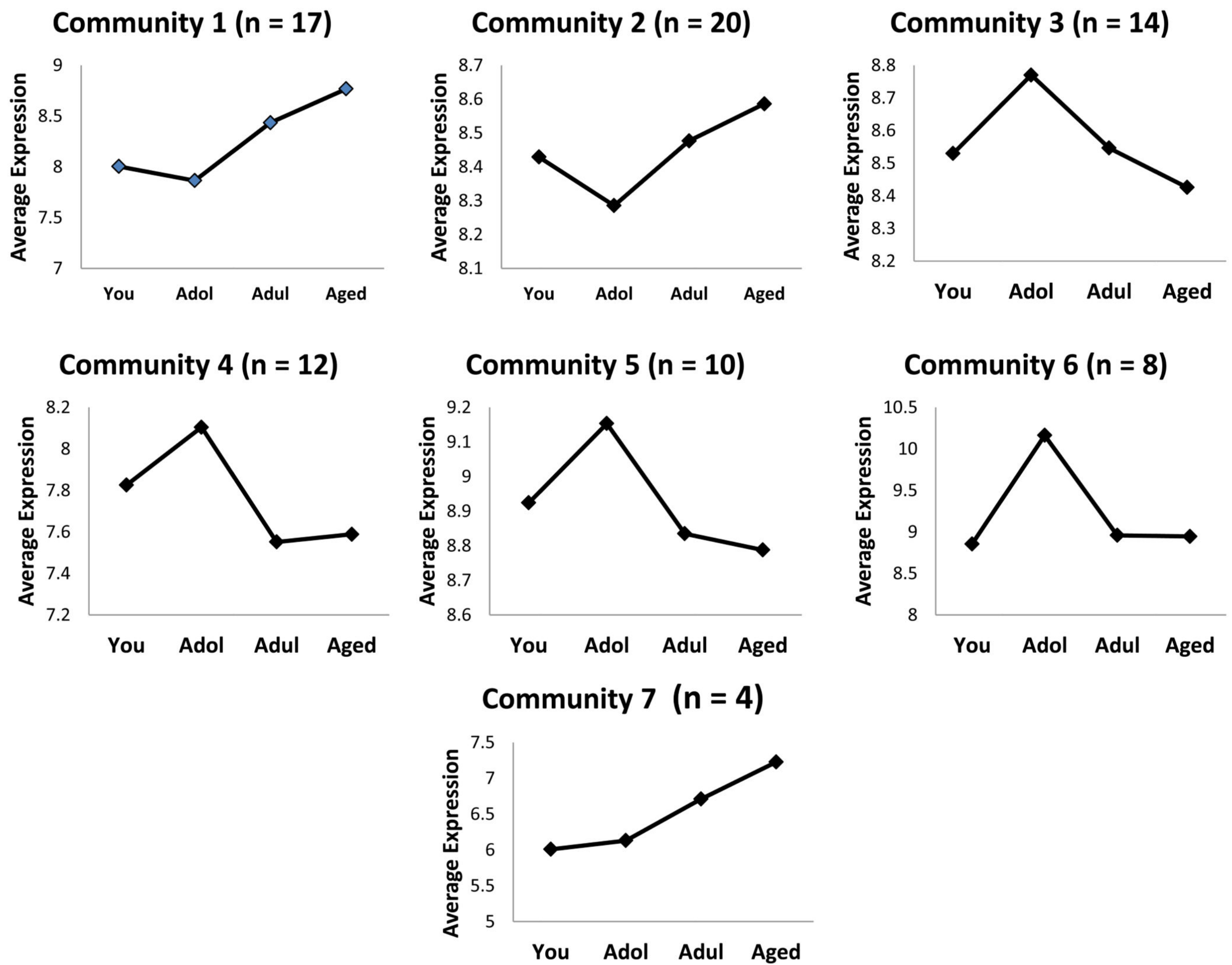

Figure 2.

Average expression profiles of the genes across the age groups of healthy gingival tissues. Points denote mean expression for each group for the genes in each Community.

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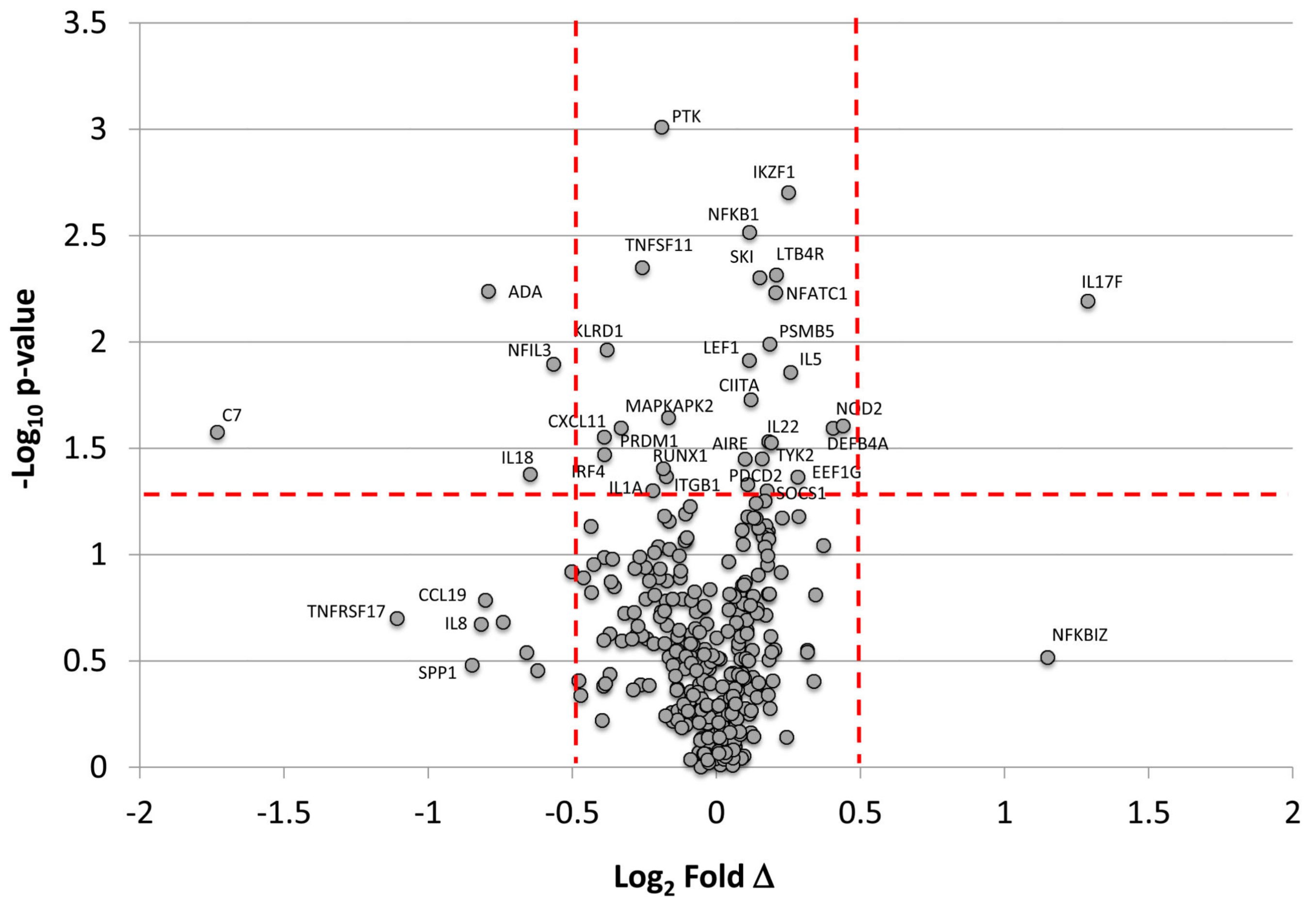

Figure 3.

Volcano plot identifying gene expression profiles between young tissues and all other combined age groups based upon p-value and fold expression. The red horizontal dashed line denotes p-value <0.05 and the vertical dashed lines denote differences in expression between young and other age groups at ±1.4 fold ($\log_2 = \pm 0.5$).

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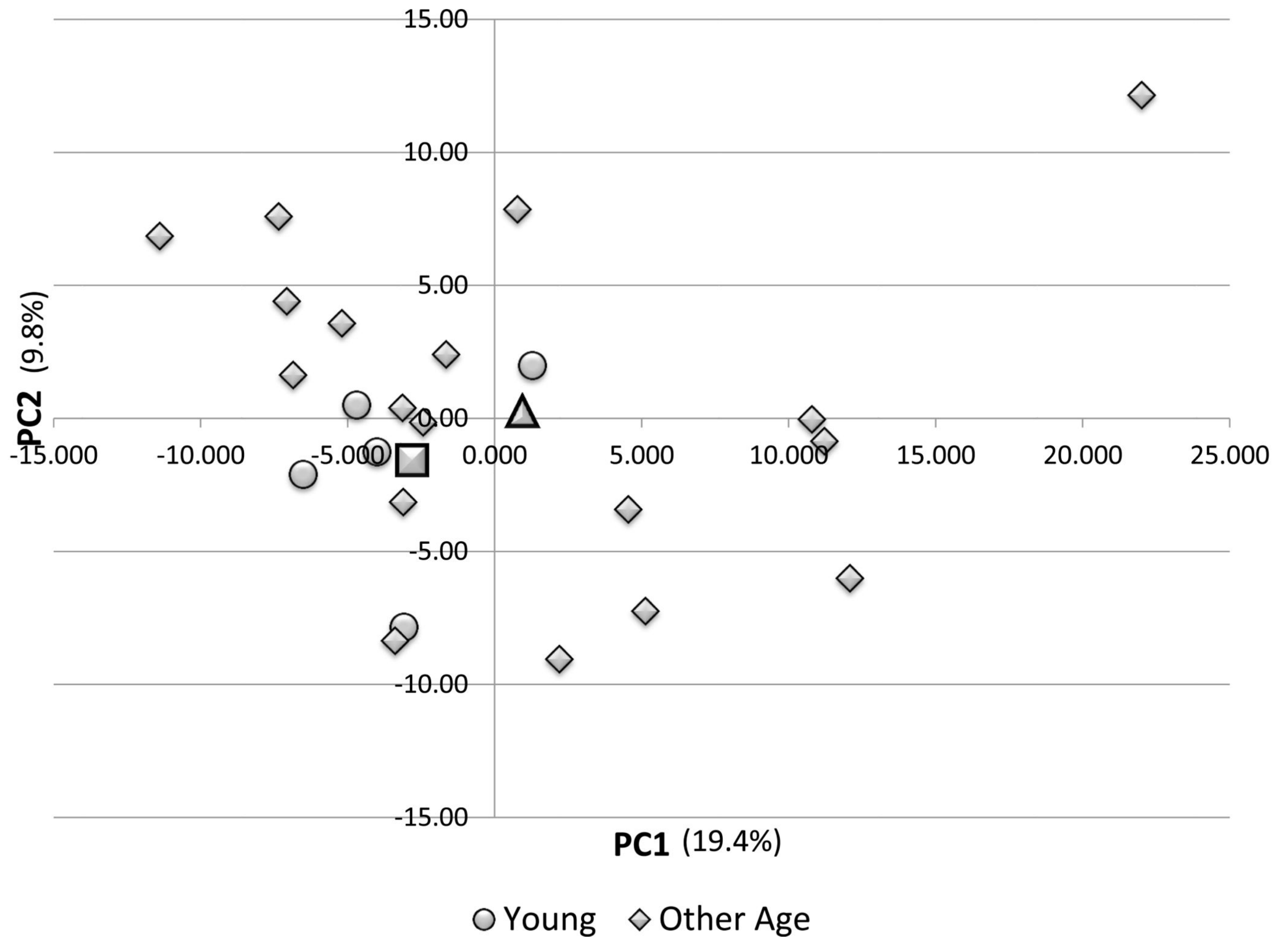

Figure 4.

Principal Components Analysis of immunology gene set for young and other age groups of animals. Each point denotes the PC1 and PC2 metagene position for an animal. The square denotes the mean PC values for the young group and the triangle signifies the mean for the other age groups.s

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Table 1

Listing of genes that were networked in the various communities based upon differences among the age groups in healthy gingival tissues. The asterisk (*) denotes those genes related to host responses and immune functions.

Gene ID	Function
COMMUNITY 1	
PIM27	*Pim-2 oncogene; cell survival
IGJ	*Ig J (joining) chain
NAP1L3	*Nucleosome assembly protein 1-like 3
BCL2A1	*BCL2-related protein; apoptosis IL-3
TNFAIP6	*TNFa induced protein 6; inflammation
PLAT	*Tissue plasminogen activator
MZB1	*Marginal zone B/B1 cell protein
FAM46C	*Family with sequence similarity 46, member C; interferon/viral regulation
IGLL1	*Ig lambda-like polypeptide 1
RYR3	Ryanodine receptor 3; Ca^{+2} homeostasis
IRF4	*Interferon regulatory factor 4 (LOC10042412)
VSIG6	V-set and Ig domain containing 6
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
POU2AF1	*POU class 2 associating factor 1; B cells
CCL19	*Chemokine (C-C motif), ligand 19
C3	*Complement component 3
SELL	*L-selectin
COMMUNITY 2	
SOSTDC1	*Sclerostin domain containing 1; enhances Wnt/inhibits TGFβ
CD179B	*IGLL1, preB cell receptor
SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
SELE	*E-selectin
IL19	*Interleukin 19
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein 1)
AADACL2	*Arylacetamide deacetylase-like 2
IGLL1	*Immunoglobulin lambda-like polypeptide 1 precursor, isoform 7
IGLL1	*Immunoglobulin lambda-like polypeptide 1-like, isoform 4
SBSPON	*Somatomedin B and thrombospondin, type 1 domain containing
LGALS4	Lectin, galactoside-binding, soluble, 4
ADAMTSL3	A Disintegrin-Like And Metalloprotease Domain With Thrombospondin Type I –like 3 (LOC714346)
LINC00592	Long intergenic non-protein coding RNA 592
KRT222	Ketatin 222
IL7R	*Interleukin 7 receptor
XDH	Xanthine dehydrogenase

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Gene ID	Function
EAF2	ELL (elongation factor RNA polymerase II) associated factor 2
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
H19	H19, imprinted maternally expressed transcript (non-protein coding)
NDRG4	N-Myc Downstream-Regulated Gene 4 (LOC712742)
COMMUNITY 3	
AGR3	Similar to breast cancer membrane protein 11
IL18	*Interleukin 18
SEC16B	SEC16 homolog B (similar to regucalcin gene promotor region related protein)
SLC28A3	Solute carrier family 28, member 3
ASB5	Ankyrin repeat and SOCS box-containing 5
SERPINB12	Serpin peptidase inhibitor, clade B (ovalbumin), member 12
CPA4	Carboxypeptidase A4
OLFM4	*Olfactomedin 4
KLHDC8A	Kelch domain containing 8A
ASPRV1	Aspartic peptidase, retroviral-like 1
NSG1	Neuron specific gene family member 1 (D4S234E)
IL1F9 (IL-36g)	*Interleukin 1 family, member 9
SPRR2F	Small proline rich protein 2F (LOC717894)
UPP1	Uridine phosphorylase 1
COMMUNITY 4	
CXCL6	*Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
FNDC1	Fibronectin type III domain containing 1
LAMB4	Laminin, beta 4
SNCAIP	Synuclein, alpha interacting protein
FAM30A (KIAA0125)	Family with sequence similarity 30, member A
BBOX1	Gamma-butyrobetaine hydroxylase 1
PPTC7	*T-cell activation protein phosphatase
LYPD6	LY6/PLAUR domain containing 6
LCE2D	Late cornified envelope 2D (LOC100423831)
HS3ST3B1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1
ZNF385B	Zinc finger protein 385B-like
STK32A	Serine/threonine kinase 32A
COMMUNITY 5	
SAA1	*Serum amyloid A1
PTN	*Pleiotrophin
LINC00302	Long-intergenic non-protein coding RNA 302
MAP7D2	MAP7 domain containing 2
HLA-DOB	*Major histocompatibility complex, class II, DO beta
TNIP3	*Tumor necrosis factor alpha induced protein 3 (TNFAIP3) interacting protein 3

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Gene ID	Function
ILK	*Epithelial Integrin-linked kinase
AMY2A	Amylase, alpha 2A (pancreatic)
FABP3	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
CXCL14	*Chemokine (C-X-C motif) ligand 14
COMMUNITY 6	
CHI3L2	*Chitinase3-like 2
MST1R	*Macrophage stimulating 1 receptor (LOC100423330)
SERPINA12	Serpin peptidase inhibitor, clade A (α1 antiproteinase)
SOD2	*Manganese, superoxide dismutase
LCE1A	Late cornified envelope 1A (LOC713600)
FLG2	Filaggrin family member 2
LCE2D	Late cornified envelope 2D (LOC100423831)
PRR9	Proline rich 9
COMMUNITY 7	
KCNA3	Potassium voltage-gated channel, shaker-related subfamily, member 3
CDKN2A	Cyclin-dependent kinase inhibitor 2A (LOC709988)
FAM65B	Family with sequence similarity 65, member B (LOC715354)
CYTIP	Cytohesin 1 interacting protein

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Table 2

Genes that were significantly over- or under-expressed in gingival tissues between young and other age groups of animals, and those that were significantly positively correlated (decreased in young) or negatively correlated (increased in young) with aging.

Gene	Over	Under	Positive	Negative
Cytokines/Chemokines				
CCL19			0.6140	
CCL20			0.4322	
CCL3			0.4597	
CCL5			0.5583	
CCL8			0.5277	
CXCL11		0.020	0.5550	
CXCL13			0.5584	
IL16			0.5861	
IL17F	0.007			
IL18		0.032		
IL1A		0.040	0.6732	
IL1B		0.033		
IL1RN			0.4761	
IL22	0.033			
IL5	0.013			
IL6			0.6274	
IL7			0.5367	
PPBP			0.5351	
TGFB1				-0.4540
TNFSF13B			0.5694	
Transcription				
AIRE	0.035			-0.6258
CIITA	0.018			
ETS1			0.4680	
GFI1			0.6021	
IKBKB			0.6028	
IKZF1			0.5819	
IRF1			0.4252	
IRF4		0.033	0.6231	
IRF5			0.5550	
LEF1			0.6124	
NFATC1	0.005			-0.4607
NFIL3		0.012		
NFKB1	0.003			-0.4359

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Gene	Over	Under	Positive	Negative
RELA			0.4361	
RUNX1		0.039		
SKI	0.004			
SOCS1				-0.4269
STAT2		0.049		
STAT4			0.4234	
TP53				-0.4908
Receptors				
CCR5			0.4600	
CD14			0.4853	
CD164			0.5668	
CD2			0.6286	
CD27			0.5856	
CD44				-0.5563
CD46			0.4300	
CD48			0.6025	
CD53			0.6063	
CD81				-0.5143
CD82			0.4877	
CD86			0.5423	
CD9			0.4258	
CLEC4E			0.5048	
CSF2RB			0.5327	
CSF3R			0.4318	
CTLA4			0.4918	
CXCR4			0.4365	
ICOS			0.5962	
IFNAR2			0.4696	
IL1R2			0.4802	
IL2RG			0.5369	
IL4R	0.043			
KLRD1		0.010	0.6280	
LTB4R	0.004			
LTBR			0.5662	
LY96			0.6142	
MASP2			0.4263	
NOD2	0.0248			-0.4654
PRDM1			0.5863	
PSMB5	0.010			

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Gene	Over	Under	Positive	Negative
PSMD7			0.4541	
PTAFR			0.7595	
TLR2			0.4581	
TLR4			0.4865	
TMEM173			0.6165	
TNFRSF17			0.5393	
Signaling				
MAPKAPK2		0.022	0.6809	
PTPN22			0.4734	
SH2D1A			0.4304	
SMAD5				-0.7383
SYK			0.4723	
TAGAP			0.6237	
TRAF3			0.4566	
TYK2	0.029			
ZAP70			0.6092	
Cell Communication				
ICAM1			0.4560	
ICAM2			0.5432	
ICAM3			0.5057	
ITGA6			0.4463	
ITGB1		0.042		
ITGB2			0.5712	
NCAM1				-0.6141
PDGFB				-0.5024
PTK2		0.0009	0.4439	
SELE			0.5393	
SELL			0.5939	
SPP1			0.5130	
TNFAIP6			0.4670	
Cell Development				
ADA		0.003	0.6407	
BATF			0.7221	
CSF1			0.4530	
EEF1G	0.036			-0.5507
G6PD			0.4359	
HFE			0.4957	
LCP2			0.5376	
MME			0.4448	

Gene	Over	Under	Positive	Negative
MS4A1			0.4275	
OAZ1			0.6009	
TNFSF11		0.004	0.6345	
Complement				
C1QA			0.5234	
C1S			0.4498	
C7		0.026	0.7253	
Apoptosis				
CASP10			0.4783	
CLU			0.4359	
PDCD1LG2			0.4224	
PDCD2	0.050			-0.4793
TNFSF10				-0.4293
TNFSF15				-0.4691
Antimicrobial				
CAMP			0.4756	
CTSC			0.5175	
CYBB			0.4818	
DEFB4A	0.025			
IFIT2				-0.5051
IFNB1			0.5326	

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Table 3

Comparison of gene expression profiles using qPCR and microarray analyses. Values represent fold-difference comparing Young Healthy to Adult Healthy tissue message levels assigned a value of 1.0.

Gene ID	Fold Difference
PSMB5	
qPCR	1.50 ± 0.15
GeneChip	1.38 ± 0.11
IL1A	
qPCR	-1.69 ± 0.11
GeneChip	-2.36 ± 0.08
IL22	
qPCR	11.94± 5.53
GeneChip	3.59 ± 0.33
IL17F	
qPCR	20.30 ± 12.72
GeneChip	2.43 ± 1.17
TNFSRSF17	
qPCR	-1.88 ± 2.04
GeneChip	-2.15 ± 0.14
CXCR4	
qPCR	2.01 ± 0.38
GeneChip	1.38 ± 0.12
SAA1	
qPCR	3.56 ± 0.21
GeneChip	1.81 ± 0.16

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Table 4

Gene expression contribution to Principal Component separation of gingival tissue profiles in young versus other age groups of animals. The values denote loading values for the PC analysis and are listed from highest positive to lowest negative value in each category. Data are presented on genes derived from all 319 evaluated with positive values 0.6 and negative values -0.4

Gene ID	PC1	Gene ID	PC2
Cytokines/Chemokines		**Cytokines/Chemokines**	
CCL19	0.8780	CXCL12	0.7486
IL16	0.8559	IL1B	0.7296
TNFSF13B	0.8558	IL2	0.6187
IL6	0.8216	IL28B	0.6058
CXCL13	0.7823	IL1A	-0.4395
CCL5	0.6851	CXCL11	-0.5044
TNFSF8	0.6403	IL1RN	-0.5748
IL7	0.6270	IL7	-0.6532
IL12B	0.6265	**Transcription Factors**	
CXCL10	0.6053	IRF4	0.6059
Transcription Factors		TBP	0.6573
IRF8	0.9001	NFKBIZ	-0.4875
IKZF1	0.8905	**Receptors**	
GFI1	0.7589	CD58	0.8584
IRF1	0.7531	IL11RA	0.7479
STAT4	0.6977	IL2RA	0.7279
LEF1	0.6913	FCGRT	0.6945
STAT2	0.6376	FCER1A	0.6427
JAK2	-0.5575	RORC	0.6294
Receptors		TGFBR2	0.6291
CD53	0.9094	TGFBR1	0.6094
CTLA4	0.9032	IL1R1	-0.4092
IL2RG	0.8815	B2M	-0.4193
LY96	0.8763	CD82	-0.4461
CD74	0.8747	PSMB10	-0.4668
ICOS	0.8617	TNFRSF1B	-0.4859
TNFRSF17	0.8486	PSMB5	-0.6201
CCR7	0.8464	PSMD7	-0.6984
CD2	0.8218	TLR7	-0.7420
IFNAR2	0.8183	**Signaling**	
CD27	0.8135	IRAK1	0.6093
CXCR4	0.7999	IL1RAP3	0.6052
TMEM173	0.7605	SMAD3	-0.5263

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Gene ID	PC1	Gene ID	PC2
KLRD1	0.7374	MAP4K4	-0.7219
CLEC4E	0.7113	UBE2L3	-0.5158
CSF1R	0.6909	**Cell Communication**	
TLR4	0.6841	ICAM3	-0.6066
CXCR3	0.6469	**Cell Development**	
CD209L2	0.6260	FYN	0.6619
CD83	0.6059	KIT	0.6189
PSMB5	-0.4194	OAZ1	-0.4615
CD44	-0.5552	**Apoptosis**	
CD36	-0.6934	CDKN1A	-0.4657
Signaling		BCL3	-0.4719
MAPKAPK2	0.7262	PDCD2	-0.5063
PTPN22	0.7410	**Antimicrobial**	
ARHGDIB	0.6972	CTSG	-0.4105
TAGAP	0.8666	DEFB4A	-0.4847
SMAD5	-0.4312		
Cell Communication			
ICAM2	0.8101		
ITBG2	0.8869		
SPP1	0.7957		
ENTPD1	0.7123		
SELE	0.6757		
SELL	0.6010		
ITGA6	-0.4206		
NCAM1	-0.6459		
Cell Development			
LCP2	0.8750		
MS4A1	0.7718		
BTK	0.8517		
BATF	0.6428		
EEF1G	-0.4622		
Complement			
C1S	0.7640		
C1QA	0.8634		
SERPING1	0.7395		
C5	-0.6314		
Apoptosis			
BCL3	0.6031		
PDCD1LG2	0.6668		

Gene ID	PC1	Gene ID	PC2
PCDC2	-0.4150		
Antimicrobial			
CYBB	0.9140		
IFITM1	0.8173		
IFI35	0.7135		

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