Gene expression profiling of periodontitis-affected gingival tissue by spatial transcriptomics

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Periodontitis is a highly prevalent chronic inflammatory disease of the periodontium, leading ultimately to tooth loss. In order to characterize the gene expression of periodontitis-affected gingival tissue, we have here simultaneously quantified and localized gene expression in periodontal tissue using spatial transcriptomics, combining RNA sequencing with histological analysis. Our analyses revealed distinct clusters of gene expression, which were identified to correspond to epithelium, inflamed areas of connective tissue, and non-inflamed areas of connective tissue. Moreover, 92 genes were identified as significantly up-regulated in inflamed areas of the gingival connective tissue compared to non-inflamed tissue. Among these, immunoglobulin lambda-like polypeptide 5 (IGLL5), signal sequence receptor subunit 4 (SSR4), marginal zone B and B1 cell specific protein (MZB1), and X-box binding protein 1 (XBP1) were the four most highly up-regulated genes. These genes were also verified as significantly higher expressed in gingival tissue of patients with periodontitis compared to healthy controls, using reverse transcription quantitative polymerase chain reaction. Moreover, the protein expressions of up-regulated genes were verified in gingival biopsies by immunohistochemistry. In summary, in this study, we report distinct gene expression signatures within periodontitis-affected gingival tissue, as well as specific genes that are up-regulated in inflamed areas compared to non-inflamed areas of gingival tissue. The results obtained from this study may add novel information on the genes and cell types contributing to pathogenesis of the chronic inflammatory disease periodontitis.

Periodontal disease is initiated by a biofilm forming in connection to the gingiva, and its release of substances such as lipopolysaccharides and toxins. This process elicits a local immune response with subsequent stimulation of various cells and production of pro-inflammatory mediators, including chemokines, cytokines, matrix metalloproteinases, and prostaglandins, which leads to tissue and bone destruction10–12. A few studies have reported on gene expression in gingival tissue from patients with periodontitis, using microarrays or RNA sequencing (RNA-seq)13–17. These studies have revealed differences in gene expression between periodontitis-affected and healthy tissue, where gene expression related to inflammation, apoptosis, and cell death are strongly represented in periodontitis-affected tissue13–17.

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However, a drawback with standard RNA-seq used in the above-mentioned studies is that the experiments are performed on whole biopsies and therefore produce an average of the gene expressions of all cells present in the tissue. Thus, it is not possible to localize and associate gene expression with corresponding cells in the tissue. Therefore, the aim of this study was to analyze the gene expression in periodontitis-affected gingival tissue using a novel spatial transcriptomic sequencing method that allows for analysis of gene expression in a tissue section, simultaneously associating gene expression with specific cell types.

**Results**

**Gene activity in different compartments of the gingival tissue.** To investigate the gene activity in the gingival tissue section, the amount of transcripts detected in each spot was visualized in the context of their localization within the tissue. Our results, visualized in Fig. 1, revealed that the gene activity was highest in the epithelium compartment and in the areas of connective tissue with infiltrating inflammatory cells. The gene activity was lowest in areas of the connective tissue without inflammatory cells (Fig. 1). In total, the analysis identified 15,965 unique genes expressed within the gingival tissue section.

**Specific gene expression signatures within the gingival tissue.** To explore gene expression profiles within the tissue section, the dimension reduction technique t-distributed stochastic neighbor embedding (t-SNE) was applied to the obtained gene expression data. Three distinct signatures of gene expression could be identified by the t-SNE analysis as demonstrated in Fig. 2a. Each dot in the t-SNE projection represents the gene expression of a single spot in the spatial transcriptomics array. To identify the corresponding tissue of each dot, the dots were first color-coded according to their clustering in the t-SNE and thereafter visualized according to their coordinates on the spatial transcriptomics array and overlaid an image of the tissue. The results of the analysis revealed that the three gene expression signatures were attributed to three distinct regions in the tissue section; the epithelium, areas in the connective tissue with infiltrating inflammatory cells, and areas in the connective tissue without infiltrating inflammatory cells (Fig. 2b).
Gene expression in inflamed and non-inflamed connective tissue. In order to investigate genes differentially expressed between inflamed and non-inflamed areas of the connective tissue obtained from a patient with periodontitis, gene expression from spots covered by connective tissue with infiltrating inflammatory cells were compared to gene expression from spots covered by connective tissue without inflammatory cells. Differential expression analysis revealed ninety-two genes that were up-regulated, as well as three genes that were down-regulated, in inflamed areas as compared to the non-inflamed areas. The ten most highly up-regulated genes, as well as the three down-regulated genes, are demonstrated in Table 1. The four genes up-regulated to the greatest extent in the areas of inflammatory cells, compared to the areas without infiltrated inflammatory cells, were immunoglobulin lambda-like polypeptide 5 (IGLL5), signal sequence receptor subunit 4 (SSR4), marginal zone B and B1 cell specific protein (MZB1) and X-box binding protein 1 (XBP1). Distributions of the gene activities of the top four genes were visualized on top of the tissue image as demonstrated in Fig. 3, which confirmed elevated expression of these three genes in areas of the connective tissue with inflammatory cells, compared to connective tissue without infiltrated inflammatory cells.
The biological processes of the up-regulated genes were further investigated (Table 2). The gene expression distribution of immunoglobulin lambda-like polypeptide 5 (IGLL5), signal sequence receptor subunit 4 (SSR4), marginal zone B and B1 cell specific protein (MZB1) and X-box binding protein 1 (XBP1) are shown. Yellow spots correspond to low expression of IGLL5, SSR4, MZB1, or XBP1, whereas red spots correspond to high gene expression. All count values have been normalized and log2 transformed.

![Figure 3](https://www.nature.com/scientificreports/)

**Figure 3.** Distribution of gene activity of the four genes up-regulated to the greatest extent in areas of inflammatory cells compared to areas without inflammatory cells. The gene expression distribution of immunoglobulin lambda-like polypeptide 5 (IGLL5), signal sequence receptor subunit 4 (SSR4), marginal zone B and B1 cell specific protein (MZB1) and X-box binding protein 1 (XBP1) are shown. Yellow spots correspond to low expression of IGLL5, SSR4, MZB1, or XBP1, whereas red spots correspond to high gene expression. All count values have been normalized and log2 transformed.

| GO term                  | Biological process                                                                 | Fold enrichment | Adjusted p-value |
|--------------------------|------------------------------------------------------------------------------------|-----------------|------------------|
| GO:0006614               | SRP-dependent cotranslational protein targeting to membrane                         | 19.8            | 1.34E-6          |
| GO:0002474               | Antigen processing and presentation of peptide antigen via MHC class I              | 43.5            | 4.17E-5          |
| GO:0002480               | Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent | 103.7           | 2.52E-5          |
| GO:0034976               | Response to endoplasmic reticulum stress                                           | 19.9            | 2.85E-5          |
| GO:0000184               | Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay                 | 14.1            | 3.65E-5          |
| GO:0006413               | Translational initiation                                                           | 12.3            | 8.98E-5          |
| GO:0036498               | IRE1-mediated unfolded protein response                                            | 22.1            | 7.99E-5          |
| GO:0043066               | Negative regulation of apoptotic process                                           | 5.7             | 8.03E-5          |
| GO:0019083               | Viral transcription                                                                | 13.3            | 1.97E-4          |
| GO:0006412               | Translation                                                                        | 7.4             | 6.42E-4          |

**Table 2.** The top ten most significantly enriched biological processes in areas of inflammatory cells. The ten gene ontology (GO) categories, with regard to biological processes, that were identified as most significantly associated with genes up-regulated in inflamed areas compared to non-inflamed gingival connective tissue. GO term, biological process, fold enrichment and Benjamini-Hochberg adjusted p-value are demonstrated.

The biological processes of the up-regulated genes were further investigated (Table 2). The three most significantly enriched categories in the inflamed areas compared to the non-inflamed areas were SRP-dependent cotranslational protein targeting to membrane (GO:0006614), antigen processing and presentation of peptide antigen via MHC class I (GO:0002474), and antigen processing and presentation of exogenous peptide antigen via MHC independent (GO:0002480). The ten most significantly enriched gene ontology categories among the up-regulated genes are demonstrated in Table 2.

**Validation of up-regulated genes and their protein products.** In order to validate genes as differentially expressed between inflamed and non-inflamed areas of the connective tissue, we also investigated gene expression levels in gingival biopsies obtained from patients with periodontitis and healthy controls using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The levels of the four most highly up-regulated genes, IGLL5, SSR4, MZB1 and XBP1, were investigated in gingival RNA samples from ten subjects (six with periodontitis and four healthy controls). All four genes were significantly up-regulated (p < 0.05) in the gingival biopsies from patients with periodontitis compared to controls (Fig. 4).

Furthermore, we explored the protein expression of the most highly up-regulated genes, using immunohistochemistry. The immunohistochemical analysis confirmed that SSR4, MZB1 and XBP1 proteins were expressed in gingival tissue from patients with periodontitis (Fig. 5). The proteins SSR4 and XBP1 were observed in both epithelium and connective tissue, whereas MZB1 localized mainly in connective tissue in the areas with inflammatory cells. The protein product for IGLL5 could not be validated because specific antibodies were not commercially available. Control stainings with the isotype-matched irrelevant antibodies were negative (figure not shown).
The immune response to periodontitis involves the up-regulation of genes related to protein production and control. This process includes the processing and trafficking of secreted and membrane-bound proteins, which are key components of the immune response. The most highly up-regulated genes in the inflamed area were involved in B-cell development. The MZB1 protein regulates surface presentation and secretion of IgMs.

Regarding the expression of genes in periodontitis-affected gingival tissue, our analysis revealed 442 genes that were up-regulated in inflamed areas, whereas no genes could be identified as up-regulated in non-inflamed areas. This is likely due to the higher density of cells in the inflamed area compared to the non-inflamed area. The four most highly up-regulated genes in the inflamed area were IGLL5, SSR4, MZB1, and XBP1. The genes IGLL5 and MZB1 have previously been reported as up-regulated in gingival tissue from patients with periodontitis. However, little is known about the function of IGLL5, which is homologous to IGLL1, and MZB1.

In response to inflammation, the inflamed regions have a larger activity in the inflamed area than in the non-inflamed area. The four genes most up-regulated in the inflamed area, compared to the non-inflamed area, were IGLL5, SSR4, MZB1, and XBP1. The genes IGLL5 and MZB1 have previously been reported as up-regulated in gingival tissue from patients with periodontitis. However, little is known about the function of IGLL5, which is homologous to IGLL1, and MZB1. The fourth most up-regulated gene, XBP1, is a transcription factor reported to be necessary for differentiation of plasma cells and its protein expression is required for the transcription of class II major histocompatibility genes. The most highly enriched gene ontology terms associated with the genes up-regulated in the inflamed area were related to functions associated with localization and targeting to the ER, which is responsible for the folding, processing, and trafficking of secreted and membrane-bound proteins.
The drawback with standard RNA-seq is that it is performed on whole biopsies and it is not possible to localize the gene expression within the biopsy without post-hoc tests such as fluorescent in situ hybridization, or investigating the genes’ protein products using immunohistochemistry, which are low-throughput techniques in

**Figure 5.** Protein expression in gingival tissue biopsies by immunohistochemistry. Gingival tissue sections obtained from patients with periodontitis were stained for signal sequence receptor subunit 4 (SSR4), marginal zone B and B1 cell specific protein (MZB1) and X-box binding protein 1 (XBP1). The sections were counterstained with Mayer’s hematoxylin. Images were taken with x20 objective. Representative images of gingival biopsies from four patients with periodontitis are shown. Scale bar: 50μm. CT: connective tissue; E: epithelium; IC: inflammatory cells.
comparison to RNA-seq. The strength of the current study is that we can simultaneously quantify and localize gene expression within gingival tissue in a high-throughput manner.

In summary, in this study we report distinct gene expression signatures within periodontitis-affected gingival tissue, as well as specific genes that are up-regulated in inflamed areas compared to non-inflamed areas of gingival tissue using the novel technology spatial transcriptomics. The results obtained from this study may improve our understanding of the genes and cell types contributing to the chronic inflammatory disease periodontitis.

**Methods**

**Ethics approval.** This study was performed in accordance with the Declaration of Helsinki and current Swedish legislation. The gingival tissue collection was approved by the Regional Ethics Board in Stockholm with reference number 2008/1935 – 31/3 and written informed consent was obtained from all individuals included in the study.

**Gingival tissue collection.** Gingival tissue biopsies were collected from subjects with periodontitis and healthy controls without periodontitis as previously described17,32. The inclusion criteria for periodontitis comprised tooth sites with probing depth \(\geq 6\) mm, clinical attachment level \(\geq 5\) mm, and bleeding on probing. Healthy control subjects showed no signs of periodontal disease, with no gingival inflammation, a probing depth \(\leq 3.0\) mm, a clinical attachment level \(\leq 3.5\) mm, and no bleeding on probing17.

**Generation of sequencing libraries.** Sequencing libraries were prepared as previously described33,34. Briefly, the gingival biopsies were snap-frozen, embedded in OCT and sectioned on a cryostat at a thickness of 10 \(\mu\)m. Six sections were selected and mounted on a glass slide where barcoded probes had been deposited in 1,007 spots in an array-format. The tissue sections were fixed with formaldehyde, stained with haematoxylin and eosin, and imaged under a microscope. For subsequent analysis, the section of the tissue with most intact morphology was chosen. Following permeabilization of the tissue section, reverse transcription was performed in situ with the probes attached on the glass slide functioning as primers, resulting in complementary DNA (cDNA) coupled to the barcoded probes on the array. The tissue was thereafter enzymatically digested and removed from the glass slide, after which the probes were enzymatically cleaved. The barcoded cDNA was collected and libraries were generated, which were sequenced on the Illumina NextSeq platform using paired-end sequencing.

**Sequence alignment and generation of gene expression database.** For each pair of reads, one read contained the positional barcode and the other contained the gene transcript. The read containing the positional barcode was converted into its corresponding x and y coordinates on the array, whereas the read containing the transcript was aligned against the human genome. The gene alignment and x and y coordinates were added to a database and this database was used for all following analyses. Detailed information on sequence processing is available in a recently published study34.

**Statistical analysis and visualization of gene expression data.** A series of analyses investigating the gene expression together with the spatial information was conducted. All analyses were performed using R software, version 3.3.335, if not otherwise specified. The data was filtered to only contain gene expression from spots covered by tissue. For analyses associating gene expression with morphology, spots were plotted according to their x and y coordinates and overlaid on an image of the tissue section, so that the spots appeared on their original positions in the tissue section.

First, the total amount of gene transcripts in each spot was visualized by plotting the spots color-coded according to their total transcript content (yellow to red corresponding to low to high transcript content). For this analysis, log2 transformed gene expression data was used. In the next analysis, a truncated singular value decomposition (SVD) was applied to the gene expression data, followed by t-distributed stochastic neighbor embedding (t-SNE), using the scikit-learn package in python36. The t-SNE algorithm was employed since it is widely used for single-cell RNA-seq data analysis37-41, which is more similar to our spatial transcriptomics data than is standard RNA-seq data. In addition, this dimension reduction technique has previously been reported to model spatial transcriptomics RNA-seq data well34. The truncated SVD decomposed the gene expression data into ten components, which together explained 98.19% of the total variance, and these components were used as input for the t-SNE. Three clusters of gene expression patterns were readily visible after constructing a t-SNE plot, and k-means clustering was run to assign the spots to three groups. The groups were assigned a color each and the spots were thereafter plotted and overlaid onto the image of the tissue section, as described above.

Differential gene expression analysis was performed between spots covered by inflamed connective tissue, and spots covered by non-inflamed tissue, using DESeq242, with a modified zero-tolerant computation of the geometric mean and an adjusted p-value cutoff of 0.05. For this analysis, an additional adjacent section from the same biopsy was included. Moreover, due to that the inflamed connective tissue had markedly higher transcript content per spot than the non-inflamed areas, the transcript counts were summed for the spots in the non-inflamed connective tissue in each section. In the DESeq2 analysis, the two summed groups of spots from the non-inflamed areas were treated as two samples, and all spots in the inflamed areas were considered a sample each. The distribution of gene expression for the three most highly up-regulated genes was visualized at the corresponding locations in the tissue section. Log2 transformed normalized counts (as obtained from the DESeq2 analysis) was plotted for each gene, color-coded yellow to red, corresponding to low to high gene expression.

Gene ontology category analysis was performed on the differentially expressed genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool44 with the “GOTERM_BP_ALL” option selected.
Quantitative RT-qPCR. Total RNA was isolated from gingival tissue biopsies of ten subjects with and without periodontitis (six patients with periodontitis and four healthy controls) as previously described\(^1\) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The amount of total RNA was quantified using a Qubit spectrophotometer (Molecular Probes, Eugene, Oregon, USA). cDNA synthesis was performed from 1 μg of total RNA per 20 μl of reaction using the iScript\textsuperscript{TM} cDNA Synthesis Kit (BioRad, Hercules, CA, USA), according to the manufacturer’s instructions. The gene expression analysis was performed by quantitative reverse transcription PCR (qRT-PCR) using TaqMan Gene Expression Assays together with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). TaqMan probes were used as follows: IGLL5 (Hs04330879_g1), MZB1 (Hs00196721_m1), MZB1 (Hs00414907_m1), XBP1 (Hs00231936_m1) and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1). All qRT-PCR reactions were run in duplicates on the 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was calculated according to the \( \Delta\Delta \text{Ct} \) method, where each periodontitis sample was normalized to the healthy control sample and to GAPDH (reference gene) of corresponding sample. Statistical significance was calculated using Wilcoxon signed rank test and \( p<0.05 \) was considered significant.

Immunohistochemistry staining. Gingival tissue samples were fixed and embedded, following standard procedures as previously described\(^1\). For immunohistochemistry experiments, the sections (4 μm thick) were deparaffinised and antigen retrieval was performed using citrate buffer, pH 6.0. The R&D Systems Cell and Tissue Staining Kit (CTS005) was used for the blocking steps, secondary antibodies and chromogenic staining. Primary antibodies for SSFR1 (1:200, Atlas Antibodies, Stockholm, Sweden), MZB1 (1:500, Atlas Antibodies, Stockholm, Sweden), and XBP1 (1:500, Abcam, Cambridge, UK) were applied overnight. All washing steps and incubations with primary antibodies were performed in PBS with 0.1% saponin. The sections were counterstained with Mayer’s hematoxylin (Histolab Products AB, Gothenburg, Sweden), dehydrated and mounted with PERTEX\textsuperscript{®} (Histolab Products AB, Gothenburg, Sweden). All steps were carried out at room temperature. As negative isotype control polyclonal rabbit IgG (0.0005 μg/ml, DAKO, Agilent Technologies, Santa Clara, CA, USA) was used.

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Author Contributions
A.L. wrote the manuscript together with T.Y.L. The authors N.G., T.B., A.J., A.M., and J.L. contributed to the writing process and reviewed the manuscript. T.Y.L., J.L., T.B., designed and supervised the clinical part of the study. A.L. performed experiments for spatial transcriptomics parameter optimizations and N.G. performed qRT-PCR and immunohistochemistry experiments. A.M., A.J. and F.S. performed the final spatial transcriptomics experiment and library preparation. A.L. together with N.G. analyzed the data.

Additional Information
Competing Interests: The authors declare no competing interests.

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