

IL-34 Expression in Gingival Fibroblasts, Gingival Crevicular Fluid and Gingival Tissue

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ABSTRACT

IL-34 is a protein associated with bone degenerative diseases but the role in periodontal disease is unknown. The aim of this study was to assess the expression of IL-34 in primary human gingival fibroblasts (GF) and investigate if the expression is regulated by the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α). We also investigated if IL-34 is detectible in gingival crevicular fluid (GCF) in healthy, gingivitis and periodontitis sites. Furthermore, we examined if healthy and inflamed gingival tissue contains IL-34.

GF were stimulated by IL-1 β 300 pg/ml and TNF- α 10 ng/ml. IL-34 mRNA was measured by quantitative real-time PCR (qPCR). GCF was collected from 11 healthy, 10 gingivitis, and 21 periodontitis gingival crevices. IL-34 protein was quantified using enzyme-linked immunoabsorbent assays (ELISA). Healthy and inflamed gingival tissue biopsies were collected and examined using immunohistochemistry (IHC).

IL-34 mRNA was expressed in GF and the expression was enhanced 12x fold-change versus control by TNF- α 10 ng/ml and 4x fold-change versus control by IL-1 β 300 pg/ml. IL-34 was also present in GCF but no significant difference in IL-34 protein was detected between the healthy, gingivitis, and periodontitis groups. Healthy and inflamed gingival tissue showed equal amounts of IL-34 protein in the epithelium while sub-epithelially the inflamed tissue showed higher levels of IL-34 protein.

Pro-inflammatory cytokines stimulate IL-34 mRNA expression in GF. IL-34 protein is present in GCF and gingival tissue which demands further investigation about the eventual role of IL-34 in the pathogenesis of periodontitis.

INTRODUCTION

The periodontium is the support system that nourishes and attaches the teeth to the jaw bone and it consists of the gingiva, the periodontal ligament (PDL), the root cementum and the alveolar bone. The PDL consist of mainly collagen fibers that attach the root to the alveolar bone. The most abundant cells in the periodontium are the gingival fibroblasts (GF), which are present in the gingival connective tissues (Lindhe *et al.*, 2008).

The most common disease affecting the periodontium is gingivitis. An inflammation of the soft gingival tissue initiated by bacteria in tooth associated biofilm (Dahlén *et al.*, 2008). In some individuals gingivitis progress to periodontitis, an inflammatory degeneration of the periodontium including loss of alveolar bone and ultimately tooth loss (Schätzle *et al.*, 2004). Some individuals can harbor gingivitis for long time periods without any loss of tooth support and it is unknown why certain individuals are more prone to get periodontitis (Löe *et al.*, 1986).

Inflammation induced bone loss is a common factor in diseases like periodontitis and rheumatoid arthritis (RA) (Lerner, 2006). The molecular mechanisms for tissue degeneration in periodontitis are not completely understood, but the general view is that cytokines, kinins and prostaglandins are locally responsible for activation of bone resorbing osteoclasts. It is generally assumed that infiltrating leukocytes produces osteoclast-stimulatory cytokines. However, resident cells such as gingival- and periodontal ligament fibroblasts are also able to produce such cytokines. These cells have been found to express osteotropic cytokines, similar to synovial fibroblasts in the joint capsule. It is previously reported that human gingival fibroblasts can produce pro-inflammatory cytokines such as interleukin-6 (IL-6) and IL-11 in response to IL-1 β , tumour necrosis factor alpha (TNF- α) and toll-like receptor stimulation (Souza *et al.*, 2013).

Bone resorbing osteoclasts originates from monocyte/macrophage cell lineage. Macrophage colony-stimulating factor (M-CSF) supports osteoclast precursor survival through its receptor on the macrophage/osteoclast, colony-stimulating factor 1 receptor (CSF-1R). The receptor activator of nuclear factor κ B (RANKL) is the key molecule, which in concert with M-CSF induces osteoclast differentiation and function by binding to receptor RANK on osteoclast progenitor cells (Souza *et al.*, 2013). RANKL can be blocked by the decoy receptor osteoprotegerin (OPG) (Krajewski *et al.*, 2009). Pro- and

anti-inflammatory cytokines, among other paracrine and endocrine factors can control genesis and activity of osteoclasts via the RANKL/RANK/OPG-system (Lerner, 2006).

It has been shown that the group of cytokines having resorption stimulating capacity includes IL-1 α and β , IL-6, IL-11, IL-17, TNF- α , leukemia inhibitory factor and oncostatin M. Because some cytokines have inhibitory capacity on the resorption process, e. g. IL-4, IL-10, IL-13 and interferon- γ , the balance between stimulatory and inhibitory cytokines are important for the degree of resorption stimuli (Souza *et al.*, 2013).

IL-34 is a newly identified ligand of CSF-1R, which mediates macrophage differentiation/proliferation (Chen *et al.*, 2011; Baud'Huin *et al.*, 2010). Interestingly, IL-34 is expressed in synovial tissue and synovial fluid of RA patients, and is expressed by synovial fibroblasts. IL-34 expression is associated with synovitis severity in RA patients. Moreover, the formation of IL-34 by RA-synovial fibroblast is stimulated by TNF- α suggesting a role of IL-34 in the pathogenesis of inflammatory diseases (Hwang *et al.*, 2012). RA is an inflammatory autoimmune disease affecting the joints and the surrounding tissue (Dumusc *et al.*, 2014). The similarities between RA and periodontitis are the tissue and bone degenerations that are caused by inflammation and cells like fibroblasts that trigger the inflammation by producing inflammatory mediators (Berthelot *et al.*, 2010). Both states are considered to be chronic disorders that run in relapse. It is therefore interesting to elucidate if IL-34 are synthesized by cells in the periodontium, if the expression are regulated by inflammatory cytokines and thereby could contribute to the pathogenesis of periodontitis.

We hypothesized in this study that GF have the ability to express IL-34 and that the expression is enhanced by the key mediators in periodontitis, IL-1 β and TNF- α . Later we examined the amount of IL-34 protein present in gingival crevicular fluid (GCF) and if there are differences between the amounts of IL-34 in healthy, gingivitis and periodontitis sites. After that, we examined if IL-34 are detectible in healthy and inflamed gingival tissue.

MATERIALS AND METHODS

Fibroblast Cultures

Gingival fibroblasts were isolated from gingival papillar explants obtained from three voluntary donors with clinically healthy gingiva as previously described (Lerner *et al.*, 1987).

Cells were cultured in α modification of Minimum Essential Medium (α -MEM) and 10% foetal calf serum (FCS, GIBCO-BRL/Life Technologies, Paisley, UK) for 7-10 days. The cells were detached and seeded at a density of 50 000 cells/cm² in 24- wells plates for 24 hours. After 24 hours the medium was changed and the cells were incubated for 24 hours in absence (control) or presence of TNF- α and IL-1 β .

RNA Isolation and First-stranded cDNA Synthesis

Total RNA from gingival fibroblast cell cultures was isolated by using the RNAqueous™-4PCR kit (Ambion, Austin, TX) according to instructions by the manufacturer. High Capacity cDNA Reverse Transcription Kit (Foster City, CA) was used to transcribe mRNA to cDNA.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To detect and analyze the gene expression of h-RPL13a, hM-CSF, hIL-6 and hIL-34 Taq-man (API PRISM 7900HT Sequence Detection System) were used. The mRNA of each gene was analyzed by specific primer probe mix (Applied Biosystem, Foster City, CA, USA).

Study population, inclusion and exclusion criteria

GCF samples were collected from patients undergoing periodontal treatment at the specialist clinic, county council of Västerbotten or at the dental students' clinic at Umeå Dental School, Umeå University. In total number 42 samples were collected. 11 samples collected at tooth sites with no sign of inflammation, 10 with gingivitis and 21 with severe periodontitis.

Patients with autoimmune diseases or who have taken antibiotics during the last 3 months were excluded. All the samples taken were from periodontally untreated sites.

Definitions of healthy gingiva, gingivitis and periodontitis (Armitage, 2000, 2004):

Healthy: absence of disease: no bleeding on probing, no periodontal pocket >4mm.

Gingivitis: bleeding on probing, bitewing x-ray revealing no loss of bone, pseudo pockets may occur.

Severe periodontitis: bleeding on probing, pocket depth >6mm, bitewing x-rays showing bone loss > 3mm from the enamel-cement junction.

The sample collecting procedure

Microtubes with perforated cups and Whatman 3MM chromatographic paper points were used to collect the GCF and transfer from the dental clinic to the laboratory. Before the samples were taken we polished the supragingival tooth surface by Youngs rubber cup without chemical medicaments, dried and isolated the surface from saliva with cotton pellets and cotton rolls. The points were inserted in the crevice for 30-40 seconds, 1 minute (min) at healthy sites. In 20 min the sample was transferred to the laboratory to elute GCF. 50 µl physiological NaCl-solution were added to the paper point and centrifuged for 20 min at 3600 times the gravitation force (g) at 5 degrees Celsius (°C). The procedure was repeated. After the second centrifuge the cup and paper point were removed and the 100 µl was transferred to a sterile microtube and 100 µl NaCl was added. All the samples were eluted and stored in a -80°C box within 60 min.

Enzyme-Linked Immunoabsorbent Assay (ELISA)

ELISA technique was used to detect IL-34 protein in GCF-samples. DuoSet ELISA Development kit (R&D Systems Europe, Ltd. Abingdon, UK) specifically to human IL-34 (catalog number DY5265) was used. The materials provided by the manufacturer was used; Capture Antibody (Part 843501), Detection Antibody (Part 843502), Standard (Part 843503), Streptavidin-HRP (Part 890803). Solution required prepared at laboratory; Phosphate buffer saline (PBS), Wash buffer (R&D System catalog #WA126), Reagent Diluent (R&D System catalog #DY995), Substrate solution (R&D System catalog #DY999), Stop Solution (R&D System catalog #DY994). The kit was used according to the instruction given by the manufacturer.

Immunohistochemistry (IHC)

IHC was used to detect IL-34 protein in healthy and inflamed gingival tissue. The tissue was given by a donor undergoing periodontal surgery at Umeå Dental School. Inclusion criteria and definitions as stated under the heading “study population” was used.

IHC protocol

The collected tissue was fixated in 4% neutral buffered formalin and after dehydration steps embedded in paraffin, sliced with microtome and affixed onto microscope slides. The paraffin was eliminated from the slides by xylene (2x5 min) and later rehydrated by ethanol 99% (2x2.5 min), ethanol 95% (2x2.5 min), ethanol 70% (1x2.5 min) and ultrapure water (3x5 min). Sodium citrate buffer was used for antigen retrieval by incubating the slides at 100 °C for 20 min, this step was repeated for 10 min using fresh citrate solution. The slides were cooled down to room temperature for 1 hour. Later the slides were washed in PBS for 5 min and blocked in 5% swine serum in PBS (Dako/cat.no X0901) for at least 1 hour. The slides were incubated with 1:200 diluted polyclonal rabbit anti-IL34 antibody in 5% swine serum over night at 4°C and later washed with PBS (2x5 min), incubated in 3% H₂O₂ in darkness for 10 min at 4°C and washed with PBS for 5 min. The secondary antibody goat anti-rabbit-horseradish peroxidase (Dako/cat.no P0448) was applied to the slides with dilution 1:200 in 5% swine serum for 2 hours in room temperature and later washed with PBS (3x5 min). Sections were developed with 3,3'-diaminobenzidine (DAB) for 3-4 min, washed in water and counterstained with hematoxylin (HMT) for 1 min followed by dehydration steps: ultrapure water (4x5 min), ethanol 70% (1x2.5 min), ethanol 95% (2x2.5 min), ethanol 99% (2x2.5 min) and xylene (2x5 min). Slides mounted with Pertex medium and dried over night.

IHC is a method used for detection of specific proteins and in this study we examine the presence of IL-34 protein in gingival tissue. Therefore we used the primary polyclonal rabbit anti-IL-34 antibody which specifically binds to IL-34 protein. A secondary antibody is added to bind to the primary antibody, after that DAB is used to detect the horseradish peroxidase-bound secondary antibody by giving brown color. HMT was used for counterstaining. In the histological pictures IL-34 protein is showed as brown coloration in the tissue samples. To make sure the secondary antibodies only binds to the primary antibodies and do not bind un-specifically we used controls where only secondary antibodies were applied on tissue sections. In these samples brown coloration should not be detected.

Ethic considerations

Ethical permission for this study was applied for and approved of the local Ethical Committee of Umeå University, reference number 2013-337-31M. The Ethics Forum at

the Department of Odontology found that appropriate ethics considerations have been integrated into this degree project.

GF-cells

The cells were freely given by volunteer donors after informed consent and the samples were coded to exclude the possibility to connect a cell-line to a particular individual. Donors' rights are protected by the local Ethical Committee of Umeå University. Written agreements were received.

GCF-samples

All the participants were informed about the procedure orally and signed a written agreement before the samples were taken. The test is non invasive, painless and short procedure. The benefits are much greater than the risks.

All the samples were coded directly at the clinic by a number. The numbers are grouped into healthy, gingivitis or periodontitis. One list exists that can link the numbers to the participants and it's locked in a safety box at the tutors office. The tutor is the only one who has got the key-code.

Gingival tissue biopsies

The donor was informed and agreed to donate healthy and inflamed tissue during periodontal surgery. Donor's rights are protected by the local Ethical Committee of Umeå University, Umeå. A written agreement was received. The patient is only known by the surgeon that performed the surgery. The tissue samples are coded in healthy and diseased tissue and can't be connected to the patient.

Statistical analysis

GraphPad Prism was used to analyze the data from qPCR and ELISA. Means \pm standard deviations (SDs) were calculated for all conditions, and differences between means were analyzed using Student's t-test/unpaired t-test. A p-value < 0.05 was considered significant.

Literature search

The PubMed database was used for locating articles, following Mesh terms were used: Periodontitis, prevalence, etiology, IL-34, rheumatoid arthritis, osteoclastogenesis, inflammation-induced osteoclastogenesis, cytokines, gingival crevicular fluid, bone remodeling, bone loss, gingival health, tooth loss, risk factors, diagnoses, criteria.

RESULTS

Cytokine expression in gingival fibroblasts stimulated by IL-1 β and TNF- α

The purpose of the first experiments was to examine if GF express IL-34 mRNA and if the expression is effected by the pro-inflammatory cytokines TNF- α and IL-1 β . IL-6 is known to be up-regulated by pro-inflammatory cytokines and was therefore used as a positive control. The concentrations of TNF- α and IL-1 β were tested out in previous studies at the department of Molecular Periodontology and was shown to be optimal in GF experiments. The cells were stimulated by TNF- α 10 ng/ml and IL-1 β 300 pg/ml for 24 hours. Thereafter, RNA was isolated and the mRNA expression was detected using qPCR. The housekeeping gene hRPL-13A was used as an internal standard in each experiment. The experiments were repeated three times and data from one representative experiment are shown.

The results from qPCR showed that gingival fibroblasts express IL-34 mRNA. TNF- α 10 ng/ml up-regulated IL-34 mRNA expression 12x fold-change versus control and IL-1 β 300 pg/ml up-regulated IL-34 mRNA 4x fold-change versus control (figure 1A).

IL-6 is known to be up-regulated by TNF- α and IL-1 β and as expected TNF- α 10 ng/ml up-regulated IL-6 mRNA expression 24x fold-change versus control and IL-1 β 300 pg/ml 27x fold-change versus control (figure 1B). M-CSF mRNA was up-regulated 3x fold-change versus control by TNF- α 10 ng/ml and less than 2x fold-change versus control by IL-1 β 300 pg/ml (figure 1C).

IL-34 protein in GCF

GCF from dental pockets in individuals with healthy gingiva (n=11), gingivitis (n=10) and periodontitis sites (n=21) were collected in patients at Umeå Dental School, Umeå University. The samples were collected before any periodontal treatment was given. The IL-34 protein levels in the GCFs were later analyzed using ELISA. The results show that IL-34 proteins are detectable at a low level in GCF at all sites but no significant differences in IL-34 protein content was detected between periodontally healthy, gingivitis and periodontitis sites (figure 2).

Presence of IL-34 in periodontal tissue

Healthy and inflamed tissue samples were taken from the same donor undergoing periodontal surgery at Umeå Dental School. The tissues collected were fixated in 4%

neutral buffered formalin, later embedded in paraffin and sliced to be mounted on microscopic slides and examined by IHC.

Primary polyclonal rabbit anti-IL-34 antibody was used and as secondary antibody a goat anti-rabbit-HRP. HMT was used for counterstaining and DAB to specifically bind to secondary antibodies and color IL-34 protein brown.

IL-34 protein is detected in both healthy (figure 3A-D) and inflamed tissue (figure 4A-F). The IL-34 protein staining was equal in the epithelium, under both healthy (figure 3C, D) and inflamed conditions (figure 4E, F). In the connective tissue the staining in healthy are less (figure 3A, B) in comparison to the staining in the inflamed tissue (figure 4A, B). In the inflamed tissue the brown coloration is also seen around adipocytes and blood vessels (figure 4C-D).

Control staining with only the secondary antibodies was performed to show that the secondary antibody only bind to the primary antibody and do not bind antigens un-specifically (figure 3E, F, 4G, H).

DISCUSSION

There is evidence that inflammation and osteoclastogenesis are triggered by pro-inflammatory cytokines which in periodontitis plays a major role by contributing to alveolar bone resorption (Bloemen *et al.*, 2011). The newly detected cytokine IL-34 is able to directly affect the osteoclastogenesis by substituting for M-CSF (Chen *et al.*, 2011) and therefore there are reasons to investigate if IL-34 also plays a role in the periodontitis pathogenesis. Therefore, the aims of this study are to investigate if GF are able to express IL-34 and if pro-inflammatory cytokines modulate the expression. Moreover, we investigate if IL-34 protein is present in the GCF and gingival tissue.

In this report we show that GF do express IL-34 mRNA and the expression is enhanced by TNF- α and IL-1 β . Moreover, M-CSF, an important molecule in the formation of bone resorbing osteoclasts was also enhanced by TNF- α and IL-1 β . The effects of TNF- α and IL-1 β on M-CSF and IL-34 in GF have only been published once before (Boström *et al.*, 2013). IL-6 mRNA was also up-regulated by TNF- α and IL-1 β as expected (Palmqvist *et al.*, 2008) which ensure the pro-inflammatory effect of TNF- α and IL-1 β in the GF-experiments. The fact that primary human GF are able to express IL-34 and M-CSF and

the fact that the expressions are enhanced if the cells are stimulated by pro-inflammatory cytokines point at a possible role for these molecules in periodontitis pathogenesis.

Earlier studies show that by collecting and analyzing GCF the possibilities are given for detection and quantification of inflammatory cytokines present in the crevices fluid (Rasmussen *et al.*, 2000). Therefore we used this method to investigate IL-34 protein levels in GCF from healthy and inflamed crevices. To our knowledge there is no information about if IL-34 is present in GCF. We could detect low levels of IL-34 protein in GCF but the results show no differences between the healthy, gingivitis and periodontitis groups. Because we found increased levels of IL-34 mRNA when GF was stimulated with pro-inflammatory cytokines, we hypothesized that the levels of IL-34 should be higher in GCF under inflammatory conditions. The reason why we couldn't prove the hypothesis could be many. Firstly, the GCF is substituted continuously resulting in a high protein turn-over so the time-point for collection to catch GCF when the fluid reflects the inflammatory status may have been wrong. Secondly, IL-34 may not leak out to GCF in a significant extent. Thirdly, it could be that we were not able to elute all of the proteins from the strips. It is known that there are some cytokines that are hard to detect in GCF such as TNF- α and IL-6 (unpublished data) and the same difficulties may apply for IL-34. The sample collecting procedure takes time and if the samples are not frozen quick enough, the IL-34 protein can be degraded.

To our knowledge, we are the first to show that IL-34 is present in periodontal tissue. Interestingly, our IHC analyses indicate a higher amount of IL-34 protein in the lamina propria of inflamed gingival connective tissue in comparison to that in the lamina propria of periodontally healthy tissue. In the epithelium of both periodontally healthy and inflamed tissue sections, equal amounts of IL-34 staining were seen. These results need to be confirmed in analyses of more tissue sections but indicate that IL-34 is increased sub-epithelially in periodontitis and therefore could be a part of the pathogenic process.

Our study show that primary human GF express IL-34 and that pro-inflammatory cytokines increase the levels of IL-34 mRNA. This together with our finding that the amount of IL-34 is increased in the inflamed connective gingival tissue in periodontitis lesions indicate that IL-34 may be involved in the complex pathogenesis of periodontitis but further studies are required.

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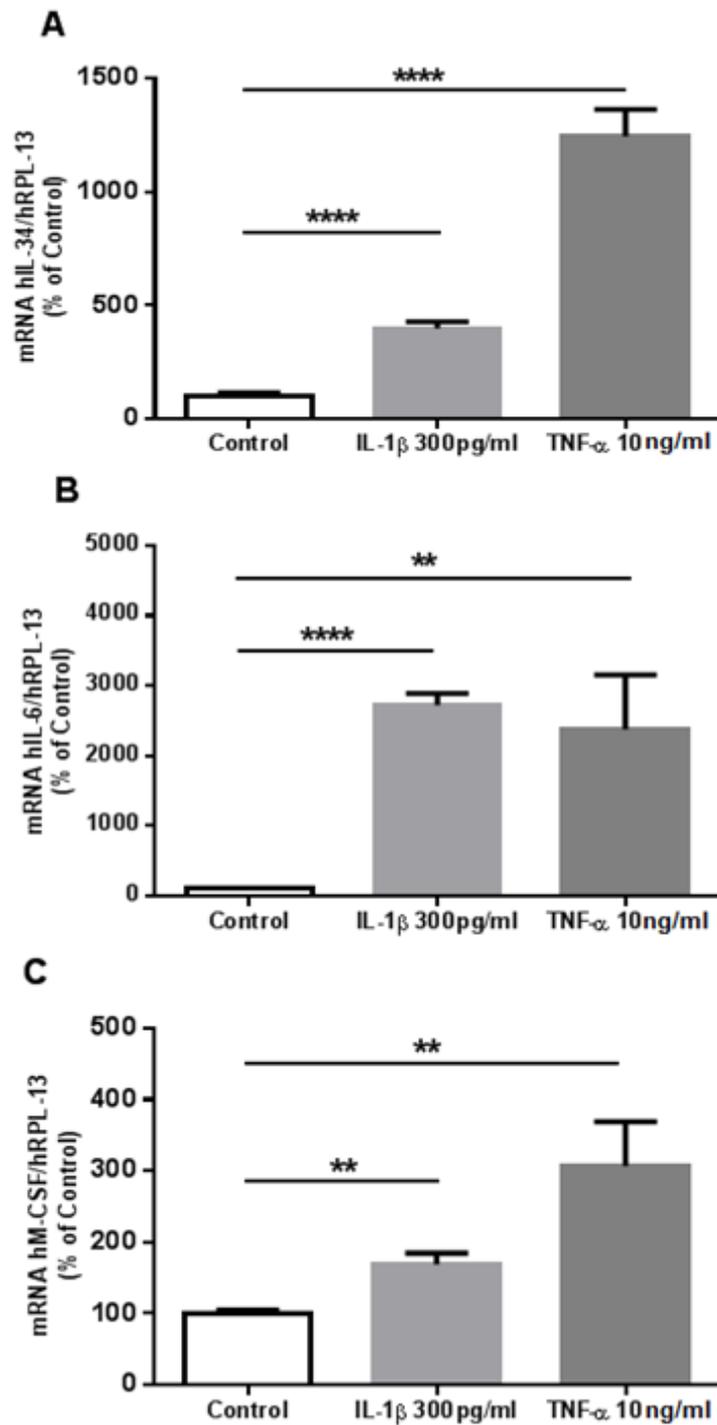


Figure 1. Gingival fibroblasts cultured for 24 hours in 24- well plates, 50 000 cells/cm². The expressions of hIL-34, hIL-6 and hM-CSF mRNA were measured by qPCR in absence of inflammatory cytokines (control) and in presence of pro-inflammatory IL-1 β and TNF- α . The diagrams show the mRNA expression of A) hIL-34, B) hIL-6 C) hM-CSF. IL-1 β and TNF- α are compared versus control. The asterisk show the statistical significance compared versus control group (** p<0,01 **** p<0,0001). One representative experiment out of three is presented.

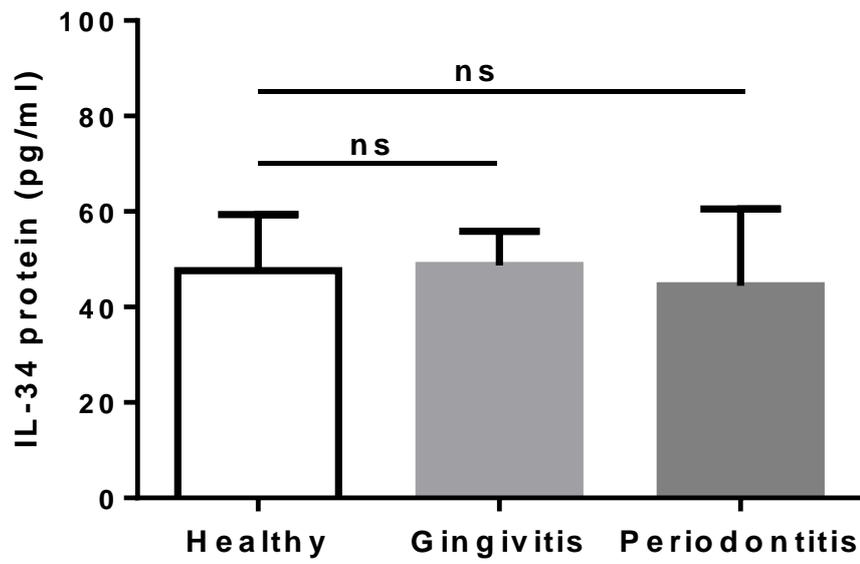


Figure 2. IL-34 protein measured in gingival crevicular fluid by ELISA. 11 healthy, 10 gingivitis and 21 periodontitis samples were collected. The results show no significant difference between healthy versus gingivitis nor between healthy versus periodontitis.

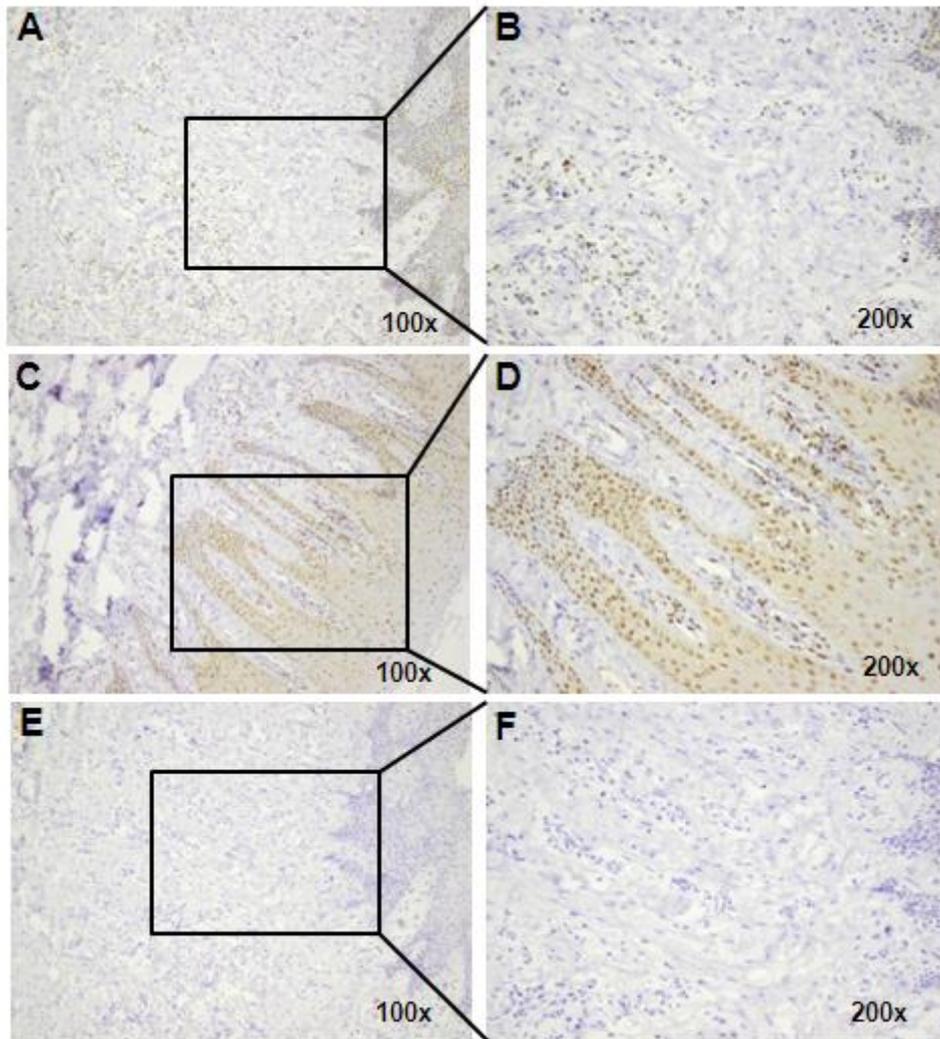


Figure 3. Localization of IL-34 in healthy gingival tissue. A) IL-34 is present in the connective tissue of the gingiva B) Higher magnification (200x) of squared area indicates IL-34 positive cells (brown color). C) Positive staining for IL34 is detected in epithelial layer of the gingiva. D) Higher magnification of squared area indicates localization of IL-34 in epithelial layer and connective tissue. E and F) Control for unspecific binding, when only secondary antibody applied on the tissue, is negative.

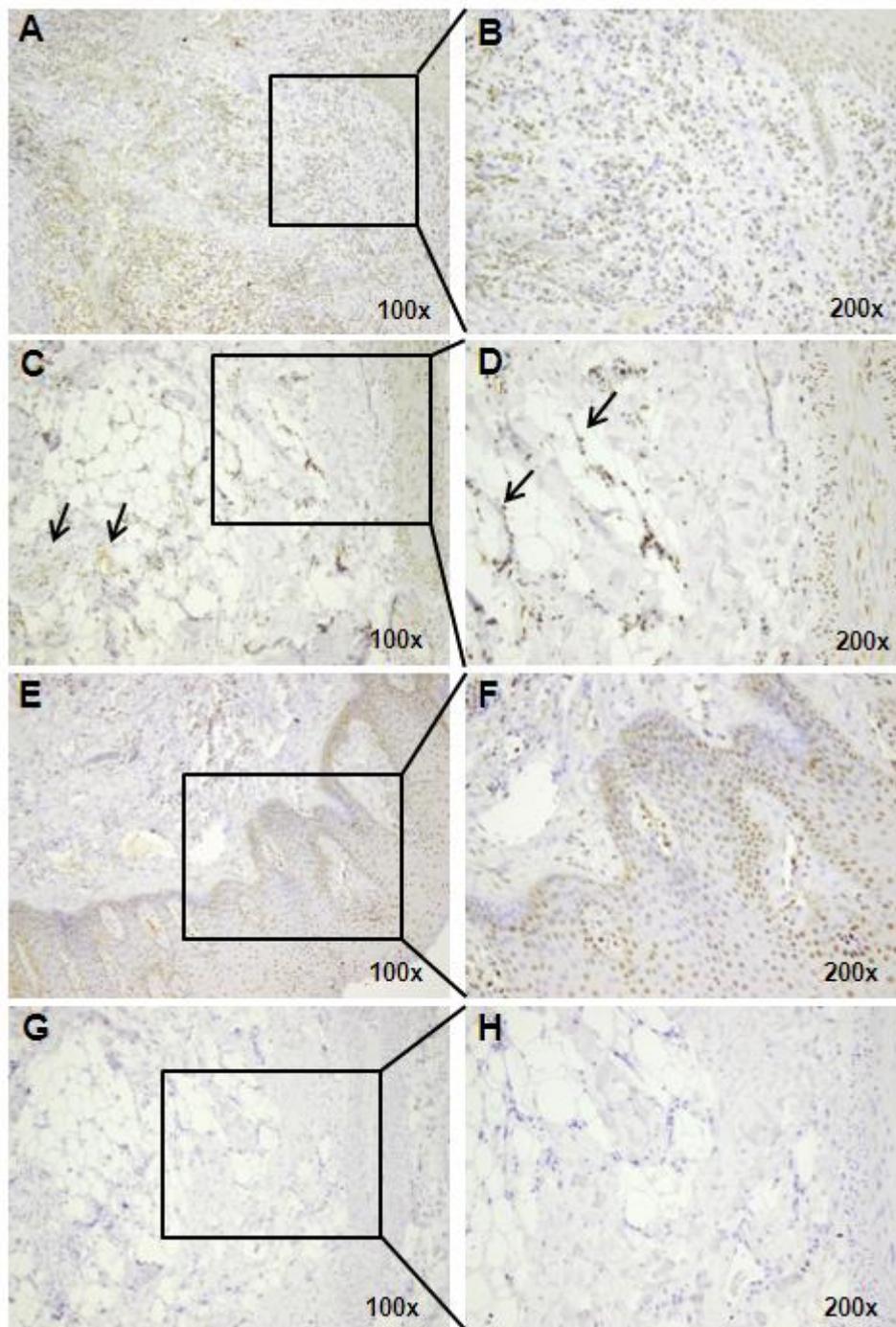


Figure 4. Localization of IL-34 in inflamed gingival tissue. A) A high level of IL-34 is detected in the connective tissue. B) Higher magnification of squared area indicates IL-34 positive cells (brown color). C) Positive staining for IL-34 is detected around adipocytes and blood vessels (arrows). D) Higher magnification of squared area indicates IL-34 positive staining around adipocytes (arrows). E) Positive staining for IL-34 is detected in epithelial layer of the gingiva. F) Higher magnification of squared area indicates localization of IL-34 in epithelial cells. G and H) Control for unspecific binding, when only secondary antibody applied on the tissue, is negative.