Title

Stem cell-like populations and immuno-regulatory molecules in periodontal granulation tissue

Running title

Periodontal granulation tissue characterisation

Conflict of interest and source of funding statement

The authors declare that no conflicts of interest exist. No external funding was obtained for the study.

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Abstract

Aim: Determine the presence of mesenchymal stem cells (MSCs) in healthy periodontal tissue and periodontal granulation tissue (GT) and explore associations between immuno-regulatory molecules and select subgingival microorganisms.

Methods: Mesenchymal stem cells were isolated, propagated and characterised by flow cytometry from a region of healthy gingival tissue and inflamed GT of 10 systemically healthy non-smokers with chronic periodontitis. Tissue levels of immunoregulatory molecules were determined by qPCR and Gingival Crevicular Fluid (GCF) levels by ELISA. Subgingival plaque levels of periodontal pathogens were determined by qPCR

Results: Cells with MSC-properties were isolated from both inflamed GT and healthy gingival (G) tissue. A pro-inflammatory process predominated in GT which was partly reflected in GCF and putative periodontal pathogens were higher at diseased sites. However, there was no significant difference in surface levels of mesenchymal (CD90, CD73, CD146, CD271, STRO-1), endothelial (CD105, CD106), hematopoietic (CD34, CD45) and embryonic (SSEA-4) stem cell markers between MSCs isolated from GT and G tissue.

Discussion: Periodontal lesions, albeit inflamed, retain healing potential as inferred by the presence of MSC-like cells with similar immunophenotypic characteristics to those found in healthy periodontal tissue. Therefore, there might be merits for healing in preserving sufficient GT *in-situ* during periodontal surgery.

Introduction

In periodontal surgery, the excision of granulation tissue (GT) at intra-bony defects is aimed at improving wound healing and promoting new attachment.[1-2] However, gingival curettage and GT removal did not improve periodontal clinical indices more than did scaling and root-planing alone.[3] In line with this finding, flap surgery in conjunction with GT excision was not a critical measure for promoting healing of periodontal tissues.[4] Granulation tissue formation seems to be a hallmark of the pathogenesis of periodontal disease. Cells residing in GT release numerous mediators, which modulate the processes of osteoclastogenesis and osteoblastogenesis, including pro-inflammatory cytokines and anti-inflammatory mediators.[5]

Mesenchymal stem cells are multipotent cells that can replicate as undifferentiated cells and have the potential to differentiate into several lineages of the mesenchymal tissue.[6] Stem cells (SCs) expressing embryonic markers have been isolated from GT removed mainly from infrabony defects of four systemically healthy non-smokers, implying that these cells may contribute to periodontal healing once the infection is controlled.[7] However, the role of SCs in gingival tissue homeostasis and/or pathologies remains unclear. Multipotent stromal SCs were isolated from human periodontal GT and these cells improved osseous repair in critical size defects in mice.[8] Mesenchymal SC (MSC)-like populations have been found within inflamed gingival tissue to be functionally equivalent to MSCs derived from healthy gingival tissue[9] and gingival-derived MSCs have been shown to exhibit immune-modulatory and anti-inflammatory activity.[10-11] Taken all together, it is speculated that MSC populations within infected GT promotes healing whilst dampening inflammation. Human MSCs were isolated from palatal connective tissue and periodontal GT to comparatively evaluate their properties.[12] Despite their differences in colony-forming unit fibroblasts (CFU-Fs), population doubling times, migration potential and level of surface marker expression, MSCs

from both sources were relatively uniform in their ultrastructure and successfully differentiated into osteogenic, adipogenic, and chondrogenic lineages.

However, comparative studies on the characteristics of MSC populations derived from matched samples of healthy vs. diseased periodontal tissues of the same individuals, as well as correlations with the inflammatory "micromilieu" of residual periodontal pockets are systematically lacking. The aim of this study was to determine the presence and properties of cell populations with MSC characteristics in the GT of periodontal lesions compared to those isolated from healthy gingival tissue of the same individuals and determine whether ongoing bacterial driven inflammation alters their basic immunophenotypic profiles.

Materials and methods

Study cohort and clinical interventions

Fifteen systemically healthy non-smokers with chronic periodontitis who were scheduled to receive periodontal surgery were recruited at the Department of Preventive Dentistry, Periodontology and Implant Biology (PDP&IB), Aristotle University of Thessaloniki (AUTh), Greece. The study was approved by the School's Ethical Committee (22/11-01-2016) and all participants provided informed consent.

Inclusion criteria: periodontal pockets of probing pocket depth (PPD) and clinical attachment levels (CAL) >5mm with bleeding on probing (BOP) and radiographic evidence of advanced bone loss. Exclusion criteria: >65 years of age, history of systemic disease, compromised medical conditions requiring prophylactic antibiotic coverage, antibiotic therapy within the last three months, bisphosphonate medication, bone metabolic diseases or disorders that compromise wound healing, use of anti-inflammatory drugs, radiation or immunosuppressive

therapy, narrow zone/absence of attached gingiva, pregnancy/lactation, smoking, previous periodontal surgery.

Patients were screened for eligibility by a single calibrated examiner (DAA), who also performed the clinical (PPD, CAL, BOP, Plaque Index) and radiographic assessment. Eligible subjects underwent cause-related periodontal treatment which comprised strict plaque control measures (re-iterated over the study period) and full-mouth hand/power-driven instrumentation under local anaesthesia. At 6-months, the subjects were scheduled to undergo periodontal surgery.[13] Prior to anaesthesia, GCF and subgingival plaque samples were collected from a diseased and a clinically healthy (PPD $\leq$3mm, absence of BOP, no radiographic evidence of bone loss) site in each individual. The designated sites were gently air dried and isolated from saliva by placing cotton rolls and using a saliva ejector. Supragingival plaque was carefully removed and GCF samples were obtained by using paper strips (Periopaper, OraFlow Inc., Smithtown, NY, USA). The strips were placed in the periodontal pocket/sulcus until mild resistance was felt and were left in-situ for 30s, taking care to avoid mechanical trauma. Subsequently, subgingival plaque was collected by inserting two sterile paper points (ROEKO ISO-40, Coltène-Whaledent GmbH, Co. KG, Germany) in each site for 30s. They were then stored in Eppendorf tubes (Eppendorf, Hamburg, Germany) at -80^{o}C until further processed. Granulation tissue was surgically harvested from the denoted sites that were preferably in the same quadrant and non-adjacent. An internal bevel combined with an intra-sulcural incision demarcated a collar of soft tissues and following flap elevation, GT was sharply excised from the apical part of the pocket; 50% of the tissue was used for cytokine analysis and the remaining 50% for isolation/expansion of MSCs. A thin zone of the soft tissues lining the designated healthy sulcus was also harvested and was processed in a similar manner to the GT biopsy.

MSCs culture and characterisation

The GT biopsy designated for MSC culture (GT-MSCs) and the analogous disease-free specimen (G-MSCs) were enzymatically digested as described previously,[14] seeded in 25cm^2 flasks, expanded at 37ºC in 5% CO_2 in α-MEM (alpha-Minimum Essential Medium, Life Technologies, Thermo Fisher Scientific, Paisley, UK) supplemented with 15% FBS (Life Technologies), 100 units/ml of penicillin, 100 mg/ml of streptomycin and 0.25 mg/ml of Amphotericin-B (all from Life Technologies). GT-MSCs and G-MSCs were characterised by mesenchymal (CD90, CD73, CD146, CD271, STRO-1), endothelial (CD105, CD106), hematopoietic (CD34, CD45) and embryonic (SSEA-4) SC-markers at passage-2 by flow cytometry, using a Guava® easyCyte-8HT Benchtop Flow Cytometer (Merck Millipore, Billerica, Massachusetts, U.S.A).[14] A total of 50,000 events were acquired per sample. Data were analysed using GuavaSoft-3.1.1 and Summit-5.1 software.

Gene expression in tissue

The section of the biopsy sample was placed into RNA-later (Sigma–Aldrich, Dorset, UK), immediately after collection and stored at -20ºC until required. Tissue was cut into 1.0mm pieces and transferred into buffer-RLT (Qiagen, Crawley, UK) and homogenised for 1min with a disposable pestle. RNA was extracted from tissue using the RNeasy® mini kit for fibrous tissue (Qiagen, UK), the yield was assessed spectrophotometrically and 1.0µg of RNA was converted to cDNA using the SuperScript™ First-Strand Synthesis Kit (Life Technologies) according to the manufacturers' instructions. Real-time PCR analysis of the expression of IL-1β, IL-6, IL10, TNF-α, IL-17A, IL-17E, TGF-β and two reference genes, GAPDH and RNA polymerase-II, was performed using TaqMan assay on demand assays (ABI/Life Technologies). The PCR efficiency was determined using a previously described method[15] and all primer-probe sets exhibited efficiencies of 0.90-1.05. For each gene, the

level of expression was adjusted by the geometric mean of the two-reference gene critical threshold-values.

Cytokine levels in GCF

Enzyme-linked immunosorbent assay and specific kits for IL-1β, IL-6, IL-10, TNF-α, IL-17A, IL-17E (Pepro Tech, London UK), TGF-β (Mabtech AB, Stockholm Sweden) were used for cytokines measurement in GCF. Minimum detection limits were: IL-1β, IL-6, IL-10, TNFα and IL-17A, all 1.9pg/ml; IL-17E and TGF-β, both 3.9pg/ml.

Quantities of subgingival plaque microorganisms

The Epicentre Masterpure Gram-positive DNA isolation kit (Cambio, Cambridge, UK) was used to prepare genomic DNA from the plaque samples and from known quantities of laboratory strains of the target microorganisms. The amount and purity of DNA was checked spectroscopically. Real-Time-PCR analysis was used to determine copy numbers of *P. gingivalis*,[16] *A. actinomycetemcomitans, F. nucleatum, T. forsythia*,[17] *T. denticola*[18] and *Streptococcus mitis*[19] in subgingival plaque. Known numbers of the target microbes were used to create standard curves from which copy numbers of unknown samples were determined.

Statistical analyses

Statistical power calculation was based on the assumptions that the data were dependent and either normally distributed or log-normally distributed. For an effect size of 1, utilising the paired t-test the estimated minimum number per group required to exceed 80% statistical power (α=0.05) were n=10. Q-Q plot analysis for each parameter determined that data were not normally distributed. To facilitate non-parametric analyses and multiple comparisons in cytokines/bacteria and to allow for covariates the sample size was increased to n=15. All 30

GCF and plaque samples were analysed for cytokine measurements and quantification of select bacteria, respectively, 30 tissue specimens were analysed for cytokine gene expression but MSCs could only be isolated/expanded from 20 specimens (N_{GT}=$N_{disease\text{-}free}$=10), resulting in samples not being perfect pairs. Analysis of this data set was performed using 1000 random data combinations in a Permutation test based on the Wilcoxon test[20] using R-Project for Statistical Computing. Otherwise the paired data sets (GT and disease-free tissues) were analysed using SPSS Inc. version 21 (IBM, Chicago, USA and Graphpad Prism V5, La Jolla, USA). Correlation between the biochemical and microbial parameters were evaluated with the Kendal Tau test. Biological specimens and clinical data were coded, so that the laboratory analysis was blind to the clinical details.

Results

Clinical data

All periodontal defects (N=15) were located in posterior segments. Demographic details of the participants and clinical data are shown in Table 1.

MSC cultures

Few morphological differences were observed between GT-MSC and G-MSC cultures. Both types of cells presented a typical fibroblast-like, spindle-shaped morphology. Phenotypic characterisation indicated similar profiles for GT-MSCs and G-MSCs, characterised by high expression (>95%) of CD73 and CD90 and low expression (<1-2%) of STRO-1, CD45, CD34 and CD271 in both GT-MSCs and G-MSCs (N=10) (Fig. 1). However, a high inter-subject variability was noted in the expression of CD105, CD146, CD106, CD73, CD90 and SSEA-4 with no significant differences between GT-MSCs and G-MSCs (all $p>0.5$; Fig. 2).

Cytokine and bacterial profiles

The expression of several pro-inflammatory cytokines was significantly higher in GT compared with the disease-free biopsy tissue (IL-1β: p=0.007; TNF-α: p=0.0001; IL-6: p=0.011; IL-17A: p=0.0001; Fig. 3A-B-C-E, respectively). Despite the observation that median levels were higher for IL-10-, IL-17E-, or TGF-β mRNAs inside GT, this failed to reach statistical significance (all p>0.1; Fig. 3D-F-H). There was a significantly higher ratio of IL-17A:IL-17E mRNA in the GT compared with the disease-free tissue specimens (p=0.001; Fig. 3G).

The amounts of TNF-α were significantly higher in GCF sampled from the GT-associated site than the disease-free site (p<0.039; Fig. 4B). Amounts of IL-17E were significantly lower in the GCF samples from the diseased sites, in parallel with a significantly higher IL-17A:IL-17E ratio (p<0.0054, p=0.008, respectively; Fig. 4F-G). In contrast, there was no significant difference in the amounts of the other cytokines measured in the GCF of diseased sites *versus* disease-free sites (p>0.10; Fig. 4A-C-D-E-H).

The median copy numbers of *P. gingivalis* (p=0.021), *F. nucleatum* (p=0.003), *T. denticola* (p=0.035), and *T. forsythia* (p=0.0052) were significantly higher in the GT-associated plaque samples compared to the disease-free samples (Fig. 5A-B-C-D, respectively). In contrast, the median copy numbers of *A. actinomycetemcomitans* and *S. mitis* tended to be lower in the GT samples but failed to reach statistical significance (both p>0.1; Fig. 5E-F).

Correlation analyses

Significant correlations were observed between cytokines in GCF and the CD markers on MSCs isolated from GT (Table 2). For instance, IL-6 correlated positively with CD105 (Tau=0.556, p=0.025) and IL-17E with CD106 (Tau=0.600, p=0.016) and IL-17A with CD146 (Tau=0.511, p=0.04). There were also statistically significant correlations between gene transcript levels in the GT and CD markers; IL-17E mRNA correlated positively with

CD105 (Tau =0.667, p=0.012) and the *IL-17A:IL-17E* transcript ratio negatively with CD105 (Tau=-0.556, p=0.037).

The bacteria *F. nucleatum* and *T. denticola*, correlated positively with CD90 (Tau=0.648, p=0.016; Tau=0.535, p=0.046, respectively), whereas *T. forsythia* and *S. mitis* correlated negatively with CD90 (Tau=-0.592, p=0.028; Tau=-0.535, p=0.046, respectively) (Table 2).

The copy numbers of *P. gingivalis* correlated positively with the amounts of TGF-β and TNF-α in GCF of the GT-associated sites and also positively with the ratio of *IL-17A:IL-17E* mRNA (Tau=0.302, p=0.024; Tau=0.321, p=0.017; Tau=0.302, p=0.024, respectively; Table 2), but *P. gingivalis* correlated negatively with TGF-β mRNA in GT (Tau=-0.368, p=0.007). Copy numbers of *F. nucleatum* and *T. forsythia* correlated positively with the ratio of IL-17A:IL-17E mRNA in GT (Tau=0.296, p=0.027; Tau=0.307, p=0.022, respectively) and copy numbers of *S. mitis* correlated negatively with GCF IL-17A and with the ratio of IL-17A:IL-17E mRNA in GT (Tau=-0.278, p=0.038; Tau=-0.278, p=0.038, respectively).

Discussion

The healing process of a wound occurs in at least four phases, coagulation, inflammation, proliferation and maturation.[21] Granulation tissue (GT) has a large cell-infiltrate incorporating fibroblasts, macrophages and leukocytes, as well as randomly organised collagen fibres.[22] From a clinical perspective, GT excision controls bleeding locally and thus contributes to a more efficient debridement of a periodontal osseous lesion while allowing space for the placement of graft materials.[23-24] In both homeostasis and disease/injury, the perivascular regions in the periodontal ligament are enriched with stem/progenitor cells[25] that can migrate to the wound site, attach to the denuded root surface, proliferate and mature to tissue-forming cells (osteoblasts, cementoblasts and fibroblasts) and also interact with other host cells by releasing regenerative signals, cytokines, growth factors, chemokines, etc.[26]

There is growing evidence that GT-derived MSCs possess high regenerative potential and may be used as autologous transplants for subsequent periodontal reconstructive operations.[7-8,12]

In this study the GT was excised from residual periodontal pockets and the inflammatory infiltrate and presence of MSCs observed within this infected tissue reflected a chronic disease state, which remained unresolved. The primary interest of the current study was to address the question whether following cause-related periodontal treatment and inflammation control the surgical retention of GT has advantages in terms of promoting the healing processes. The GT biopsies and the disease-free connective tissue specimens contained MSC-like cells that expressed CD105, CD146, CD106 (MSC-markers), as well as SSEA-4 (embryonic marker). Cells at passage 2 expressed low levels of STRO-1 (<2%)[27-28] -albeit also found by other studies-[29] and of CD105 (49.2-61.3%).[28-29] The tissues demonstrated a high inter-subject variability regarding the expression of these markers possibly reflecting heterogeneity of the cell population and respective variability in "stemness".[30] This could be attributed to several factors, i.e. culture conditions, disease conditions,[31] or reflect the individual genetic background and not be necessarily linked to the presence or absence of inflammation.[32] Within the limitations of the present study this marker expression variability was consistent between the diseased and healthy tissue specimens failing to support the notion that inflammation overtly affects the immunophenotype of stem cell-like populations isolated from GT. Gingival-MSCs have been shown to retain their proliferative ability after prolonged culture, to differentiate into multiple different cell lineages and release growth factors and cytokines that may facilitate repair of damaged tissues, while exhibiting unique immunomodulatory properties suggesting that they may play a potential role in tissue regenerative protocols.[33-34] However, while the current study did not sought to determine whether inflammation altered the function and properties of the stem cell populations, there

were no apparent differences in the morphology or immunophenotype between the GT-MSCs and G-MSCs. This finding partially concurs with previous data demonstrating that SC characteristics mainly in terms of morphology,proliferation and migration rate anddifferentiation potential remain largely unaffected by the inflammatory processes,[7,32,34-37] although different patterns in phenotypic and functional properties of cell subsets in health *vs.* disease are also seen.[12,38-39]

Inflammatory cells in periodontal GT express pro-inflammatory cytokines that are related to bone resorption.[40] A higher level of pro-inflammatory cytokine gene expression including *TNF-α, IL-1β, IL-6* and *IL-17A* was found in GT than disease-free connective tissue, verifying presence of inflammation at periodontitis tissue sites.[41-43] Despite greater expression of *TNF-α, IL-1β, IL-6* and *IL-17A* genes in inflamed GT compared to disease-free samples, only TNF-α was significantly increased in GCF from diseased sites. Where the amount of TNF-α was high, the amount of IL-17E was low. In contrast to IL-1 or IL-6, it has been shown that TNF-α increases the expression of SC markers on dental pulp-derived MSCs, telomerase activity and the capacity for migration, proliferation, and differentiation.[44] These authors suggested that during the initial stage of wound healing, the inflammatory state may be of fundamental importance for both the attraction of MSCs and modulation of their "stemness", while there is evidence to support that inflammatory conditions in the culture conditions mildly influence the MSC properties of stem/progenitor cells residing in periodontal connective tissues.[28] The present study showed that GCF levels of IL-6 from diseased sites correlated with the expression of CD105. IL-6 is thought to maintain the proliferative and undifferentiated state of bone marrow-derived MSCs, which secrete copious amounts of IL-6, until they undergo osteogenic differentiation.[45] IL-6 family cytokines have been implemented in the maintenance of embryonic and adult SCs,[46-50] and in the increase of the capacity for *in vitro* wound healing.[45]

There is contradicting evidence regarding IL-17A levels in GCF,[51] saliva,[51-52] and serum[51] of periodontitis patients, but these investigations were performed on samples collected from periodontitis patients and matched healthy controls. Of note was that in the current study the matched samples originated from an apparently disease-free site and a single diseased site from the same individual, which during the healing process may show alterations in inflammatory regulators before overt clinical improvement occurs. A reduction of IL-17E in GCF at diseased sites supports previous observations from our group that IL-17E levels in biofluids were reduced in periodontitis[51] and increased following successful periodontal therapy.[53] Median levels of IL-17E-, IL-10- and TGF-β mRNAs tended to be higher in GT than disease-free tissue. Whether this is an early indication of changes within the GT to an anti-inflammatory and wound healing cytokine profile is unclear. Anti-inflammatory cytokine levels fall immediately after and remain depressed for several days following periodontal flap surgery, while levels of IL-1β and TNF-α are increased during the initial phases of wound healing.[54] Anti-inflammatory cytokines have been shown to play a key role in the disease process,[41-42] and along with the pro-inflammatory cytokines they are also implicated in the healing process.[55] The inflammatory phase of a wound drives tissue healing as pro-inflammatory cytokines activate the proliferative phase of wound healing for the restoration of the vascular network and GT formation,[56] followed by tissue maturation and remodelling as inflammation is regulated by pro- and anti-inflammatory cytokines.[5] MSC-like populations may assist in maintaining a balance of pro-inflammatory and anti-inflammatory processes favouring an anti-inflammatory reaction that drives wound healing. MSC-like cells derived from inflamed gingival tissues exhibit a similar phenotypic profile -in line with current findings-, *in vitro* differentiation capacity and *in vivo* developmental potential to MSC-like cells from healthy gingival tissues,[34] although it has been shown that MSCs from inflamed tissues can exhibit altered stemness and immunomodulatory properties[57-59] that may

contribute to an imbalance in the local immune response and advancement of alveolar bone loss. On the premise that MSC-like cells promote tissue healing it is proposed that retaining the soft tissue wall of a periodontal defect during periodontal surgery and flap elevation would reduce soft tissue recession and promote periodontal wound healing and regrowth of lost tissues. There is evidence to support the clinical superiority of tissue-friendly and patient-centred surgical techniques that minimise tissue trauma and retain the soft tissue architecture.[60-62] The intrinsic healing potential of a wound occurs when ideal conditions are provided by the surgical approach,[60-61] implying that substantial periodontal clinical improvements are attainable without the use of any regenerative graft materials. A clinical study in humans (Dept. of PDP&IB; ClinicalTrials.gov ID: NCT02449005) is underway and is expected to complement current findings regarding the premise of periodontal GT preservation during flap surgery.

While there is no direct link between the microbes at the sulcus or periodontal pocket they probably contributed to the inflammatory response in the tissues, as there appears to be some correlation between certain microbes and cytokine expression, the cytokines, in turn could have had an impact on the SC population. *In vitro* experiments have shown that dental stem cells are less likely to interact directly with putative periodontal pathogens regarding adherence and internalization than differentiated cells in an anaerobic environment.[63] Key periodontal pathogens were higher at the GT-sites compared to the disease-free sites as is in accord with the literature,[64-65] whereas *A. actinomycetemcomitans* and *S. mitis* were similar at GT and disease-free sites. *A. actinomycetemcomitans* appears to be less prevalent in deep periodontal pockets[66] and Streptococci are suggested to attenuate the pro-inflammatory response,[67-69] while the literature suggests that MSCs may contribute to microbial defense.[70]

F. nucleatum, *T. denticola*, and *S. mitis* correlated positively with CD90, whereas *T. forsythia* correlated negatively with this marker. Depressed expression of CD90 antigens associated with a diminished immunosuppressive activity of MSCs[71]. GCF IL-17E was correlated with the proportion of MSCs expressing CD106, an immunomodulatory molecule on the surface of MSCs.[72] The associations between the microbes and local cytokines with the markers on MSCs detected in this investigation should be explored further. Although in comparisons of cells isolated from GT and disease-free tissue samples factors measured in this study had no detectable effect on the morphology and expression of CD markers on MSC-like cells. Further studies are required to document whether chronic inflammation alters the properties of MSCs isolated from periodontal GT and also to isolate and thoroughly characterise potential MSCs residing in periodontal GT before any therapeutic intervention is initiated

Conclusion

Periodontal lesions, albeit inflamed, retain healing potential as inferred by the presence of MSC-like cells with similar immunophenotypic characteristics to those found in clinically healthy periodontal tissues. The bacterial-driven inflammation and cytokine expression both in GCF of residual periodontal pockets and within the GT appears to correlate with SC markers of MSCs-like populations isolated from the GT; current data suggest there might be merits for healing in preserving sufficient GT in-situ during periodontal surgery, but the mode of healing and whether the process of healing would be repair or regeneration remains to be elucidated.

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References

1. Widman L. The Operative Treatment of Pyorrhea Alveolaris, a New Surgical Method: a Paper Read At the Fifty Years' Jubilee of the Scandinavian Dental Association (Skandinaviska Tandläkareföreningen) 1916. Stockholm: C. A. Löjdquists boktryckeri, 1918.

2. Nyman S, Lindhe J, Karring T. Reattachment- new attachment. In textbook of Clinical Periodonlologv. ed. Lindhe J., Copenhagen, Denmark: Munksgaard, 1983: 409-432.

3. Echeverria JJ, Caffesse RG. Effects of gingival curettage when performed 1 month after root instrumentation. A biometric evaluation. J Clin Periodontol. 1983; 10: 277-286.

4. Lindhe J, Nyman S. Scaling and granulation tissue removal in periodontal therapy. J Clin Periodontol. 1985; 12: 374-388.

5. Kantarci A, Hasturk H, Van Dyke TE. Host-mediated resolution of inflammation in periodontal diseases. Periodontol 2000. 2006; 40: 144-163.

6. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143–147.

7. V, Belibasakis GN, Schmidlin PR, Bostanci N. Infected periodontal granulation tissue contains cells expressing embryonic stem cell markers. A pilot study. Schweiz Monatsschr Zahnmed. 2013; 123: 12-6.

8. Hung TY, Lin HC, Chan YJ, Yuan K. Isolating stromal stem cells from periodontal granulation tissues. Clin Oral Investig. 2012; 16: 1171-1180.

9. Ge S, Mrozik KM, Menicanin D, Gronthos S, Bartold PM. Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy. Regen Med. 2012; 7: 819-832.

10. Zhang Q, Shi S, Liu Y, et al. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol. 2009; 183: 7787-7798. Erratum in: J Immunol. 2010; 184:1656.

11. Chen M, Su W, Lin X, et al. Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. Arthritis Rheum. 2013; 65: 1181-1193.

12. Páll E, Florea A, Soriţău O, Cenariu M, Petruţiu AS, Roman A. Comparative Assessment of Oral Mesenchymal Stem Cells Isolated from Healthy and Diseased Tissues. Microsc Microanal. 2015; 21: 1249-1263.

13. Ramfjord SP, Nissle RR. The modified widman flap. J Periodontol. 1974; 45: 601-607.

14. Bakopoulou A, Papachristou E, Bousnaki M, et al. Human treated dentin matrices combined with Zn-doped, Mg-based bioceramic scaffolds and human dental pulp stem cells towards targeted dentin regeneration. Dent Mater. 2016; 32: e159-175

15. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 2000; 25: 169–193.

16. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH. Comparison of real-time PCR and culture for detection of Porphyromonas gingivalis in subgingival plaque samples. J Clin Microbiol. 2003; 41: 4950-4954.

17. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH. Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. FEMS Immunol Med Microbiol 2005; 45: 191-199.

18. Guglielmetti MR, Rosa EF, Lourenção DS, et al. Detection and quantification of periodontal pathogens in smokers and never-smokers with chronic periodontitis by real-time polymerase chain reaction. J Periodontol. 2014; 85: 1450-1457.

19. Seow WK, Lam JH, Tsang AK, Holcombe T, Bird PS. Oral Streptococcus species in pre-term and full-term children - a longitudinal study. Int J Paediatr Dent. 2009; 19: 406-411.

20. Dubnicka SR, Blair R. Rank-based procedures for mixed paired and two-sample designs. J Mod Appl Stat Meth 2002; 1: 32-41.

21. Hunt TK, Hopf H, Hussain Z. Physiology of wound healing. Adv Skin Wound Care. 2000; 13 (2 Suppl): 6-11.

22. Clark RAF. Wound repair. Curr Opin Cell Biol 1989; 1: 1000-1008.

23. Harrel SK, Rees TD. Granulation tissue removal in routine and minimally invasive procedures. Compend Contin Educ Dent. 1995; 16: 960, 962, 964 passim.

24. Wennström JL, Heijl L, Lindhe J. Periodontal surgery: access therapy. In: Lindhe J (ed) Clinical periodontology and implant dentistry, 4th edn. Blackwell Munksgaard, Oxford, 2003: 519–560.

25. Gould TR, Melcher AH, Brunette DM. Migration and division of progenitor cell populations in periodontal ligament after wounding. J Periodontal Res. 1980; 15: 20–42.

26. Wada N, Tomokiyo A, Gronthos S, Bartold PM. Immunomodulatory Properties of PDLSC and Relevance to Periodontal Regeneration. Curr Oral Health Rep. 2015; 2: 245–251.

27. El-Sayed KM, Paris S, Graetz C, Kassem N, Mekhemar M, Ungefroren H, Fändrich F, Dörfer C. Isolation and characterisation of human gingival margin-derived STRO-1/MACS(+) and MACS(-) cell populations. Int J Oral Sci. 2015; 7: 80-88.

28. Yang H, Gao LN, An Y, Hu CH, Jin F, Zhou J, Jin Y, Chen FM. Comparison of mesenchymal stem cells derived from gingival tissue and periodontal ligament in different incubation conditions. Biomaterials. 2013; 34: 7033-7047.

29. Wu SM, Chiu HC, Chin YT, Lin HY, Chiang CY, Tu HP, Fu MM, Fu E. Effects of enamel matrix derivative on the proliferation and osteogenic differentiation of human gingival mesenchymal stem cells. Stem Cell Res Ther. 2014; 5: 52.

30. Mindaye ST, Ra M., Lo Surdo JL, Bauer SR, Alterman MA. Global proteomic signature of undifferentiated human bone marrow stromal cells: Evidence for donor-to-donor proteome heterogeneity. Stem Cell Res. 2013; 11: 793–805.

31. Lv FJ, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. Stem Cells. 2014; 32: 1408-1419.

32. Pereira LO, Rubini MR, Silva JR, et al. Comparison of stem cell properties of cells isolated from normal and inflamed dental pulps. Int Endod J. 2012; 45: 1080–1090.

33. Tang L, Li N, Xie H, Jin Y. Characterization of mesenchymal stem cells from human normal and hyperplastic gingiva. J Cell Physiol. 2011; 226: 832-842.

34. Ge S, Mrozik KM, Menicanin D, Gronthos S, Bartold PM. Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy. Regen Med. 2012; 7: 819-832.

35. Park JC, Kim JM, Jung IH, et al. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: in vitro and in vivo evaluations. J Clin Periodontol. 2011; 38: 721–731.

36. Liao J, Al Shahrani M, Al-Habib M, Tanaka T, Huang GT. Cells isolated from inflamed periapical tissue express mesenchymal stem cell markers and are highly osteogenic. J Endod. 2011; 37: 1217-1224.

37. Dokić J, Tomić S, Cerović S, Todorović V, Rudolf R, Colić M. Characterization and immunosuppressive properties of mesenchymal stem cells from periapical lesions. J Clin Periodontol. 2012; 39: 807-816.

38. Alongi DJ, Yamaza T, Song Y, et al. Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. Regen Med. 2010; 5: 617-631.

39. Tang H-N, Xia Y, Yu Y, Wu R-X, Gao L-N, Chen F-M. Stem cells derived from ‘‘inflamed’’ and healthy periodontal ligament tissues and their sheet functionalities: a patient-matched comparison. J Clin Periodontol 2016; 43: 72–84.

40. Kwan Tat S, Padrines M, Théoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev. 2004; 15: 49-60.

41. Lappin DF, MacLeod CP, Kerr A, Mitchell T, Kinane DF. Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. Clin Exp Immunol. 2001; 123: 294-300.

42. Seymour GJ, Gemmell E. Cytokines in periodontal disease: where to from here? Acta Odontol Scand. 2001; 59: 167-173.

43. Takahashi K, Azuma T, Motohira H, Kinane DF, Kitetsu S. The potential role of interleukin-17 in the immunopathology of periodontal disease. J Clin Periodontol. 2005; 32: 369-374.

44. Ueda M, Fujisawa T, Ono M, et al. A short-term treatment with tumor necrosis factor-alpha enhances stem cell phenotype of human dental pulp cells. Stem Cell Res Ther. 2014; 5: 31.

45. Pricola KL, Kuhn NZ, Haleem-Smith H, Song Y, Tuan RS. Interleukin-6 maintains bone marrow derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. J Cell Biochem 2009; 108: 577–588.

46. Gimble JM, Wanker F, Wang CS, et al. Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. J Cell Biochem 1994; 54: 122–133.

47. Rougier F, Cornu E, Praloran V, Denizot Y. IL-6 and IL-8 production by human bone marrow stromal cells. Cytokine 1998; 10: 93–97.

48. Liu CH, Hwang SM. Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine 2005; 32: 270–279.

49. Li D, Wang GY, Dong BH, Zhang YC, Wang YX, Sun BC. Biological characteristics of human placental mesenchymal stem cells and their proliferative response to various cytokines. Cells Tissues Organs 2007; 186: 169–179.

50. Song HY, Jeon ES, Kim JI, Jung JS, Kim JH. Oncostatin M promotes osteogenesis and suppresses adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells. J Cell Biochem 2007; 101: 1238–1251.

51. Awang R, Lappin DF, Macpherson A, et al. Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. Inflamm Res. 2014; 63: 1001-1012.

52. Özçaka O, Nalbantsoy A., Buduneli N. Interleukin-17 and interleukin-18 levels in saliva and plasma of patients with chronic periodontitis. J Periodontal Res. 2011, 46: 592-598.

53. Nile CJ, Apatzidou DA, Awang RA, Riggio MP, Kinane DF, Lappin DF. The effect of periodontal scaling and root polishing on serum IL-17E concentrations and the IL-17A:IL-17E ratio. Clin Oral Investig. 2016; 20: 2529-2537.

54. Kaner D, Soudan M, Zhao H, Gaßmann G, Schönhauser A, Friedmann A. Early Healing Events after Periodontal Surgery: Observations on Soft Tissue Healing, Microcirculation, and Wound Fluid Cytokine Levels. Int J Mol Sci. 2017; 18: pii: E283.

55. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008; 16: 585-601.

56. Reinke JM, Sorg H. Wound repair and regeneration. Eur Surg Res. 2012; 49: 35-43.

57. Liu D, Xu J, Liu O, et al. Mesenchymal stem cells derived from inflamed periodontal ligaments exhibit impaired immunomodulation. J Clin Periodontol 2012; 39: 1174–1182.

58. Sun HH., Chen B, Zhu QL, et al. Investigation of dental pulp stem cells isolated from discarded human teeth extracted due to aggressive periodontitis. Biomaterials 2014; 35: 9459–9472.

59. Yazid FB, Gnanasegaran N, Kunasekaran W, Govindasamy V, Musa S. Comparison of immunodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. Clin Oral Investig. 2014; 18: 2103–2112.

60. Cortellini P, Tonetti MS. Clinical and radiographic outcomes of the modified minimally invasive surgical technique with and without regenerative materials: a randomized-controlled trial in intra-bony defects. J Clin Periodontol. 2011; 38: 365-373.

61. Trombelli L, Simonelli A, Pramstraller M, Wikesjö UM, Farina R. Single flap approach with and without guided tissue regeneration and a hydroxyapatite biomaterial in the management of intraosseous periodontal defects. J Periodontol. 2010; 81: 1256-1263.

62. Harrel SK, Wilson TG Jr, Nunn ME. Prospective assessment of the use of enamel matrix derivative with minimally invasive surgery: 6-year results. J Periodontol. 2010; 81: 435-441

63. Biedermann A, Kriebel K, Kreikemeyer B, Lang H. Interactions of anaerobic bacteria with dental stem cells: an in vitro study. PLoS One. 2014; 9 (11): e110616.

64. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005; 38: 135-187.

65. Larsen T, Fiehn NE. Dental biofilm infections - an update. APMIS. 2017; 125: 376-384.

66. Dahlén G, Claesson R, Aberg CH, Haubek D, Johansson A, Kwamin F. Subgingival bacteria in Ghanaian adolescents with or without progression of attachment loss. J Oral Microbiol. 2014; 6.

67. Hasegawa Y, Mans JJ, Mao S, et al. Gingival epithelial cell transcriptional responses to commensal and opportunistic oral microbial species. Infect. Immun. 2007; 75: 2540–2547.

68. Cosseau C, Devine DA, Dullaghan E, et al. The commensal Streptococcus salivarius K-12 downregulates the innate immune responses of human epithelial cells and promotes host-microbe homeostasis. Infect. Immun. 2008; 76: 4163–4175.

69. Kumar PS, Matthews CR, Joshi V, de Jager M, Aspiras M. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. Infect Immun. 2011; 79: 4730-4738.

70. Auletta JJ, Deans RJ, Bartholomew AM. Emerging roles for multipotent, bone marrow-derived stromal cells in host defense. Blood. 2012; 119: 1801-1809.

71. Campioni D, Rizzo R, Stignani M, et al. A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. Cytometry B Clin Cytom, 2009; 76: 225–230.

72. Yang ZX, Han ZB, Ji YR, et al. CD106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. PLoS One. 2013; 8: e59354.

Figure legends

Figure 1.

Representative flow cytometry parameter diagrams showing percentage MSC surface epitopes in granulation tissue (GT-MSC) and in disease-free gingival connective tissue (G-MSC) specimens of a single patient. Green line represents marker of interest and red line isotype control

Figure 2.

Summary data of flow cytometry, for stem cell markers expressed in granulation tissue (GT-MSC) and in disease-free gingival connective tissue (G-MSC) specimens of ten patients. Large inter-subject variability is noted and no apparent dissimilarities between the GT-MSC and G-MSC cultures for any of the markers tested according to a permutation test with a Monte Carlo simulation: CD105, $p=0.231$; CD146, $p=0.503$; CD73, $p=0.654$; CD106, $p=0.256$; CD90, $p=0.686$; SSEA-4, $p=0.506$; CD271, $p=0.179$; CD34, $p=0.563$; CD45, $p=0.128$.

Figure 3.

Quantification of cytokine gene expression in granulation tissue (GT) and in disease-free tissue specimens (H) in the same individuals (n=15). Statistical significant differences are shown in asterisks (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Figure 4.

Cytokine levels in gingival crevicular fluid (GCF) associated with the granulation tissue sites (GT) and with the disease-free sites (H) in the same individuals (n=15). Significant differences are shown in asterisks (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Figure 5.

Quantification of S. mitis and putative periodontal pathogens in subgingival plaque samples at sites associated with the granulation tissue biopsies (GT) and disease-free sites (H) in the same individuals (n=15). Significant differences are shown in asterisks (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Tables and Figures

Table 1. Patient clinical data and demographic details (N=15)

Gender	Age	No of Teeth	Full-mouth PPD (mm)	Full-mouth CAL (mm)	5-7mm sites (%)	>7mm sites (%)
7F / 8M	51.66 (7.33)	26.27 (3.06)	3.40 (0.75)	4.33 (1.16)	17.69 (12.22)	3.52 (5.21)

Mean (SD)

All participants were non-smokers and of Caucasian origin and had full-mouth plaque scores <20% 6-months following cause-related periodontal treatment. F: female; M: male; GT site: the periodontal defect from where granulation tissue and other samples (GCF, subgingival plaque) were collected; H-site: disease-free site from where a connective tissue specimen and other samples were collected.

Table 2. Kendal Tau correlations between subgingival plaque micro-organisms, GCF and mRNA levels of cytokines associated with diseased sites in 15 patients and correlations with CD markers in 10 patients

		IL-17A in GCF	TGF-β in GCF	TNF-α in GCF	IL-17A:IL-17E mRNA in GT	TGF-β mRNA in GT	F.n.	T.d.	T.f.	S.m.
P.g.	Tau=		0.302	0.321	0.302	-0.368				
	p=		**0.024**	**0.017**	**0.024**	**0.007**				
F.n.	Tau=	-0.268			0.296			0.698	0.856	
	p=	**0.046***			**0.027**			**<0.001**	**<0.001**	
T.d.	Tau=						0.698		0.72	
	p=						**<0.001**		**<0.001**	
T.f.	Tau=				0.307		0.856	0.720		
	p=				**0.022**		**<0.001**	**<0.001**		
S.m.	Tau=	-0.278			-0.278		-0.841	-0.803	-0.744	
	p=	**0.038**			**0.038**		**<0.001**	**<0.001**	**<0.001**	
CD90	Tau=						0.648	0.535	-0.592	-0.535
	p=						**0.016**	**0.046**	**0.028**	**0.046**
CD105	Tau=				-0.556					
	p=				**0.037**					
CD146	Tau=	0.511								
	p=	**0.040**								

Additional significant correlations: CD105 correlated with IL-17E mRNA in GT (Tau =0.667, **p=0.012**); CD105 correlated with IL-6 in GCF (Tau = 0.556, **p=0.025);** CD106 correlated with IL-17E in GCF (Tau=0.600, **p=0.016**).

***Significant correlations (Tau) p value is in bold.**

P.g. *= P. gingivalis,* F.n.*= F. nucleatum,* T.d*. = T. denticola,* T.f.= *T. forsythia,* S.m.*= S. mitis.*

Figure 1. Representative flow cytometry parameter diagrams showing % expression of MSC surface epitopes in granulation tissue (GT-MSC) and in disease-free gingival connective tissue (G-MSC) specimens of a single patient. Green line represents marker of interest and red line isotype control

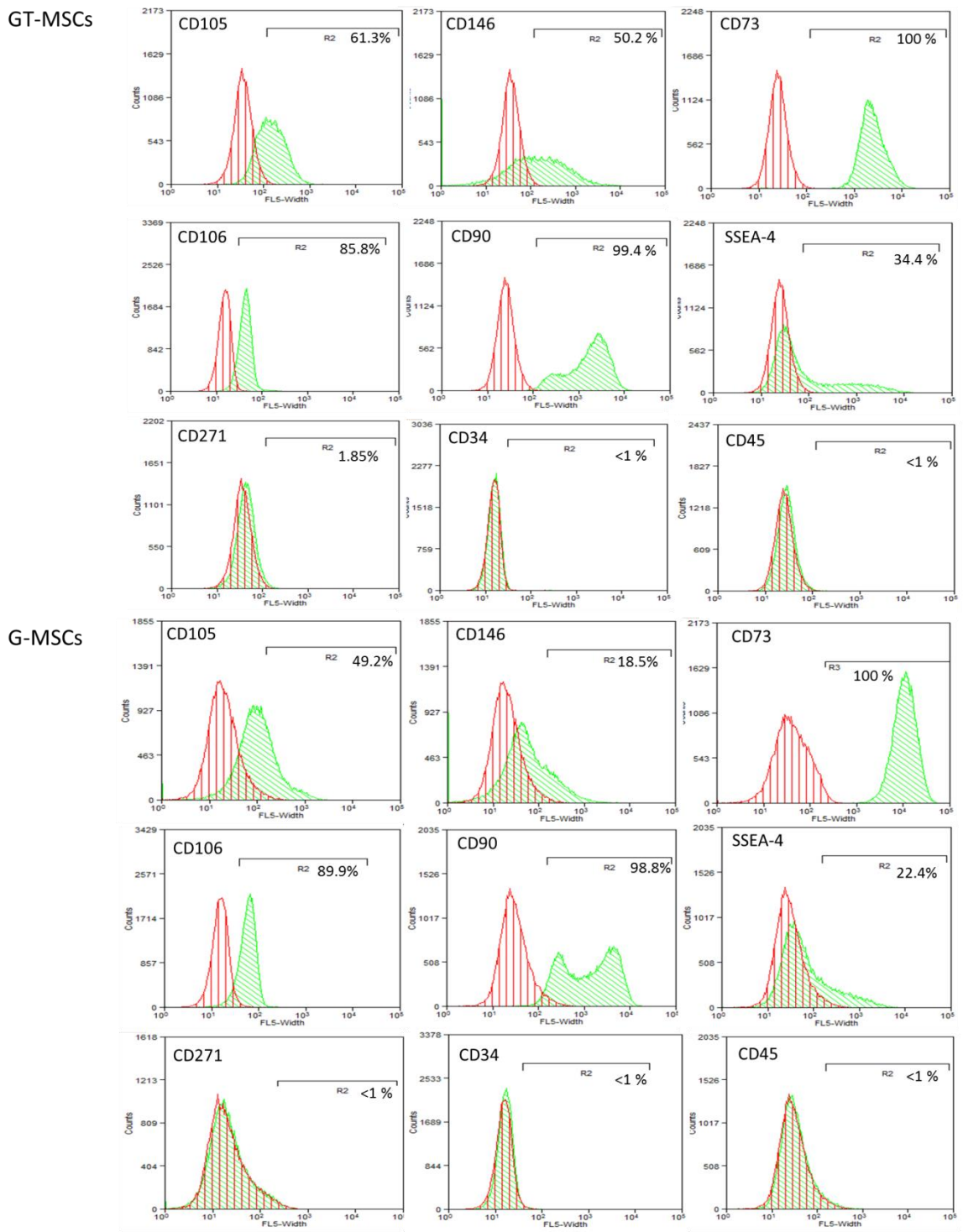
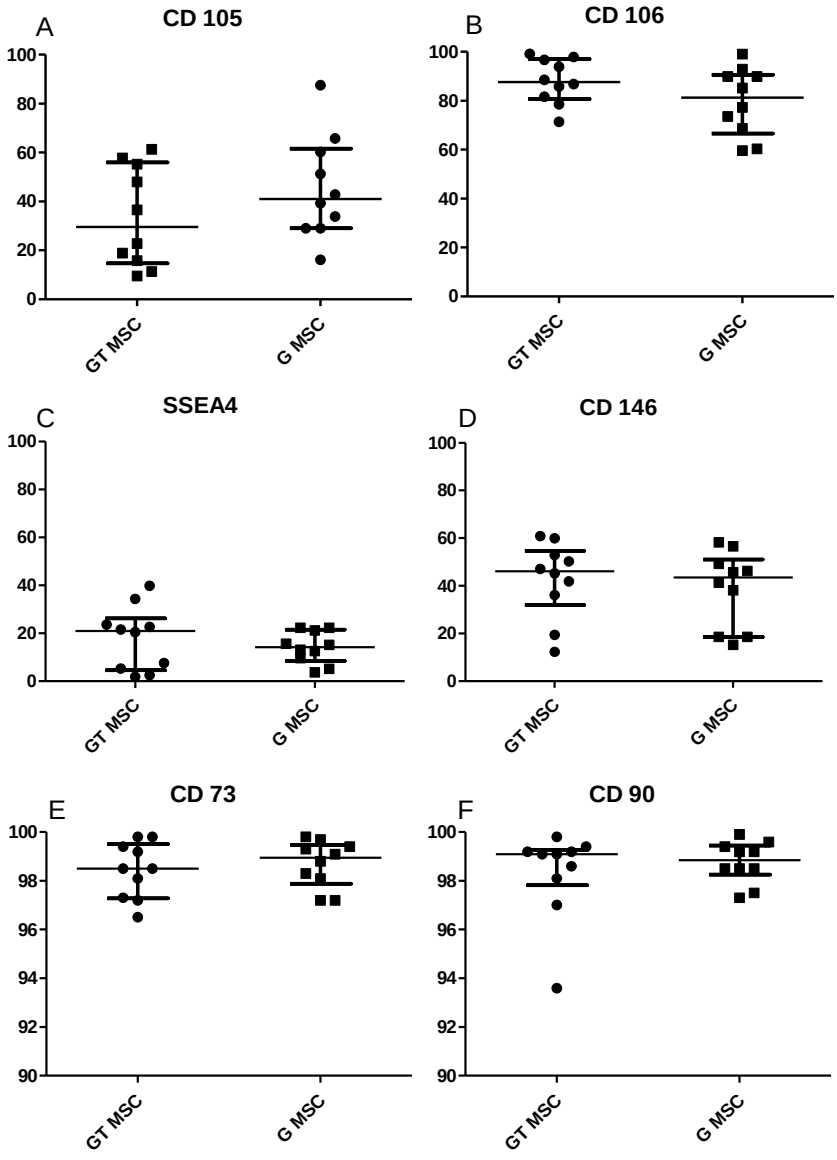


Figure 2. Summary data of flow cytometry for stem cell marker expression in granulation tissue (GT-MSC) and in disease-free gingival connective tissue (G-MSC) specimens of ten patients



Large inter-subject variability is noted and no apparent dissimilarities between the GT-MSC and G-MSC cultures for any of the markers tested according to a permutation test with a Monte Carlo simulation: CD105, p=0.231; CD146, p=0.503; CD73, p=0.654; CD106, p=0.256; CD90, p=0.686; SSEA-4, p=0.506; CD271, p=0.179; CD34, p=0.563; CD45, p=0.128.

Figure 3. Quantification of cytokine gene expression in granulation tissue (N_{GT}=15) and in disease-free tissue specimens (N_H=15); statistical significant differences are shown in asterisks (*p<0.05; **p<0.01; ***p<0.001)

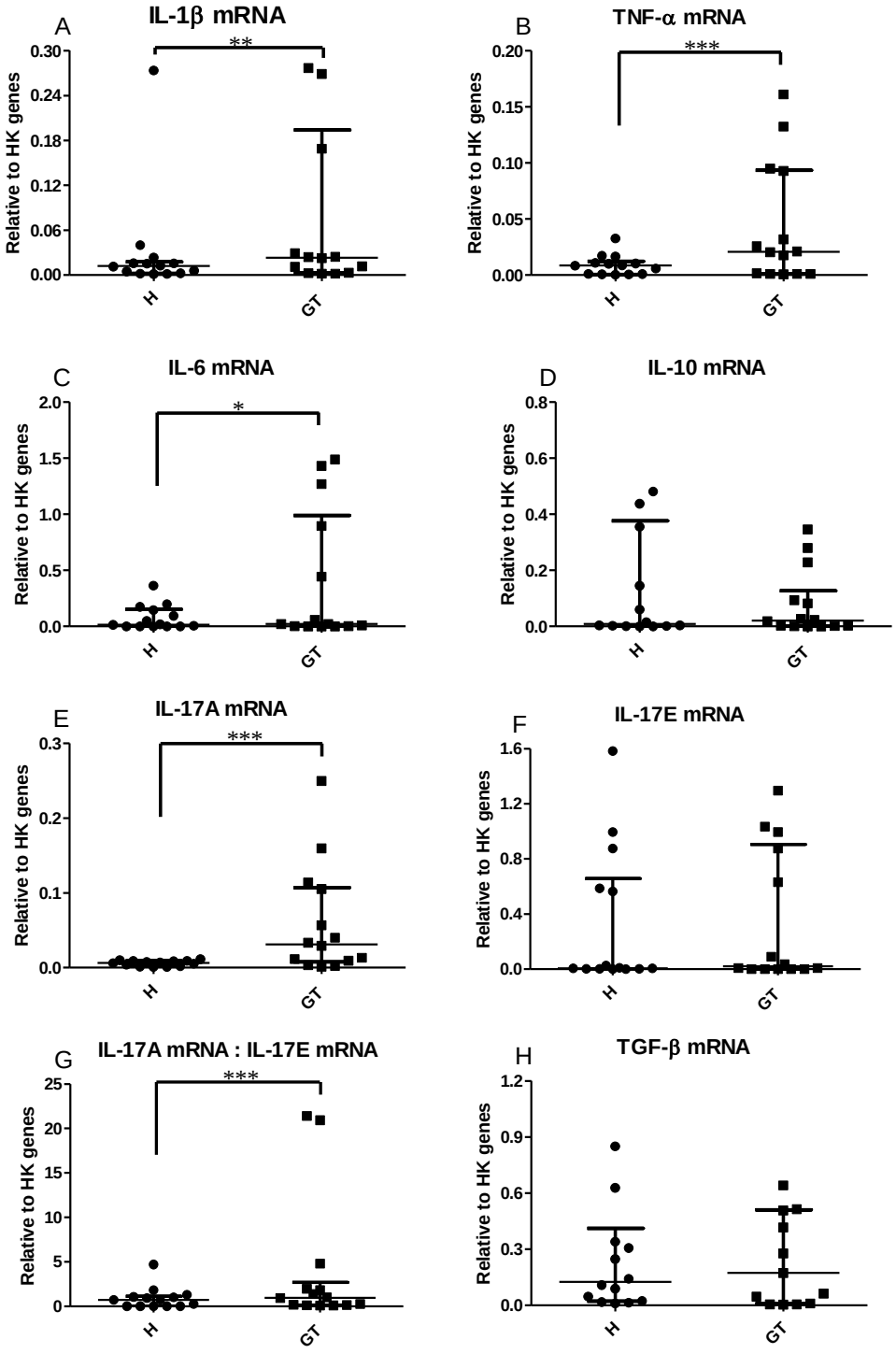


Figure 4. Cytokine levels in gingival crevicular fluid (GCF) associated with the granulation tissue sites (N_{GT}=15) and with the disease-free sites (N_H=15); significant differences are shown in asterisks (*p<0.05; **p<0.01; ***p<0.001)

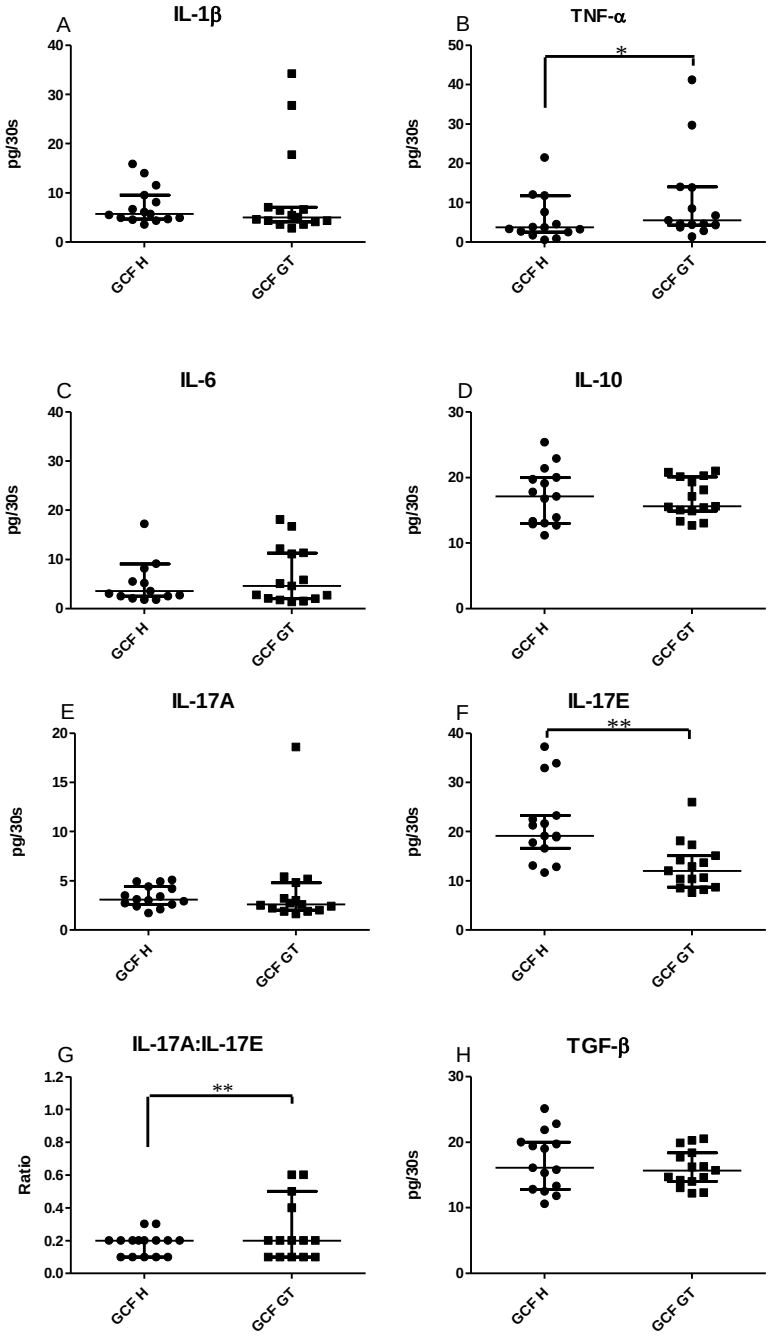


Figure 5. Quantification of *S. mitis* and putative periodontal pathogens in subgingival plaque samples at disease-free sites ((N_H=15) and at sites associated with the granulation tissue biopsies (N_{GT}=15); significant differences are shown in asterisks (*p<0.05; **p<0.01; ***p<0.001)