HDAC3 Regulates Gingival Fibroblast Inflammatory Responses in Periodontitis

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Appendix

**Appendix Figure 1.** Pan-HDACi do not induce apoptosis in gingival fibroblasts (GFs). Flow cytometric analysis of annexin V binding to GFs treated for 24 h with DMSO and 5 µM SAHA or 1 µM ITF2357 (A-B) or with staurosporine (STS) at 10 nM or 100 nM (C). STS treatment was used as a positive control for apoptosis induction. Data are presented as representative histograms (A and C) or as the mean % of annexin V-positive cells +SEM (n=3).
Appendix Figure 2. HDACi suppress inflammatory mediator expression in gingival fibroblasts (GFs) infected with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. (A) qPCR analysis of *CCL2, CCL5, IL1B* and *MMP3* mRNA levels in GFs (n=3) treated with DMSO or HDACi: ITF2357, HDAC3/6i, HDAC6i(a) (all at 500 nM) for 30 min prior to 4 h infection with *P. gingivalis* at MOI = 10 (left panel) or MOI = 25 (right panel). (B) qPCR analysis of *CCL2, CCL5, CXCL10, COX2, IL1B* and *MMP3* mRNA levels in GFs treated with DMSO or 500 nM ITF2357 for 30 min prior to 4 h infection with *F. nucleatum* at MOI = 100. Results are shown as (A) mean relative expression +SEM or (B) relative expression from a representative of two independent experiments.
Appendix Figure 3. HDAC1 silencing has no effect on inflammatory gene expression in *P. gingivalis*-infected GFs. Relative mRNA expression of *CCL2*, *IL1B* and *MMP3* in GFs (n=2) transfected with non-targeting siRNA (scrb), siHDAC3 or siHDAC1 before infection with *P. gingivalis* (MOI=100) for 4 h. qPCR data are shown as the mean relative expression +SEM.
Appendix Table 1. Clinical characteristics of healthy donors (HD) (n = 9) and patients with periodontitis (n = 4) included in the study:

| Characteristic | HD        | periodontitis |
|---------------|-----------|---------------|
| Age (y)       | 24 (18-44)| 60 (49-60)    |
| Male:female ratio (n) | 3:6 | 1:3 |
| API [%]       | 12 (0-43) | 32.5 (27.3-97) |
| SBI [%]       | 0 (0-10)  | 32.8 (24-74)  |
| PPD [mm]      | 2.0 (1.5-2.0) | 3.4 (1.3-4.5) |
| CAL [mm]      | 2.0 (1.5-2.0) | 5.9 (5.1-7)   |

The values are presented as median (range), unless otherwise indicated. API: approximal plaque index; SBI: sulcus bleeding index; PPD: probing pocket depth; CAL: clinical attachment loss.

Appendix Table 2. Specificity profile (IC₅₀ values in nM) of ITF HDACi used in this study.

| HDAC | HDAC3/6i (ITF3307) | HDAC6i(a) (ITF3926) | HDAC6i(b) (ITF3107) | HDAC8i (ITF3056) |
|------|--------------------|---------------------|---------------------|-------------------|
| HDAC1| 21                 | 2886                | 598                 | 19385             |
| HDAC2| 97                 | 11374               | 2423                | >30000            |
| HDAC3| 5                  | 2492                | 369                 | 15395             |
| HDAC8| 142                | 490                 | 178                 | 192               |
| HDAC6| 7                  | 4                   | 5                   | 8837              |
| HDAC4| 13578              | 606                 | 3918                | 164340            |
| HDAC5| 17522              | 512                 | 3505                | 119009            |
| HDAC7| 1554               | 623                 | 2466                | 59785             |
| HDAC9| 15333              | 640                 | 1896                | 220531            |
| HDAC10| 51               | 2680                | 731                 | 22745             |
| HDAC11| 32               | 1470                | 944                 | 35431             |
### Appendix Table 3. Sequences of primers used for qPCR analyses.

| Gene  | Forward primer                                  | Reverse primer                                  |
|-------|-------------------------------------------------|-------------------------------------------------|
| IL6   | GACAGCCACCTCACCTCTTCA                           | CCTCTTTGCTGCTTTACAC                            |
| IL8   | GCTCTGTGTGAAGGTGCAGT                           | CCAGACAGACCTCTCTTCCA                           |
| IL1B  | ACAGATGAAGTGCTCCTTCAA                          | GTCGGAGATTCTGAGCTGGAT                         |
| CCL2  | TCTGTGCTGCTGCTCATAG                           | GGGCATGATTGACATCTGAGC                         |
| CCL5  | ATCCTCATTGCTACTGCCCCT                         | GCCACGTTGAGAAATACCTCC                         |
| COX2  | AGCCCTTCCTCTGTGCCT                            | AATCAGGAAGCTGCTTTTTACCT                      |
| CXCL10| TGAAATTATTCCTGCAAGCCAA                         | CAGACATCTCTTCTCACCTCTTCTTCTT                 |
| MMP1  | GGGAGATCGATCGGGACACTC                         | GGGCCTGGTTGAAAAGCAT                         |
| MMP3  | GAGGACACCAGCATGAACCT                         | CACCTCAGAGTTGCAGAGT                          |
| HDAC3 | GATGGCATTTGATGCCAGAG                         | GATCACAGCCACGAGAATCA                         |
| RPLP0 | GCGTCCTCGTGAAGTGACATCG                       | TCAGGGATGCTCCACGCAGG                         |
Supplementary Methods

RNA isolation and quantitative (q)PCR

Extraction of total RNA was performed using an EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio-Basic), quantified with a Nanodrop spectrophotometer (Thermo Scientific) and equivalent amounts of RNA were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR reactions were performed using PowerUp SybrGreen PCR mix (Applied Biosystems) on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). The sequences of primers (Genomed S.A.) used in the study are listed in Appendix Table 3. The CFX Manager (BIO-RAD) was used for data analysis. The ΔΔCT method was used to calculate mRNA expression relative to RPLP0 (ribosomal protein lateral stalk subunit P0) unless otherwise indicated.

Western blot analysis

Cells were lysed in 1x Laemmli’s buffer containing 2% SDS, 10% glycerol and 125 mM Tris-HCl, pH 6.8. Protein content was measured using the Bradford assay (BioShop) and equal amounts of protein were resolved by electrophoresis on 10%, 12% or 15% polyacrylamide gels. Proteins were then transferred to the Immobilon-P SQ PVDF membranes (Millipore), which were subsequently blocked in 2% milk (BioShop) in TBS containing 0.1% Tween-20 (BioShop) (TBS/T). Membranes were then incubated with primary antibodies specific for acetyl-histone 3(Lys9/Lys14) (Ac-H3), Ac-tubulin(Lys40), Ac-lysine, H3, IκBα, phospho (p)-p38, p-ERK, p-p65, HDAC3 (all from Cell Signaling Technology) or tubulin (clone DM1A, Sigma-
Aldrich) at 4°C o/n, washed in TBS/T and incubated with horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse Ig secondary antibodies (Dako). Blots were developed using a ClarityWestern ECL Substrate (BIO-RAD) and visualized using a ChemiDocMP Imaging System. Densitometry was performed using the ImageLab software (BIO-RAD).

ELISA

GFs were treated with DMSO or HDACi for 30 min before infection with *P. gingivalis* at MOI=100 for 1 h. Cells were then washed three times with PBS and cultured in fresh medium containing antibiotics (250 µg/ml gentamycin and 200 µg/ml metronidazole) and DMSO or HDACi for another 23 h prior to collection of supernatants. Alternatively, after treatment with inhibitors, cells were stimulated with TNFα for 24 h. Concentrations of IL-8, CCL2, CCL5 and CXCL10 were determined in cell-free culture supernatants using ELISA MAX kits (BioLegend) according to the manufacturer’s instructions. An Infinite M200 microplate reader (Tecan) was used for absorbance measurements.

Measurement of cell viability

GF viability was measured using the LDH release assay (Roche). After 24 h culture in the absence or presence of HDACi or 1 µM staurosporine (STS), supernatants were collected and processed according to the manufacturer’s instructions. Supernatants from cells treated with lysis solution were used as a reference reflecting 100% cytotoxicity. Absorbance was measured at 490 nm using an Infinite M200 microplate reader (Tecan). Alternatively, after 24 h treatment
with HDACi or STS, cells were collected by trypsinization, washed and stained with annexin V-APC (BD Biosciences) as per the manufacturer’s protocol. The percentage of apoptotic cells was determined by flow cytometry using a BD FACSCalibur™ cell analyzer (BD Biosciences). Results were analysed using the FlowJo software (TreeStar).

**Transfection**

One day before transfection, GFs were seeded in 12-well plates in antibiotic-free DMEM supplemented with 10% FCS, which was then replaced with Opti-MEM I Reduced Serum Medium (Thermo Scientific). HDAC3-specific siRNA and control non-targeting siRNA (Dharmacon) (final concentration 100 nM) were mixed with DharmaFECT1 (Dharmacon) and Opti-MEM, and the mixture was incubated for 20 min at room temperature prior to transfection. 24 h post-transfection medium was replaced with DMEM containing 10% FBS and cells were cultured for an additional 24 h. Medium was then replaced with antibiotic-free DMEM containing 2% FCS and cells were infected with *P. gingivalis* at MOI = 100 for 4 h.

**Measurement of bacterial adhesion, invasion and intracellular survival: colony-forming assay and flow cytometry**

GFs were treated with DMSO or 5 µM SAHA in triplicate wells for 30 min. Cells were then infected with *P. gingivalis* at MOI = 100 for 1 h, washed three times with PBS and then were either lysed immediately in distilled sterile H₂O for 30 min or cultured in fresh medium containing DMSO or 5 µM SAHA for another 23 h prior to lysis. Alternatively, after infection
and washing, cells were cultured for 1 h in fresh medium containing gentamycin (250 µg/ml) and metronidazole (200 µg/ml) prior to lysis to eliminate bacteria that adhere to cell surface but are not internalized. Cell lysates were serially diluted and 10 µl of each dilution was plated on blood agar plates in duplicate and cultured for 5-7 days at 37°C in anaerobic conditions before counting bacterial colonies. Alternatively, bacteria were stained using the CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Life Technologies) as per the manufacturer’s instructions. After 30 min treatment with 5 µM SAHA or 500 nM ITF2357, GFs were infected with CFSE-labelled *P. gingivalis* at MOI = 100 for 60 min. Cells were then extensively washed with PBS to remove unattached extracellular bacteria, trypsinized, re-suspended in PBS and analyzed using a BD FACS Calibur™ and FlowJo software.