Suppression of αvβ6 Integrin Expression by Polymicrobial Oral Biofilms in Gingival Epithelial Cells

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Supplementary information

Methods

RT-qPCR on mouse gingiva

The gingiva around molar teeth was excised from the maxilla of 2-month-old WT and *Itgb6*$^{-/-}$ mice under a surgical microscope. Gingival tissues from three mice were pooled together as one biological replicate. The samples were ground over dry ice using a mortar and pestle, followed by extraction of total RNA using NucleoSpin RNA II kit and RT-qPCR to determine the differences in gene expression between WT and *Itgb6*$^{-/-}$ mice. Total of 18 WT and *Itgb6*$^{-/-}$ mice were used in the study per genotype (six biological replicates). PCR primer sequences are listed in Supplementary Table S**2.**

Immunohistochemistry of mouse gingiva

The gingiva around molar teeth was excised from the maxilla of 9-month-old WT and *Itgb6*$^{-/-}$ mice under a surgical microscope and processed for frozen sections and immunohistochemistry as described for the human tissue. Primary antibodies used for immunostaining were: β1 integrin (rat; mAb13; a kind gift from Dr. Kenneth Yamada, National Institutes of Health, Bethesda, MD, USA), α6 integrin (rat; mAb1378; Chemicon) and αvβ6 integrin (rabbit; β6B1). Primary antibodies were omitted from the negative control stainings. To highlight tissue structure, comparable sections were stained with hematoxylin and eosin, as described.

Culture of mouse macrophages

The murine macrophage-like cell line RAW 264.7 (ATCC) was used for confirming receptor agonist activity. Cells were cultured as periviously decribed[1] and seeded into plates at 3×10^4 cells/cm^2 for 48 h in their complete growth medium for the experiments. The cells were then rinsed once with PBS, changed to FBS-free medium and treated with receptor agonists or left untreated for 24 h. RNA isolation and RT-qPCR were performed as described.

P. gingivalis and *T. denticola* bacterial extracts

P. gingivalis (ATCC 33277) and *T. denticola* (ATCC 35405) were cultured as previously described[2]. The bacterial extracts were produced and heat-or proteinase K-treated as described for biofilm. The bacterial extracts were tested at concentrations 3-60 μg protein/ml and used in the experiments at concentration of 30 μg protein/ml (maximal efficacy without cytotoxity).

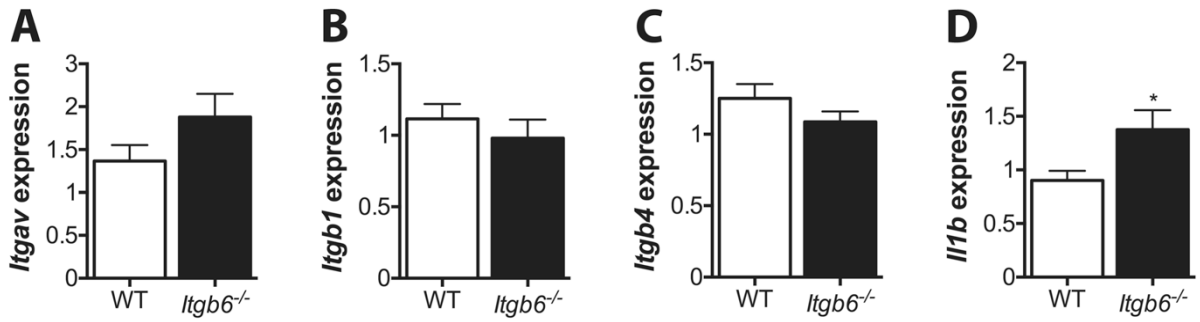
Genomic PCR of bacterial biofilms

Genomic PCR was used to determine the presence of total oral mycoplasma in the bacterial biofilms and to estimate the relative proportion of these bacteria in them using mycoplasma-specific and universal bacterial primers for 16S ribosomal RNA. The bacterial samples were boiled for 15 min. PCR amplification was performed with the CFX96 system (Bio-Rad; program for *Mycoplasma* gene: 5 min at 95°C, followed by 40 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C; program for universal bacterial gene: 5 min at 95°C, followed by 20 cycles of 30 s at 94°C, 1 min at 59°C and 1 min at 72°C). The sequences of Mycoplasma primers were: Forward: 5'-ACA CCA TGG GAG CT GGT AAT-3'; Reverse: 5'-CTT CTT CGA CTT TCA GA-CCC AAG -3'. The universal bacterial primer sequences were: Forward: 5'-CAD ACT CCT ACG GGA GGC-3'; Reverse: 5'-ATC CTG TTT GMT MCC CVC RC-3' (M=A/C, R=A/G, V=A/G/C). The PCR products were resolved by agarose gel electrophoresis (1% gel) and the images captured under UV light. ImageJ software was used for the quantification of blots.

References

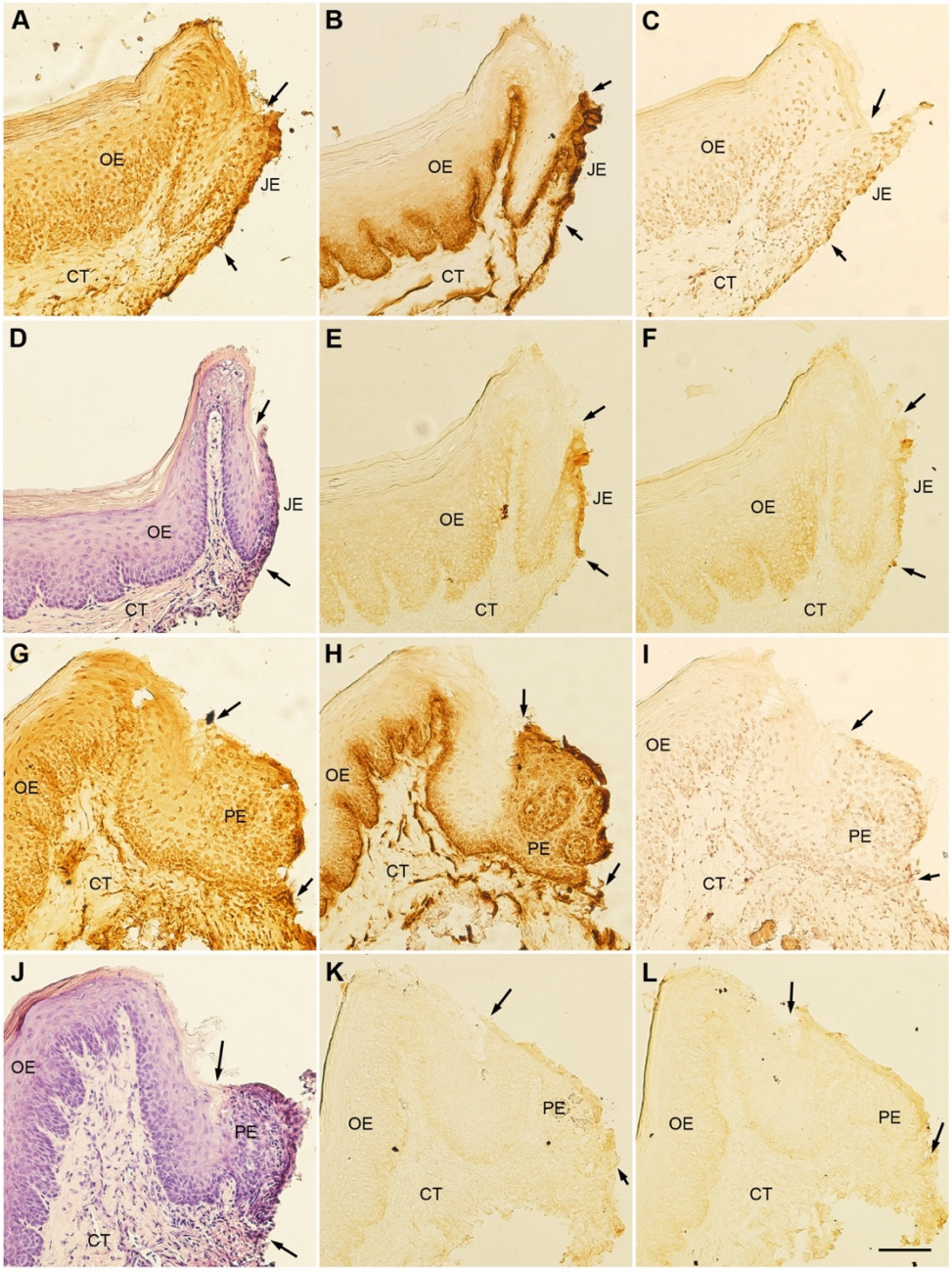
1 Barth, K. A., Waterfield, J. D. & Brunette, D. M. The effect of surface roughness on RAW 264.7 macrophage phenotype. J Biomed Mater Res A 101, 2679-2688, doi:10.1002/jbm.a.34562 (2013).

2 Bodet, C., Chandad, F. & Grenier, D. Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia. Microbes Infect 8, 27-35, doi:10.1016/j.micinf.2005.05.015 (2006).



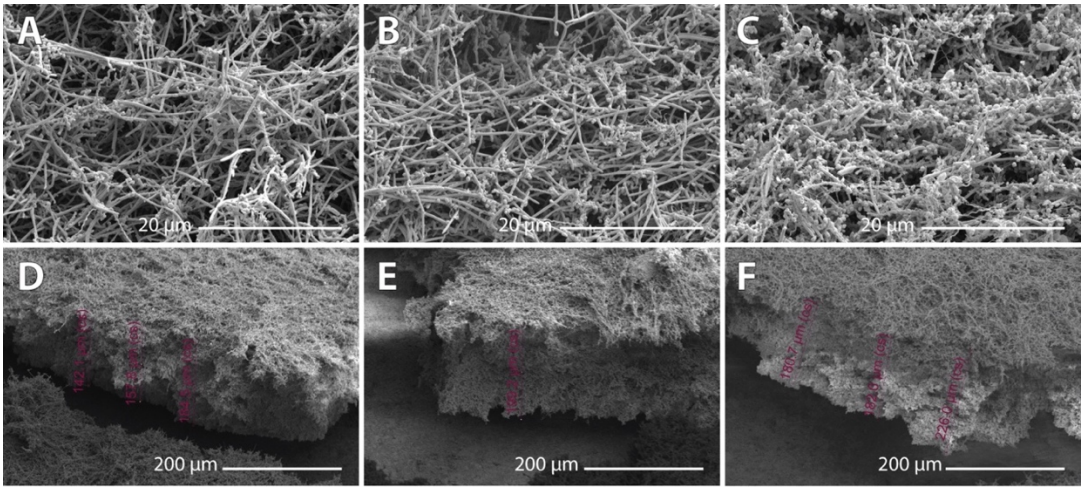
Supplementary Figure S1. Integrin and IL-1β gene expression in WT and *Itgb6*$^{-/-}$ mouse gingiva

A-D, RNA from WT and *Itgb6-/-* mouse gingiva was extracted and analyzed for *Itgav* (A), *Itgb1* (B), *Itgb4* (C) and *Il1b* (D) expression by RT-qPCR. Gingival tissue from three animals was pooled as one biological replicate. Mean ± SEM of total of six replicates (18 mice) per group is presented. *, $p<0.05$.



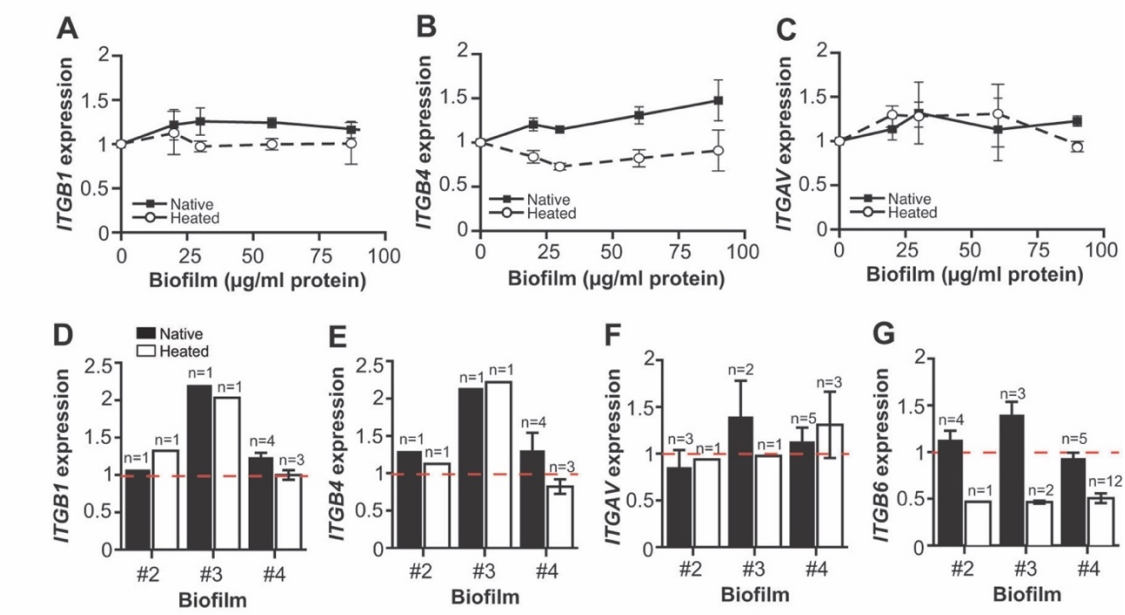
Supplementary Figure S2. Immunolocalization of integrins in WT and *Itgb6*$^{-/-}$ mouse gingiva

Immunolocalization of β1 (A, G), α6 (B, H) and αvβ6 integrins (E, K) in WT (E-F) and *Itgb6*$^{-/-}$ (G-L) mouse gingiva. Species-appropriate negative controls were performed with secondary antibodies only: anti-rat (C, I) and anti-rabbit (F, L). Comparable hematoxylin-eosin-stained sections of WT (D) and *Itgb6*$^{-/-}$ (J) mouse gingiva highlight the tissue structure. OE, Oral epithelium; CT, connective tissue; JE, junctional epithelium; PE, pocket epithelium. Arrows point the most coronal and apical aspects of the JE/PE. Bar is 50 μm.



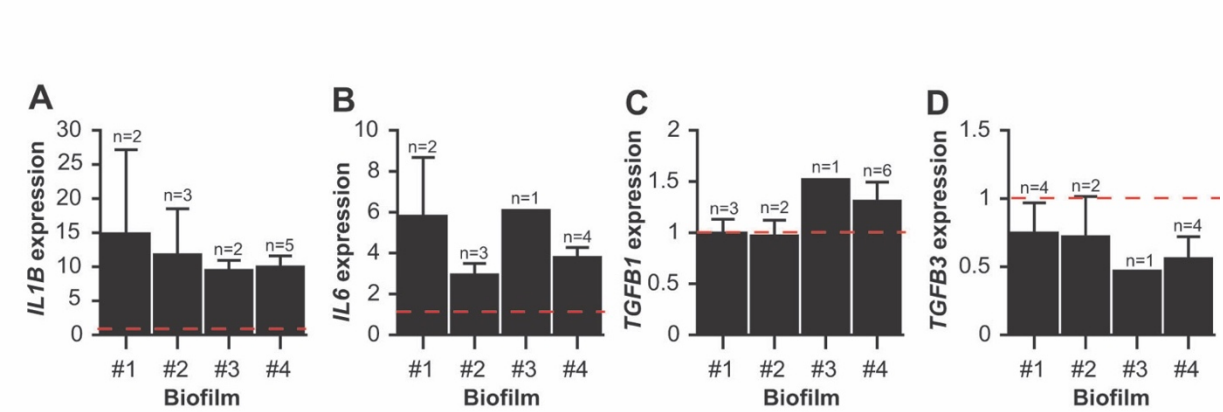
Supplementary Figure S3. Structure of oral bacterial biofilm from three donors.

A-F, The multi-species oral bacterial biofilms from three different donors were cultured for three weeks for SEM micrographs. (A-C) scale bar = 20 µm; (D-F) scale bar = 200 µm. D-F, Cross section (cs) SEM of oral bacterial biofilms reflects the thickness of biofilms of A-C, respectively.



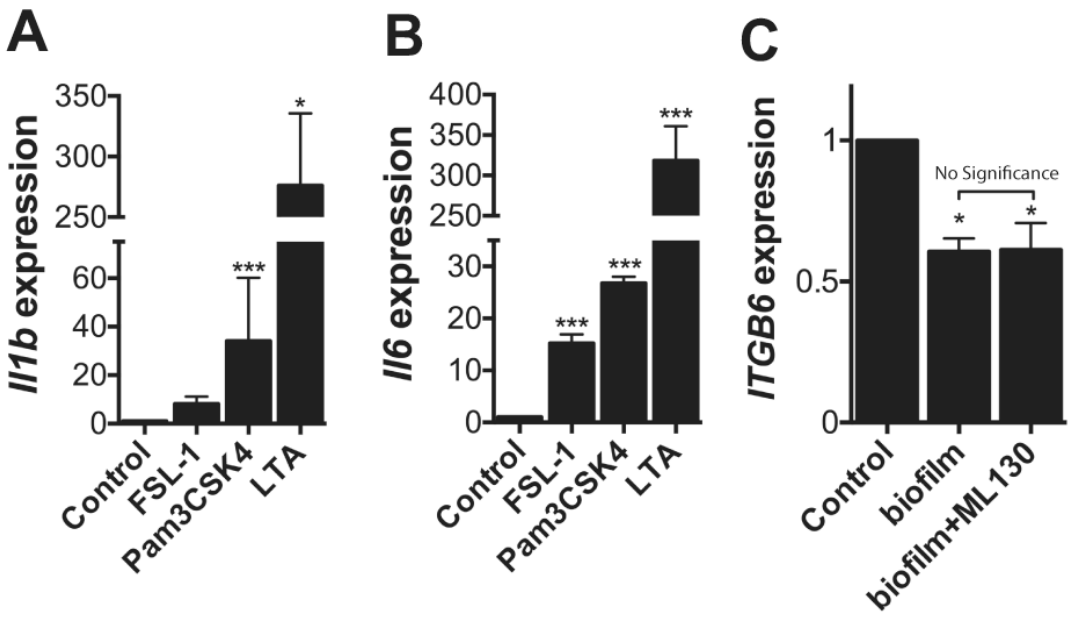
Supplementary Figure S4. Integrin gene expression in biofilm-treated GECs.

A-C, GECs were exposed to various concentrations of native or heated oral biofilm extract (#4 biofilm; 0-90 µg protein/ml) for 32 h. RT-qPCR was then performed to assess expression of *ITGB1* (A), *ITGB4* (B) and *ITGAV* (C). Mean ± SEM is presented, n=3-5. **D-G,** GECs were treated with either native or heated biofilm extract from three different donors (60 µg biofilm protein/ml) for 32 h and analyzed for *ITGB1* (D), *ITGB4* (E), *ITGAV* (F) and *ITGB6* (G) expression by RT-qPCR. Numbers of experimental repeats are indicated in the Figure. Mean ± SEM is presented. Dashed line indicates the expression level in the untreated control cells.



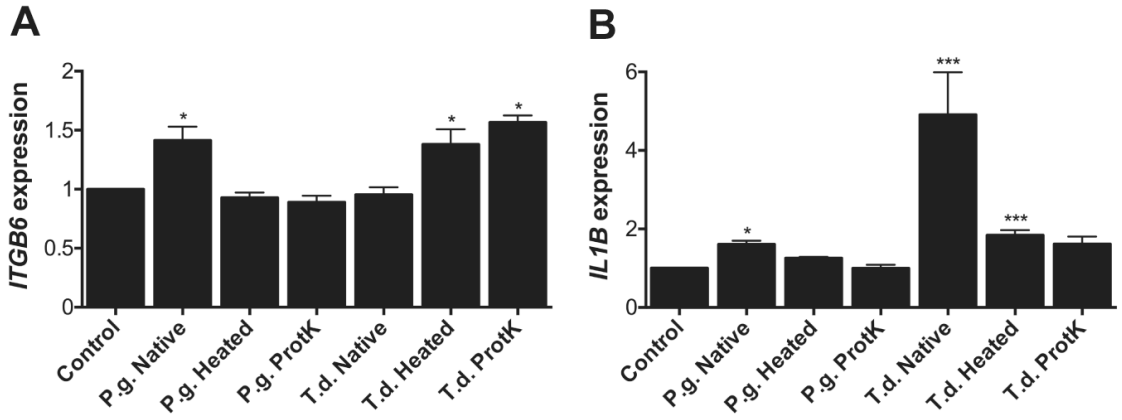
Supplementary Figure S5. Cytokine gene regulation by four biofilms.

A-D, GECs were treated with either native or heated biofilm extract from four different donors (60 µg protein/ml) for 32 h and analyzed for *IL1B* (A), *IL6* (B), *TGFB1* (C) and *TGFB3* (D) expression by RT-qPCR. Numbers of experimental repeats are indicated in the Figure. Mean ± SEM is presented. Dashed line indicates the expression level in the untreated control cells.



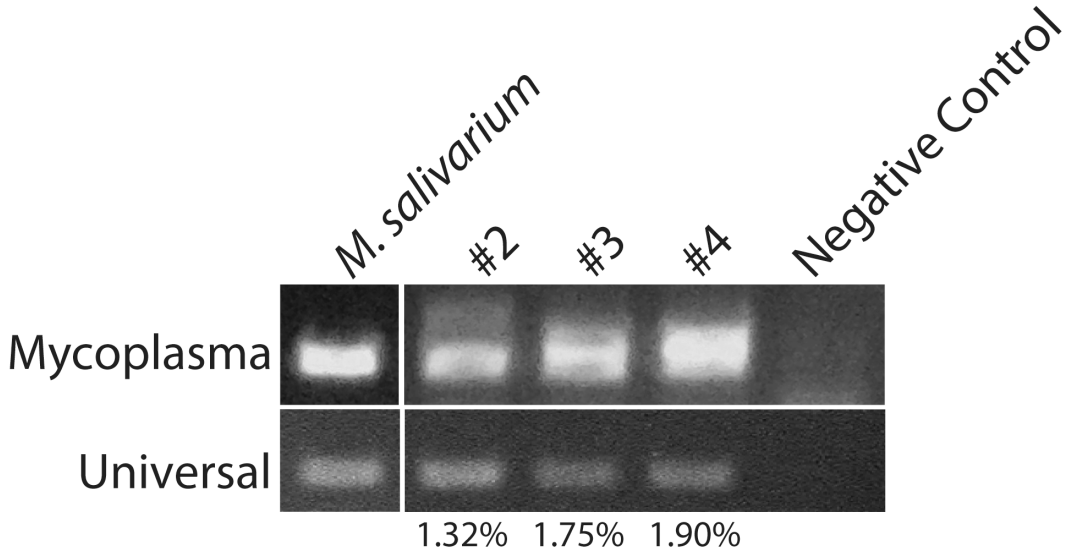
Supplementary Figure S6. Effects of TLR ligands on cytokine expression in RAW 264.7 macrophage cell line and NOD1 inhibitor on *ITGB6* downregulation in GECs.

A and B, RAW 264.7 cells were treated with FSL-1 (100 ng/ml), Pam3CSK4 (300 ng/ml) or LTA (2 µg/ml) or left untreated for 24 h, and their effect on *Il1b* (A) and *Il6* expression (B) was analyzed by RT-qPCR. Mean ± SEM is presented (n=3). **C,** GECs were pre-treated with NOD1 inhibitor ML130 for 1 h or left untreated, followed by addition of heated biofilm extract (60 µg protein/ml) for 32 h. The samples were analysed by RT-qPCR for *ITGB6* expression. Mean ± SEM is presented (n=4). *, $p<0.05$; ***, $p<0.001$.



Supplementary Figure S7. The effects of bacterial extracts of periodontal pathogens *P. gingivalis* and *T. denticola* on *ITGB6* expression in GECs.

A and B, GECs were treated with native, heated, or proteinase K-digested *P. gingivalis* and *T. denticola* extracts (30 µg protein/ml) for 32 h. *ITGB6* and *IL1B* expression was analyzed by RT-qPCR. Mean ± SEM is presented (n=3). *, $p<0.05$; ***, $p<0.001$.



Supplementary Figure S8. Agarose gel electrophoresis of oral bacterial biofilm PCR products.

Genomic PCR was performed for oral bacterial biofilms #2, #3 and #4 with mycoplasma-specific and universal bacterial primers (16S ribosomal RNA gene). The biofilm samples were diluted 1:100 for universal bacterial PCR analyses. Relative to Figure 5, *M. salivarium* was diluted 1:10 and used as a positive control for both of mycoplasma and universal bacterial PCR analysis, whereas water was used as a negative control. Agarose gel electrophoresis images were quantitated using ImageJ software, and the ratios of genomic copies for mycoplasma relative to universal genes were calculated and expressed as per cent of total genomic copies.

Gene	Cq Value	Relative expression
Il1b	24⩽Cq⩽27	Upregulation
Il2	35⩽Cq⩽38	Low expression
Il6	31⩽Cq⩽33	Low expression
Il8	37⩽Cq⩽39	Low expression
Il10	30⩽Cq⩽33	Low expression
Il17a	32⩽Cq⩽34	Low expression
Il22	35⩽Cq⩽38	Low expression
Il23	27⩽Cq⩽39	Low expression
Tnfa	27⩽Cq⩽29	No change

Supplementary Table S1. Cytokine gene expression in WT and *Itgb6*$^{-/-}$ mouse gingiva.

Gene	Cq Value	Relative expression
IL1A	28⩽Cq⩽35	Low expression
IL1B	23⩽Cq⩽27	Upregulation
IL6	24⩽Cq⩽29	Upregulation
IL7	31⩽Cq⩽34	Low expression
IL8	30⩽Cq⩽38	Low expression
IL10	35⩽Cq⩽38	Low expression
IL12A	29⩽Cq⩽31	No change
IL15	33⩽Cq⩽37	Low expression
IL18	27⩽Cq⩽29	No change
IL20	31⩽Cq⩽33	Low expression
IL24	33⩽Cq⩽41	Low expression
TNFA	27⩽Cqt⩽29	No change

Supplementary Table S2. Cytokine gene expression in biofilm-treated GECs relative to non-treated cells.

Gene	GenBank accession number (mRNA)	Forward primer	Reverse primer
Homo sapiens			
TGFB1	NM_000660	CAACGAAATCTATGACAAGTTCAAGCAG	CTTCTCGGAGCTCTGATGTG
TGFB3	NM_003239	ACACCAATTACTGCTTCCGCAA	GCCTAGATCCTGTCGGAAGTC
ITGAV	NM_002210	GAGGAAAGAGTGCAATCTTGTA	GAAGCAGACGACTTCAGAGA
ITGB1	NM_002211	ATGCCAAATCATGTGGAGAATG	GGCTTCTAAATCATCACATCGTG
ITGB4	NM_000213	CGTGTGAGGAATGCAACTTCAAGG	ACCACCTCCTCGGCTCT
ITGB6	NM_000888	AATTGCCAACCCTTGCAGTAG	AATGTGCTTGAATCCAAATGTAG
IL1B	NM_000576	CAGTGAAATGATGGCTTATT	CTTCATGTTTAGGGCCA
IL6	NM_000600	CAACCTGAACCTTCCAAAGATG	TCTGGCTTGTTCCTCACTAC
IL7	NM_001199888	ACTGAATGACTTGTGTTTCCTAAAG	ATGCAGCTAAAGTTCGTGTTTC
IL8	NM_000584	CAGAGGGTTGTGGAGAAGTT	GCTTGAAGTTTCACTGGCA
IL10	NM_000572	AGAACCTGAAGACCCTCAG	CTTATTAAAGGCATTCTTCACCT
IL12A	NM_000882	TCAAGCTCTGCATACTTCTTCAT	ATGACAACGGTTTGGAGGGA
IL15	NM_172175	GCAATGAAGTGCTTTCTCTTGG	TCCTCACATTCTTTGCATCCAG
IL18	NM_001243211	TGTAACTATCTCTGTGAAGTGTGAG	TCCTGGGACACTTCTCTGAA
IL20	NM_018724	GACATCAGAATCTTAAGGAGGAC	ATACCCTGTCCAGATAGAGTC
IL24	NM_ 006850	GAGATGTTTTCCATCAGAGACA	CAGGTCAGAAGAATGTCCAC
TNFa	NM_000594	GACCTCTCTCTAATCAGCC	TTGAGGGTTTGCTACAACA
MKI67	NM_001145966	TTCGGAAGCAAATCTGATTGT	TTGTTCATTGACCTTTGAGGA
TLR1	NM_003263	CTGTATCTGTATCAAGATGATCTG	TCTGATCTGAAGTATTAACATGAAG
TLR2	NM_003264	TGACTCCATTGAAAAGAGCCA	AAGACGGAAATGGGAGAAGTC
TLR3	NM_003265	AGCCTTACAGAGAAGCTATG	TAGGAAAGATCGAGCATAGTG
TLR4	NM_003266	AACCTCCCCTTCTCAACCA	GTTCTGGGAAACTGAAGAAGCTA
TLR5	NM_003268	GACTTTGCCCATCAATACACAGG	TCTCCAGGTTCGGACAGC
TLR6	NM_006068	AGACCTACCGCTGAAAACC	CAAGTAGCTGGATTCTGTTATGG
TLR7	NM_016562	CTCAAGCTGATCTTGGCA	TAGTCTTCTTCCAAAATGGAATG
TLR8	NM_138636	AGGTAAAAGGCTACAGGTCTC	AGCTCATTTATCACCCAGTCA
TLR9	NM_017442	AGCACCTTCTTGGCTGTG	TTGGTATGGCTGAGGGACA
TLR10	NM_001195108	ACTGGATTTATCCTATAACCTCC	AACTCTCAGTTTGGAGACAG
NOD1	NM_006092	CAGGCTTGGAAGAGACAGA	TCTGGCATCATTCTTTGAAGTTAG
NOD2	NM_001293557	GAAGGCTGCTTGATCTTGC	CATATACTTCTTGCATGTGGCA
ALG9	BC009255	GAATGACCAGAATCTAGAAGAGCCA	TCTCATGGTGTCCAAATCCACTAAA
B2M	NM_004048	TGTCTTTCAGCAAGGACTGGTCTTTC	ATGGTTCACACGGCAGGCATA
GAPDH	NM_002046	CTTTGTCAAGCTCATTTCCTGGTA	GGCCATGAGGTCCACCA
Mus musculus			
Itgb1	NM_010578	GCTGGTTCTATTTCACCTATTCA	CAACCACGCCTGCTACAA
Itgb4	NM_001005608	CCAGCTGAGACCAATGGCGA	GAGCACCTTCTTCATAGGTCCA
Itgav	NM_008402	GTCAGTCGGCAGGCTC	AGATCTTCTTTTGATCACTATTTACAG
Il1b	NM_008361	TCTATACCTGTCCTGTGTAATG	ACTCCACTTTGCTCTTGAC

Il2	NM_008366	TGAGCAGGATGGAGAATTACAG	AGAGGTCCAAGTTCATCTTCTAG
Il6	NM_031168	TCGGAGGCTTAATTACACATG	GTTTGGTAGCATCCATCATTTC
Il8	NM_011339	ACGGACATGGCTGCTCAA	CTGAATACACAGACATCGTAGCTCT
Il10	NM_010548	AGCAGGTGAAGAGTGATTTTAATAAG	CATCATGTATGCTTCTATGCAGT
Il17a	NM_010552	TGTGAAGGTCAACCTCAAAGTC	ACTGAGCTTCCCAGATCACA
Il22	NM_016971	TGACGACCAGAACATCCAGA	AGACGCAAGCATTTCTCAGAG
Il23	NM_031252	ATGGCTACCATGATAAGACTAATC	CTGCTCTGGGGTTTGTTTC
Tnfa	NM_013963	TGGGACAGTGACCTGGAC	TTC TGA GAC AGA GGC AACCT
Ubc	NM_019639	GCCACCGTGAAACAACTC	CCTCCAGGGTGATGGTCTTA
B2m	NM_009735	AAGGACTGGTCTTTCTATATCCTG	ACTCTGCAGGCGTATGTATC
Gapdh	NM_002046	CTTTGTCAAGCTCATTTCCTGGTA	GGCCATGAGGTCCACCA

Supplementary Table S3. Sequences of primers (5'-3') used for RT-qPCR

Original western blot and general PCR panels: