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Inflammasome and cytokine expression profiling in experimental periodontitis in the integrin β6 null mouse

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ABSTRACT

Epithelial αvβ6 integrin participates in immune surveillance in many organs, including the gastrointestinal track. Expression of αvβ6 integrin is reduced in the junctional epithelium of the gingiva in periodontal diseases, and mutations in the *ITGB6* gene are associated with these diseases in humans and mice. The aim of this study was to unravel potential differences in the inflammatory responses in the periodontal tissues of FVB wild-type (WT) and β6 integrin-null (*Itgb6*$^{-/-}$) mice, using a ligature-induced periodontitis model and assessing inflammation, bone loss and expression profiles of 34 genes associated with periodontal disease. Using micro-CT and histology, we demonstrated more advanced inflammation and bone loss in the control and ligatured *Itgb6*$^{-/-}$ mice compared to the WT animals. Neutrophil and macrophage marker genes were significantly upregulated by ligation in both WT and *Itgb6*$^{-/-}$ mice while the expression of T-cell and B-cell markers was downregulated, suggesting acute-type of inflammation. Expression of inflammasome NLRP3-related genes *Nlpr3* and *Il1b* was also significantly increased in both groups. However, the expression of *Il18* was significantly lower in non-ligatured *Itgb6*$^{-/-}$ mice than in the WT mice and was further downregulated in both groups by the ligatures. IL-18 mediates many effects of the AIM2 inflammasome, including regulation of the microbiome. Interestingly, expression of *Aim2* was significantly lower in both control and ligatured *Itgb6*$^{-/-}$ mice than in WT animals. Overall, ligature-induced periodontitis was associated with increased expression of pro-inflammatory cytokines, chemokines and osteoclastogenic regulatory molecules. Another significant difference between the *Itgb6*$^{-/-}$ and WT mice was that mRNA expression of the anti-inflammatory cytokine IL-10 was increased in ligatured WT mice but reduced in the *Itgb6*$^{-/-}$ mice. In conclusion, αvβ6 integrin in junctional epithelium of the gingiva appears to positively regulate the expression of the AIM2 inflammasome and anti-inflammatory IL-10, thus providing protection against periodontal inflammation.

1. Introduction

A large segment of the adult population suffers from periodontal diseases (PDs) that are also potentially associated with many systemic conditions, such as cardiovascular diseases, rheumatoid arthritis and cancer [10,13]. In the pathogenesis of chronic PD, dysbiotic bacterial dental plaque biofilm induces inflammation that leads to connective tissue degradation and alveolar bone resorption around the teeth. The relationship between the development of dysbiotic biofilm and inflammation is complicated, as biofilm can modulate inflammation, which can then reciprocally regulate biofilm formation [5]. Junctional epithelium (JE) is believed to participate in the modulation of gingival inflammation although direct evidence has been limited. JE expresses transforming growth factor-β1 (TGF-β1) that serves as a major anti-inflammatory cytokine in the periodontal disease process [11]. Integrin αvβ6 binds to and activates the latent TGF-β1 to regulate its anti-inflammatory surveillance role *in vivo* [3,31,35]. We have shown earlier that JE constitutively expresses both αvβ6 integrin and TGF-β1 and that αvβ6 integrin expression is markedly reduced in the periodontal pocket epithelium (PE) [15]. Recently, the first case of human *ITGB6* mutation associated with PD was reported [4]. Interestingly, mice deficient in β6 integrin (*Itgb6*$^{-/-}$ mice) also develop PD, including PE formation, gingival inflammation and alveolar bone resorption [15]. Our recent work further demonstrates more advanced PD and systemic

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inflammation in *Itgb6*$^{-/-}$ mice infected with four human periodontal pathogens [33]. We have recently demonstrated that *ITGB6* downregulation could be induced by bacterial biofilms [7]. Both biofilm- and siRNA-induced downregulation of *ITGB6* expression associated with upregulation of *IL1B* expression in human gingival epithelial cells, thus potentially linking αvβ6 integrin and inflammasomes. In the present study, we show that αvβ6 integrin deficiency in the gingiva leads to more advanced inflammation and bone loss characterized by reduced expression of *Aim2*, *Il18* and *Il10*.

2. Materials and methods

2.1. Animal ligature model

All animal procedures were reviewed and approved by the University of British Columbia Committee on Animal Care (Protocol number A16-0034). WT and *Itgb6*$^{-/-}$ mice (both on FVB/N background) were a kind gift from Dr. Dean Sheppard (University of California, San Francisco, CA, USA; [19]). The ligature-induced periodontitis model was used as described previously [1]. Briefly, silk sutures (5-0; Ethicon, Puerto Rico) were tied around the left second maxillary molars on 9-week-old mice. The right-side maxillary molar in the same animal was used as an untreated control. The sutures were retained in place for 14 days. The animals were then sacrificed and their jaws collected and used for analysis of bone loss, histological assessment and gene expressions studies (see below).

2.2. Assessment of bone loss using micro-CT

Total of 12 mice were used to assess the bone loss by micro-CT (6 wt and 6 *Itgb6*$^{-/-}$ mice). Maxilla specimens were collected and placed in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 h at +4 °C and then transferred into 2% formaldehyde in PBS for micro-CT scanning (Scanco CT 40; Scanco, Wayne, PA, USA). Images were produced with a voxel size of 10^3 μm^3. Alveolar bone loss was evaluated from the horizonal-angulated micro-CT images for both the control and the ligatures side of the jaw by averaging the measurements of the distances from the cemento-enamel junction (CEJ) to the alveolar bone crest in mesial and distal sites of the ligatured second molar.

2.3. Histological assessment of mouse jaw specimens

After scanning by micro-CT, the maxilla specimens were decalcified in PBS containing 2% formaldehyde and 0.4 M EDTA for five weeks followed by processing for paraffin sectioning as previously described [7]. The specimens were sectioned (8 μm) in the mesio-distal direction and stained with hematoxylin and eosin. Level of inflammation was scored between the first and second molars by blinded investigators using a sliding visual score (3 investigators; 0, no inflammation; 1, mild; 2, moderate; 3, severe). The number of neutrophil leukocytes was assessed by using naphthol AS-D chloroacetate esterase kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The number of esterase positive cells was counted between the first and second molars above the alveolar bone in each tissue slide (n = 6 per group).

2.4. Gene expression profiling by real-time quantitative polymerase chain reaction (RT–qPCR)

Gingival tissue was removed from around the 2nd maxillary molars of each mouse (ligatured and control side) using a dissecting microscope. The dissecting was performed by the same investigator for all animals to limit discrepancy in the sample collection. Tissue from three mice was pooled for each biological replicate (18 animals per group were used to obtain 6 biological replicates). The maxillae were then defleshed in 2% KOH. Gingival tissue was homogenized in a mortar on liquid nitrogen and total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Inc., Bethlehem, PA, USA). RNA samples with 1.8 to 2.0 of OD260/280 ratio were accepted for analysis. 1 μg of total RNA was reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed as previously described [6]. The mean Cq values lower than 33 were included in the gene expression analyses. Reference genes and PCR primer sequences are listed in Appendix Table 1. The comparative Ct method was used for

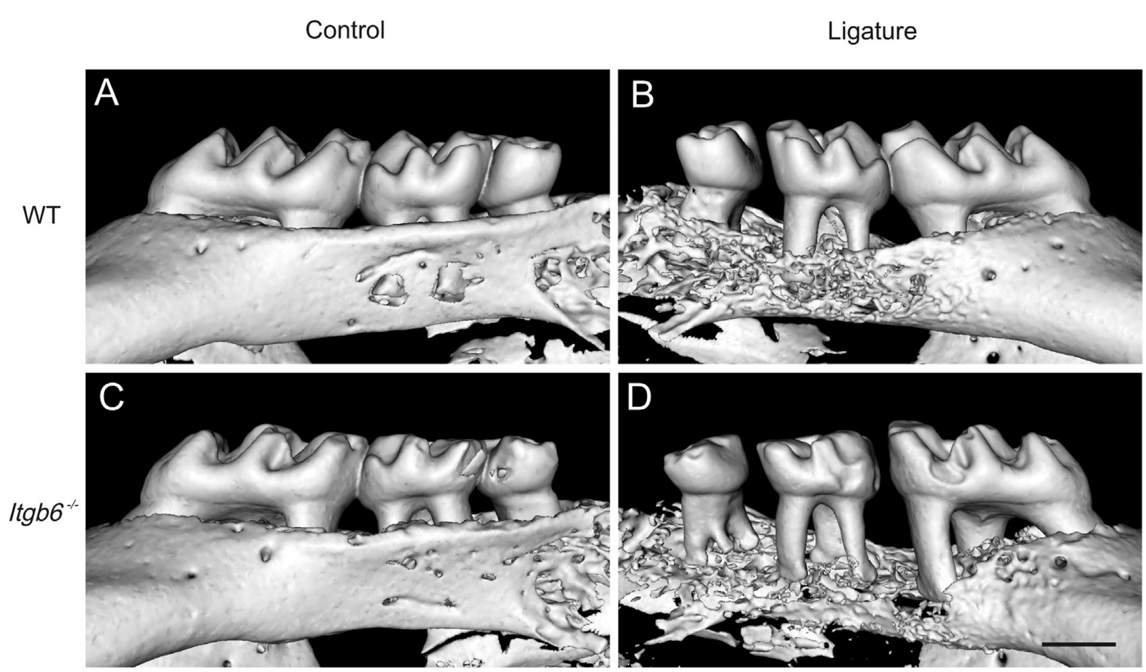

Fig. 1. Silk ligature induced bone loss in the WT and *Itgb6*$^{-/-}$ mice. Silk thread was ligatured around the left maxillary second molar for 2 weeks while the right side was left without a ligature as a non-treated control. (A and C) Non-ligatured control teeth for the WT and *Itgb6*$^{-/-}$ mice, respectively; (B and D) teeth ligatured for 2 weeks showing aggressive bone loss in the WT and *Itgb6*$^{-/-}$ mice, respectively. Scale bars = 500 μm.

data analysis (CFX Manager Software Version 2.1, Bio-Rad).

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2.5. Cell culture and RNA interference

Spontaneously immortalized human gingival epithelial cells (GECs) were maintained in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Grand Island, NY, USA) with 23 mM sodium bicarbonate, 20 mM HEPES, 1% antibiotics (50 µg/mL of streptomycin sulfate, 100 U/mL of penicillin; Gibco) and 10% heat-inactivated fetal bovine serum (Gibco) as previously described [6,7]. The small interfering RNA (siRNA) against *ITGB6* (UGGGCUGACAAGUAAUUCCdTdT) [20] and negative control siRNA (ACUUCGACACAUCGACUGCdTdT) were synthesized by Invitrogen. The siRNA transfection was performed for 48 h as previously described [7]. Alternatively, 3 µM of Smad3 inhibitor (SIS3; BioVision, Milpitas, CA, USA) was applied to the cells for 24 h in serum-free medium. The cells were then harvest for RT-qPCR as described.

2.6. Statistical analysis

Student's *t*-test for paired comparisons or one-way ANOVA followed by Tukey's post hoc test for multiple comparisons was performed. The log2-transformed data was used for the RT-qPCR statistical analysis [26]. The proportion of the 2nd molars present was analyzed by Fisher's exact test. $P < 0.05$ or lower was considered statistically significant.

3. Results

3.1. Young *Itgb6*$^{-/-}$ mice demonstrate more advanced spontaneous and ligature-induced alveolar bone loss compared to WT

We have previously shown that *Itgb6*$^{-/-}$ mice develop spontaneous signs of periodontal disease, including bone loss, as they age [7,15]. Here, we show that mice younger than three months old already present with similar changes (Figs. 1A and C and 2A). Thus, the data support the role of αvβ6 integrin in protection against PD from the time of teeth eruption. Not surprisingly, therefore, ligature-induced bone loss was much more pronounced in the *Itgb6*$^{-/-}$ mice than in the WT animals (Figs. 1B and D and 2A). In *Itgb6*$^{-/-}$ mice, furcations of 1st and 2nd molars became fully open and bone loss extended to the apical third of the roots while in WT, bone loss was mainly limited to around the 2nd molars and extended to about half of the root length. When defleshed, more 2nd molars from the *Itgb6*$^{-/-}$ maxillae (72%) were lost than from the WT (44%), confirming that the *Itgb6*$^{-/-}$ mice had more advanced PD in this model (Fig. 2B).

3.2. Histological assessment of periodontal tissues shows more intensive inflammation in *Itgb6*$^{-/-}$ mice

In the untreated WT mice, the periodontal structures between the 1st and 2nd molars showed normal architecture with JE extending to the CEJ, normal bone level and minimal inflammation (Fig. 3A and C). In the *Itgb6*$^{-/-}$ mice, JE remained at the CEJ but showed some apical extension. In addition, more inflammatory cells were present underneath the epithelium (Fig. 3E and G). The inflammatory infiltrate showed significantly higher scores in the control *Itgb6*$^{-/-}$ mouse tissue than in the WT tissue (Fig. 2C). Ligature-induced PD promoted epithelial migration apically in both groups of mice and increased inflammation scores (Figs. 3B, D, F and H and 2C). Little JE was present in ligature-treated mice. Inflammation was not limited to sub-epithelial zone but extended deep into the periodontal tissues.

3.3. Expression of Itgb6 is reduced in ligature-induced PD

To unravel potential differences in the inflammatory responses in the periodontal tissues of the control and ligatured WT and *Itgb6*$^{-/-}$ mice, we studied expression profiles of 34 genes associated with periodontal disease (Appendix Table 2). We have previously shown that αvβ6 integrin is strongly suppressed in the pocket epithelium in human periodontal disease [7,15]. Therefore, we investigated whether there were any changes in the expression levels of integrins in the gingival tissue in experimental PD in mice. There were no significant differences

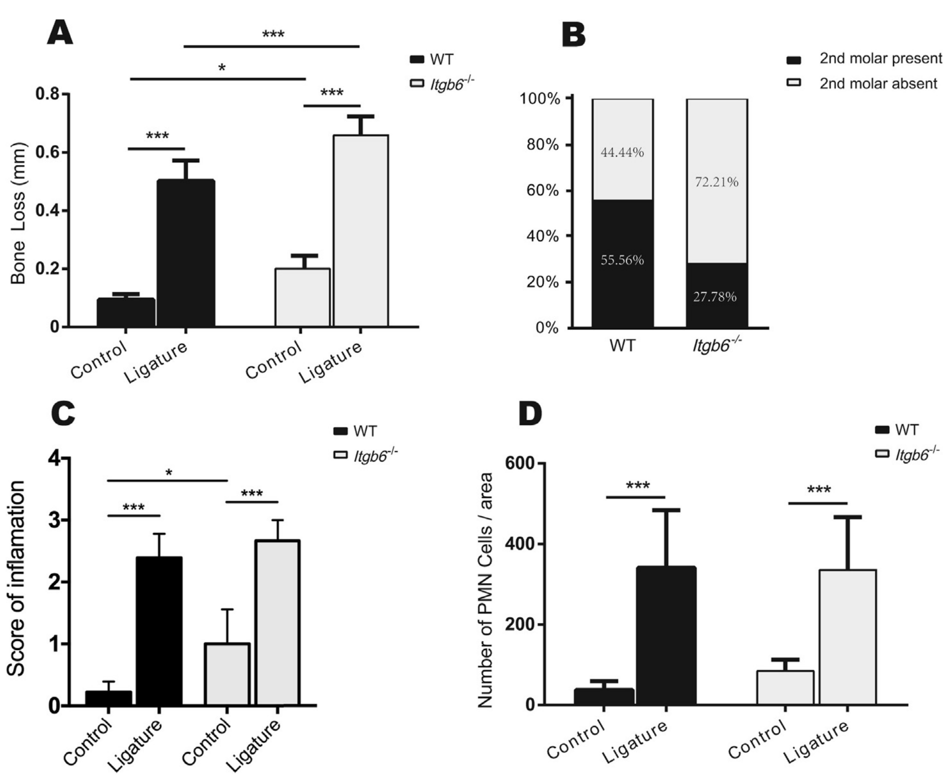

Fig. 2. Histological assessment of the periodontal tissues in experimental periodontal disease model in WT and *Itgb6*$^{-/-}$ mice. (A) Quantitative assessment of bone loss measured from Micro-CT images. (B) The proportion of the 2nd molars present after defleshing of the maxillae of WT and *Itgb6*$^{-/-}$ mice. (C) The inflammatory infiltrate adjacent to the epithelium was visually approximated in H&E-stained sections on a sliding scale (0 = no inflammation; 1, mild; 2, moderate; 3, severe). (D) The number of PMN cells present between the first and second molars above the alveolar bone in WT and *Itgb6*$^{-/-}$ mice with or without ligature. n = 6 animals per group; Mean ± SD; *, $p < 0.05$; ***, $p < 0.001$.

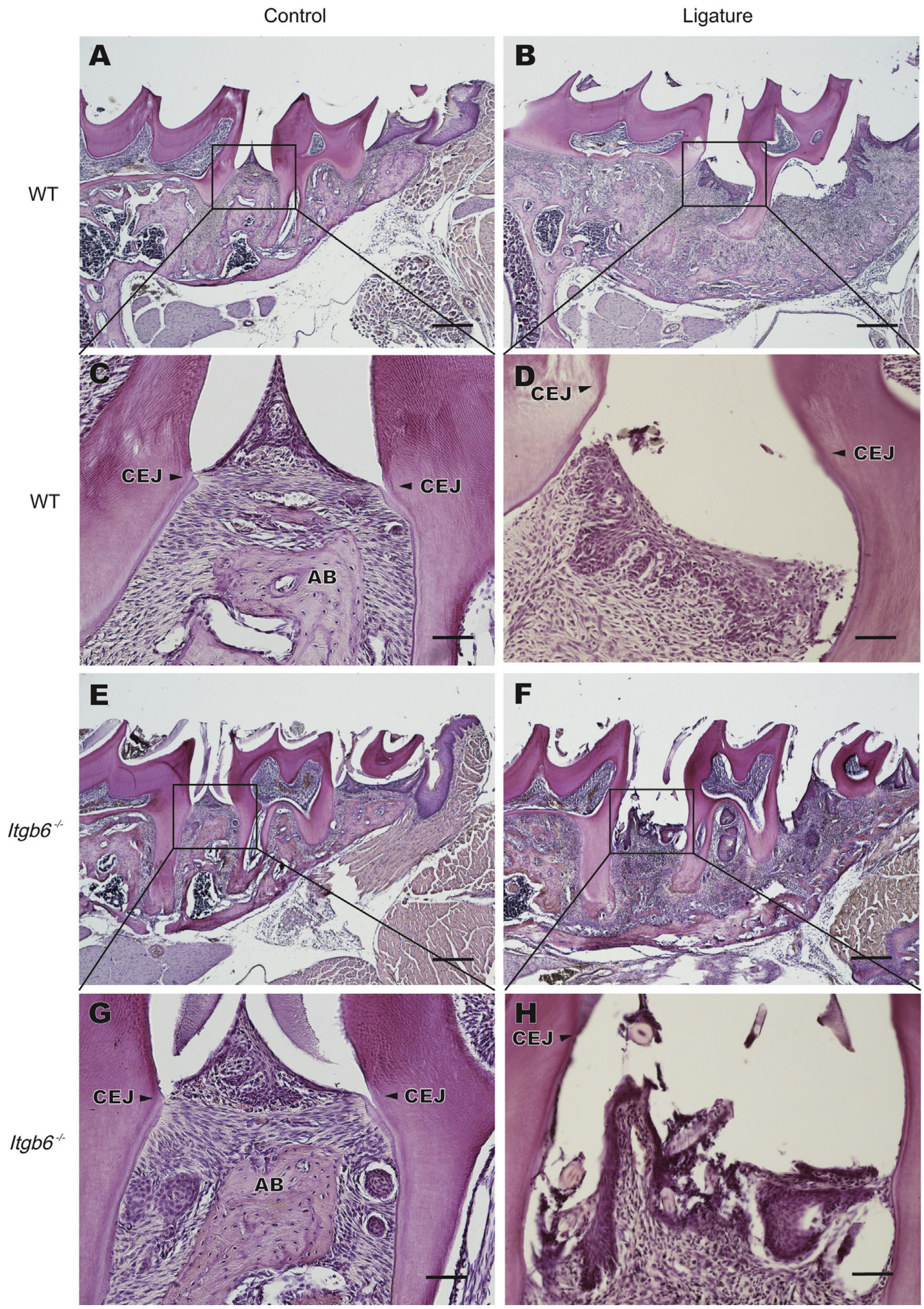

Fig. 3. Ligatured teeth in *Itgb6*$^{-/-}$ mice show more intensive inflammation in histological sections. A–H, H&E staining of the WT (A–D) and *Itgb6*$^{-/-}$ (E–H) mouse maxillae with (B, D, F and H) or without silk ligature (A, C, E and G) to the second molar for 2 weeks. The ligation induced periodontal pocket formation, inflammation, and alveolar bone resorption. Ligatured *Itgb6*$^{-/-}$ mouse tissue shows more intensive inflammation than the WT mouse. CEJ, cemento-enamel junction; AB, alveolar bone. Scale bars = 20 µm (A, B, E and F); 100 µm (C, D, G and H).

in the of *Itgav*, *Itgb1* or *Itgb4* mRNA levels between the non-ligatured WT and *Itgb6*$^{-/-}$ mice (Appendix Table 2). However, the ligation significantly reduced *Itgb6* expression in the WT animals (Appendix Table 2. In addition, the expression of *Itgb4* was reduced in both groups of ligatured mice (Appendix Table 2).

3.4. Gene profiling of cellular markers reveals dominance of acute phase inflammatory cells in ligature-induced PD

To characterize the cellular infiltrate in the ligature-induced PD, we performed RT-qPCR profiling of markers for neutrophils (PMNs), macrophages (MFs), T-cells and B-cells. In both WT and *Itgb6*$^{-/-}$ mice, the markers for PMN (Ly6G) and MFs (CD14) were significantly increased in ligature-induced periodontitis compared to controls (Fig. 4A and B; Appendix Table 2). We also tested another MF marker (F4/80) with similar results as CD14 (Appendix Table 2). However, the markers for T-cells (CD3e) and B-cells (CD20) were significantly reduced (Fig. 4C and D; Appendix Table 2), suggesting that this model represents the acute inflammatory reaction consistent with initial/early periodontal lesions dominated with PMNs and MFs, as described by [24]. We also quantified PMN cell numbers by histochemical staining. In both groups of mice, the PMN cell numbers were significantly increased after ligation (Fig. 2D), supporting the PCR marker data. Overall, the data showed no marked qualitative differences in cell type

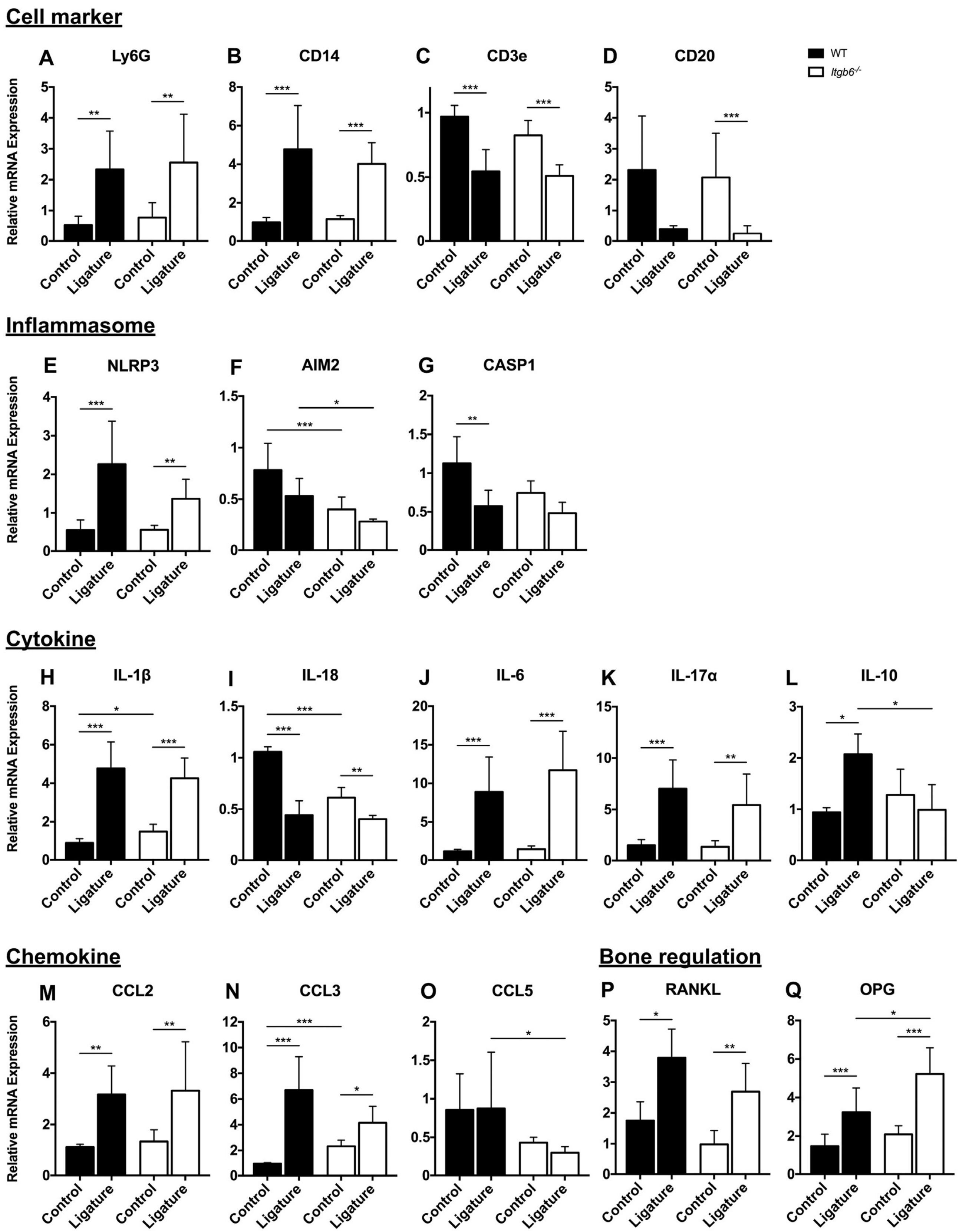

Fig. 4. The expression of genes associated with periodontal disease in the experimental periodontal mouse model. Expression of a set of significantly regulated genes associated with PD. (A–D) Cell markers for immune cells; (E–G) inflammasome-related genes; (H–L) cytokines; (M–O) chemokines; (P and Q) genes that regulate osteoclastic bone resorption. Gingival tissue from three mice was pooled for each biological replicate, and six biological replicates were analyzed for each group, representing 18 mice in each group. Mean ± SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

changes between WT and *Itgb6*$^{-/-}$ mice.

3.5. AIM 2 inflammasome is differentially regulated in Itgb6$^{-/-}$ mice compared to WT

Inflammasomes are platforms that support activation of pro-IL-1β and pro-IL-18 to their active forms by caspase-1 [14]. As these factors may be involved in the pathogenesis of PD [17,32], we performed marker profiling for the nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors (NLRs) and absent in melanoma 2 (AIM2) inflammasomes in the mouse gingival tissue. Among NLRs family members, *Nlrp1* showed low expression whereas *Nlrc4* was not regulated by ligature-induced PD (Appendix Table 2). However, the expression levels of both *Nlpr3* and *Il1b* were highly elevated in both ligatured groups (Fig. 4E and H; Appendix Table 2). Interestingly, expression levels of *Aim2*, *Casp1* and *Il18* were significantly reduced in the non-ligatured *Itgb6*$^{-/-}$ mice compared to WT (Fig. 4F, G and I; Appendix Table 2) and further downregulated in both ligatured groups (Fig. 4F, G and I; Appendix Table 2). Again, *Aim2* expression remained significantly lower in the *Itgb6*$^{-/-}$ mice. Next, we tested whether absence of αvβ6 integrin in epithelial cells could be directly linked to downregulation of *AIM2* expression using cultured human GECs. To this end, *ITGB6* in GECs was downregulated by RNA interference by over 80% (Fig. 5A). Supporting the mouse data, the expression of *NLPR3* was significantly upregulated while that of *AIM2* and *CASP1* was downregulated by the *ITGB6* siRNA. The expression level of *IL18* was not altered however (data not shown). We have previously shown that *ITGB6* downregulation is linked to higher *IL1B* expression in these cells [7]. Collectively, these results suggest that altered imflammasome expression and function could be directly linked to β6 integrin knockdown and PD in this model.

3.6. Gene expression of IL-10 and CCL3 is differentially regulated in Itgb6$^{-/-}$ and WT mice

To search for further differences in the inflammatory response between the *Itgb6*$^{-/-}$ and WT mice that could explain the advanced bone loss in the *Itgb6*$^{-/-}$ mice, we performed gene profiling for additional PD-associated inflammatory markers. The expression of pro-inflammatory *Il6* or *Il17a* was similar in non-ligatured control mice but significantly increased in ligature-induced PD in both groups (Fig. 4J and K; Appendix Table 2). Intriguingly, expression of anti-inflammatory *Il10* was significantly elevated in ligatured WT mice but not in the *Itgb6*$^{-/-}$ animals (Fig. 4L; Appendix Table 2). Expression of anti-inflammatory *tgfb1* was not significantly altered in this model (Appendix Table 2). Expression levels of other cytokines linked to PD were either low or unaltered (*Il4, Il12, Il21, Il22, Il23*; Appendix Table 2). Chemokines have also been implicated in the pathogenesis of PD [30]. The level of expression of chemokines *Ccl2* and *Ccl5* did not significantly differ in non-ligatured *Itgb6*$^{-/-}$ and WT mice (Appendix Table 2). However, the expression of *Ccl3* was elevated in non-ligatured *Itgb6*$^{-/-}$ mice compared to WT (Fig. 4N; Appendix Table 2). In ligature-induced PD, expression of *Ccl2* and *Ccl3* increased while that of *Ccl5* was significantly lower in the *Itgb6*$^{-/-}$ mice than in the WT animals (Fig. 4M, N and O; Appendix Table 2). Due to differences in bone loss, we also assessed cytokines that directly regulate osteoclastic bone resorption,

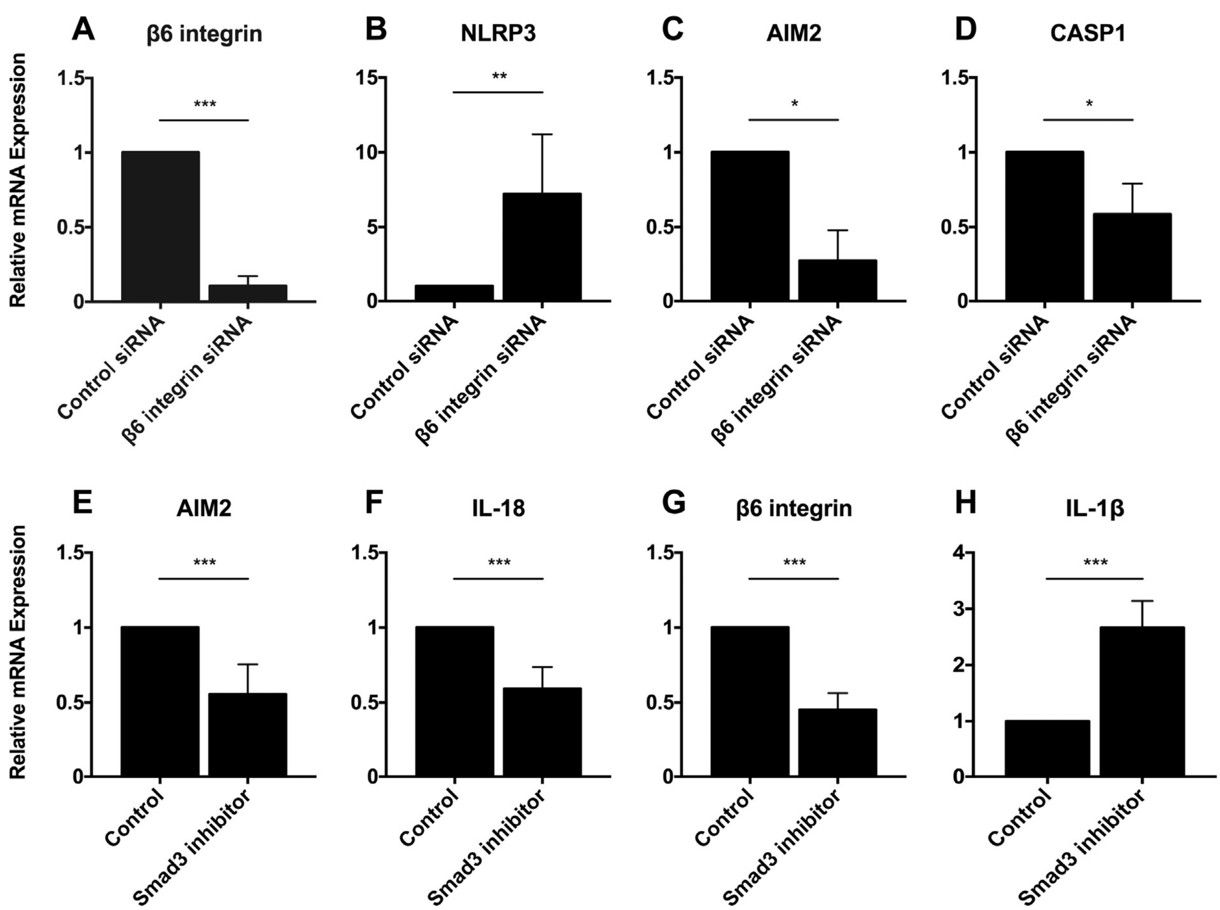

Fig. 5. β6 integrin and Smad3 involvement in the expression of AIM2 inflammasome-related genes in human gingival epithelia cells. (A) The expression of β6 integrin was reduced more than 80% after β6 integrin siRNA treatment in GECs. (B–D) The effect of β6 integrin knockdown on NLRP3, AIM2 and CASP gene expression in GECs. (E–H) The effect of TGF-β/Smad3 signaling inhibitor SIS3 (3 μM) on gene expression of AIM2, IL-18, β6 integrin and IL-1β. Mean ± SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

namely receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG). Both showed a significant increase in ligature-induced PD but the differences between *Itgb6*$^{-/-}$ and WT mice were only seen in the *Opg* levels, favoring *Itgb6*$^{-/-}$ mice (Fig. 4P and Q; Appendix Table 2).

3.7. Smad3 pathway may be involved in the AIM 2 inflammasome regulation by αvβ6 integrin

Integrin αvβ6 is a major activator of anti-inflammatory TGF-β1 *in vivo* [3,31,35]. To Investigate the link through which αvβ6 integrin could regulate *AIM2, CASP1* and *IL18* expression in GECs, we tested whether autocrine TGF-β/Smad3 signaling could be involved. The expression levels of *AIM2*, *IL18* and *ITGB6* were significantly downregulated by treatment with Smad3 inhibitor SIS3 (Fig. 5E, F and G), while *Il1B* levels were significantly upregulated (Fig. 5H), indicating involvement of autocrine TGF-β/Smad3 signaling the maintenance of ITGB6 expression in GECc and in their inflammatory gene expression. However, the expression of *CASP1* was not altered by Smad3 inhibition, and *NLRP3* showed low expression in the experiment (data not shown).

4. Discussion

We have demonstrated previously that JE expresses TGF-β1, a major anti-inflammatory cytokine, and its activator, αvβ6 integrin, and that the loss of αvβ6 integrin in the JE results in periodontal disease [15]. In the present study, we show that its loss also leads to reduced expression of *Aim2* and *Il18*, increased *Il1b* and *Ccl3* expression, higher inflammatory scores and bone loss in the *Itgb6*$^{-/-}$ mice compared to the WT animals. Remarkably, these changes can already be observed in 11-week-old mice, suggesting that PD in these mice is initiated immediately when the teeth have fully erupted. Interestingly, siRNA knockdown of *ITGB6* also strongly downregulated *AIM2* and upregulated *NLPR3* in cultured GECs, suggesting a direct connection between αvβ6 integrin and the regulation of inflammatory pathways. Detailed pathways demonstrating how the loss of β6 integrin leads to stimulation of *NLRP3* and reduction of *AIM2* expression need to be dissected in future studies. However, our results indicate that Smad3 signaling could be involved.

Interestingly, while the expression of *Nlrp3* and *Il1b* was significantly increased in both groups of mice by experimental PD, the expression of *Aim2* and *Il18* was decreased, particularly *Aim2* in the *Itgb6*$^{-/-}$ mice. Inflammasomes are crucial protein platforms that regulate inflammation by activating caspase-1 that cleaves pro-IL-1 and pro–IL-18 into mature active cytokines [17,32]. The NLRP3 inflammasome has been widely investigated in the context of many inflammatory diseases, and it can be activated by variety of signals, including bacterial components, K^+ and Ca^{2+} signaling, reactive oxygen species, mitochondrial dysfunction and lysosomal rupture [17]. AIM2 inflammasome, however, is activated by cytosolic sensing of dsDNA of pathogenic bacteria [32]. Expression of NLRP3 and IL-1β are increased in gingivitis and in various forms of PD [8,34,36], suggesting that NLPR3 inflammasome complex is involved in the PD process. Interestingly, both NLRP3 and AIM2 inflammasomes, which can equally activate both IL-1β and IL-18 through the same mechanism can be either pro-inflammatory or maintain homeostatic functions, depending how much and where they are expressed [27,32]. In the gut, for example, sufficient amounts of AIM2-generated IL-18 are required for epithelial homeostasis but its increased expression in the connective tissue can lead to intestinal inflammation [27,32]. Furthermore, AIM2 appears to protect against gut pathogens and control the commensal microbial composition [32]. Dysbiosis of the gut microflora has been observed in AIM2 knockout mice [18,23,25]. The role of AIM2 in PD is unclear, whereas IL-18 expression seems to be downregulated with disease progression [12,34]. Our data show an actual decrease of *Aim2* and *Il18* expression in experimental ligature-induced PD in mice. However, future studies in this animal model should not only investigate the link between αvβ6 integrin and AIM2 but also the possible shifts in the periodontal microbiome towards dysbiosis in the *Itgb6*$^{-/-}$ mice that develop spontaneous PD.

In general, ligature-induced PD appears to recapitulate acute periodontal inflammation mimicking initial/early periodontal lesions with the dominance of neutrophils and macrophages [24]. While ligature-induced mechanical trauma cannot be totally excluded, the biofilm accumulation on the ligatures is believed to act as the major factor causing inflammation and bone loss in this model [1]. It is not surprising, therefore, that the expression levels of many cytokines and chemokines associated with periodontal inflammation and bone loss were increased by ligatures, such as *Il6*, *Il17a*, *Ccl2*, *Ccl3*, *Rankl* and *Opg* [12,16,28]. Although there were some quantitative differences between the WT and *Itgb6*$^{-/-}$ mice, the only qualitative differences were noted in the expression of *Ccl3* in the non-ligatured samples and in the regulation of *Il10* in the experimental PD. The higher *Ccl3* expression in the *Itgb6*$^{-/-}$ mice may relate to their genetic susceptibility to PD as CCL3 levels strongly associate with PD and discriminate between periodontal health and PD in humans [2]. Expression of CCL3 is strongly suppressed by TGF-β1 [22], and whether reduced activation of TGF-β1 in the *Itgb6*$^{-/-}$ mice could explain the higher *Ccl3* expression needs to be further studied. The total gene expression level of gingival TGF-β1 did not change in our study. This is not surprising as *Tgfb1* is expressed by a variety of inflammatory cells in addition to JE, which may thus provide a relatively minor contribution to the total pool expressed. It is likely that the active TGF-β1 available in sufficient amounts in the JE could regulate periodontal inflammation far beyond the initial action site. Overall, TGF-β1 and IL-10 are considered anti-inflammatory cytokines in PD [16]. TGF-β1 participates in the regulation of IL-10 expression in variety of T-cells [21]. IL-10 functions as the control of vigorous responses of T-cells and macrophages to microbial agents. Mice deficient in IL-10 show aggravated experimental PD [29]and the increase in IL-10 in PD is believed to be limit disease severity [9]. Therefore, lack of elevated *Il10* response in the *Itgb6*$^{-/-}$ mice could be a direct consequence of reduced availability of active TGF-β1 and associate with increased PD severity in the ligatured *Itgb6*$^{-/-}$ mice.

In conclusion, this study reveals significant changes in PD-related gene expression in ligature-induced experimental periodontitis in WT and *Itgb6*$^{-/-}$ mice and formulates new questions about how αvβ6 integrin in the JE regulates inflammation. Most importantly, the direct relationship between AIM2 and αvβ6 integrin needs to be further explored, as well as the potential dysbiosis in the periodontal microbiome that could follow after downregulation of AIM2 in the *Itgb6*$^{-/-}$ mice.

5. Limitations of the study

The study largely measured gene expression, which may not completely reflect protein levels, activity or their operational structures. Also, gingival specimens collected at the end of the experiment may not truly represent inflammatory or cellular changes deeper into the periodontal tissues that experienced severe disease activity or capture the early changes in the disease process. These points need to be addressed in future studies.

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