Periodontitis-associated pathogens *P. gingivalis* and *A. actinomycetemcomitans* activate human CD14+ monocytes leading to enhanced Th17/IL-17 responses

Wan-Chien Cheng1,2,3, Saskia D. van Asten1,*, Lachrissa A. Burns1, Hayley G. Evans1,*, Gina J. Walter1,*, Ahmed Hashim4, Francis J. Hughes2,#, and Leonie S. Taams1,#

1Centre for Molecular and Cellular Biology of Inflammation, Division of Immunology, Infection & Inflammatory Disease, King’s College London, London, UK;

2Department of Periodontology, Dental Institute, King’s College London, London, UK;

3Department of Periodontology, School of Dentistry, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan;

4Centre for Immunology and Infectious Disease, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK

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Correspondence: Dr Leonie S. Taams, Centre for Molecular and Cellular Biology of Inflammation, Division of Immunology, Infection & Inflammatory Disease, King’s College London, 1st floor New Hunt's House, Room 1.26F, Guy's Campus, London, SE1 1UL, UK.
Tel: +44 20 7848 8633; Fax: +44 20 7848 8632; E-mail: leonie.taams@kcl.ac.uk

Abbreviations: Aa, Aggregatibacter actinomycetemcomitans; GCF, gingival crevicular fluid; GT, gingival tissue; HC, healthy control; MOI, multiplicity of infection; PD, periodontitis; Pg, Porphyromonas gingivalis.

*Present address: Sanquin, Amsterdam, The Netherlands (SDvA); Nuffield Department of Clinical Neurosciences, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK (HGE); Division of Translational Oncology, German Cancer Research Center, Heidelberg, Germany (GJW).

#FJH and LST contributed equally to this work.

Abstract
The Th17/IL-17 pathway is implicated in the pathogenesis of periodontitis, however the mechanisms are not fully understood. We investigated the mechanism by which the periodontal pathogens *Porphyromonas gingivalis* (*Pg*) and *Aggregatibacter actinomycetemcomitans* (*Aa*) promote a Th17/IL-17 response in vitro, and studied IL-17$^+$ CD4$^+$ T cell frequencies in gingival tissue and peripheral blood from patients with periodontitis versus periodontally healthy controls. Addition of *Pg* or *Aa* to monocyte/CD4$^+$ T cell co-cultures promoted a Th17/IL-17 response in vitro in a dose- and time-dependent manner. *Pg* or *Aa*-stimulation of monocytes resulted in increased CD40, CD54 and HLA-DR expression, and enhanced TNF-α, IL-1β, IL-6 and IL-23 production. Mechanistically, IL-17 production in *Pg*-stimulated co-cultures was partially dependent on IL-1β, IL-23, and TLR2/TLR4 signalling. Increased frequencies of IL-17$^+$ cells were observed in gingival tissue from patients with periodontitis compared to healthy subjects. No differences were observed in IL-17$^+$ CD4$^+$ T cell frequencies in peripheral blood. In vitro, *Pg* induced significantly higher IL-17 production in anti-CD3 mAb-stimulated monocyte/CD4$^+$ T cell co-cultures from patients with periodontitis compared to healthy controls. Our data suggest that periodontal pathogens can
activate monocytes, resulting in increased IL-17 production by human CD4⁺ T cells, a process that appears enhanced in patients with periodontitis. **Introduction**

Periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth, which results in progressive irreversible damage to the alveolar bone and tooth support and ultimately may result in tooth loss. It is caused by the accumulation of bacterial plaque biofilm on the teeth and the host immune responses that occur in the tissues in response to this. The adaptive T cell immune response results in the production of cytokines that may be protective against periodontal disease, but may also be involved in periodontal bone destruction [1-5]. Th17 cells are a subpopulation of T helper cells named after their signature cytokine, IL-17A (henceforth called IL-17) [6]. IL-17 may be a crucial mediator in protection against infection [7, 8]. IL-17 treatment of both immune and non-immune cells induces the production of pro-inflammatory cytokines such as IL-6, chemokines and matrix metalloproteinases, leading to activation of innate immune cells, induction of pro-inflammatory signalling pathways, and recruitment of neutrophils [9]. IL-17 has also been shown to be critical for immune homeostasis, particularly at mucosal barriers [10]. In addition,
there is increasing evidence of a critical role for IL-17 and Th17 cells in the pathogenesis of inflammatory diseases such as psoriasis, psoriatic arthritis and ankylosing spondylitis (reviewed in [11]).

In periodontitis there is evidence that IL-17 may be protective against bacterial-induced damage, but may also in some cases result in increased tissue damage. In a murine experimental periodontitis model induced by the bacterium *Porphyromonas gingivalis*, knock out of IL-17RA resulted in markedly decreased neutrophil accumulation, causing accelerated periodontal bone loss [12]. In support of this, surface-glycan altered *Tannerella forsythia* has been shown to induce a Th17-linked mobilization of neutrophils and decreased bone loss in a similar murine model of periodontal disease [13, 14].

In complete contrast, Eskan and colleagues have described a destructive role for IL-17 in the pathogenesis of murine periodontitis. In that study deficiency of the LFA-1 integrin antagonist Del-1 resulted in excessive neutrophil infiltration associated with increased periodontal bone loss. However the bone destruction was prevented in Del-1/IL-17RA
double-deficient mice showing that neutrophil infiltration and increased bone loss were associated with IL-17 signalling [15].

In addition to animal studies, emerging data show the presence of IL-17 and Th17 cells in human periodontitis [16-20]. Recent studies show that *P. gingivalis* can promote an inflammatory IL-17/Th17 response [21-23], but as yet little is known regarding the underlying mechanisms that drive and regulate an IL-17/Th17 response in human periodontitis.

In this study, we investigated how the periodontal pathogens *P. gingivalis* (*Pg*) and *Aggregatibacter actinomycetemcomitans* (*Aa*) (a periodontal pathogen particularly associated with human aggressive periodontitis) [24]), affect IL-17 production by human CD4+ T cells. Furthermore, we investigated whether there are quantitative or qualitative differences in IL-17+ CD4+ T cells in the inflamed gingival tissue and peripheral blood from patients with periodontitis when compared to healthy control subjects.

**Results**
**Pg and Aa induce IL-17 production in human monocyte/CD4+ T cell co-cultures**

To investigate whether periodontal pathogens induce an IL-17/Th17 response in vitro, CD4+ T cells and CD14+ monocytes were co-cultured with anti-CD3 mAbs in the presence or absence of either heat-killed *P. gingivalis* strain W50 (Pg) or heat-killed *A. actinomycetemcomitans* (Aa) strain Y4 (serotype b). Addition of Pg or Aa resulted in a dose- and time-dependent induction of IL-17 in co-culture supernatants (Figure 1A-F). In addition to IL-17, we also detected an increase in IFN-γ production in co-culture supernatants following Pg or Aa stimulation (Figure 1G, H).

**Pg and Aa activate human monocytes, leading to IL-17 induction in CD4+ T cells**

To investigate the underlying mechanisms associated with *Pg* and *Aa*-induced IL-17 production in CD4+ T cells, we tested the effect of *Pg* or *Aa* stimulation on monocyte activation. Stimulation of monocytes with *Pg* resulted in significantly increased expression of the activation markers CD40, CD54 and HLA-DR and increased levels of IL-1β, TNF-α, IL-6 and IL-23 (Figure 2B). Similar results were obtained for *Aa*-stimulated monocytes, which also significantly upregulated CD86 expression (Figure 2C, D). IL-12p70 levels were very

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low and not significantly increased in \( P_g \) or \( A_a \)-stimulated monocytes (\( n = 9-10 \), data not shown). Also protein levels of IFN-\( \gamma \) in cultures of \( P_g \) or \( A_a \)-stimulated monocytes were negligible (\( n = 9-10 \), data not shown).

TLR2 and TLR4 have been implicated in recognition of \( P_g \) by APCs [28, 29]. Stimulation with \( P_g \) did not affect the expression of TLR2 or TLR4 (data not shown). Blocking both TLR2 and TLR4 significantly reduced the \( P_g \)-induced IL-17 production in supernatants from CD4\(^+\) T cell/monocyte co-cultures (Figure 3A). In cultures of purified CD14\(^+\) monocytes, TLR2 and TLR4 blockade decreased the secretion of IL-1\( \beta \), TNF-\( \alpha \), IL-6 and IL-23 upon \( P_g \) stimulation (Figure 3B), but did not affect the expression of the activation markers HLA-DR, CD40, CD54 or CD86 (\( n = 4 \), data not shown). In cultures of purified CD4\(^+\) T cells, \( P_g \) addition did not lead to increased expression of the activation markers CD25, CD28 and CD69, or IL-17 or IFN-\( \gamma \) production (\( n = 4 \), data not shown). These data suggest that TLR2- and TLR4-mediated recognition of \( P_g \) by monocytes contributes to subsequent Th17 polarisation. We tested the requirement for IL-1\( \beta \), TNF-\( \alpha \), IL-6 and IL-23 in the \( P_g \)-induced IL-17/Th17 response by co-culturing CD4\(^+\) T cells with monocytes, anti-CD3
and \( P_g \) in the presence or absence of neutralising antibodies to IL-1\( \beta \), TNF-\( \alpha \), IL-6 and IL-23.

IL-17 secretion was significantly reduced by adding a cocktail of the neutralising antibodies and by separately adding blocking antibodies to IL-1\( \beta \) or IL-23, whilst blockade of IL-6 or TNF-\( \alpha \) did not significantly affect IL-17 production by CD4\(^+\) T cells (Figure 3C, D).

**Presence of IL-17-producing T cells in human gingival tissue**

Next, we investigated the presence of IL-17-producing cells in inflamed gingival tissue from periodontitis lesions. Gingival tissue cells were isolated from diseased and healthy gingival tissue samples by collagenase digestion. When gated on total viable single cells (gating strategy shown in Supporting Information Figure 1), increased proportions of IL-17\(^+\), IFN-\( \gamma \)\(^+\) and TNF-\( \alpha \)\(^+\) cells were seen in periodontitis lesions (Figure 4A). Using back-gating (gating strategy shown in Supporting Information Figure 2), the IL-17\(^+\) cells in diseased tissue were found to be predominantly CD3\(^+\) CD4\(^+\) T cells (on average 81\%), with on average 7\% and 10\% of the IL-17\(^+\) cells being CD8\(^+\) T cells and CD3\(^-\) cells, respectively, and a small percentage (\( \sim 2\% \)) of IL-17\(^+\) cells being \( \gamma \delta \) T cells (Figure 4B). In contrast, IFN-\( \gamma \)\(^+\) and TNF-\( \alpha \)\(^+\) cells
comprised on average 37% and 48% of CD4\(^+\) T cells, respectively, with the remaining proportion being predominantly CD3\(^+\) CD8\(^+\) cells and CD3\(^-\) cells (Figure 4B).

We then quantified the frequencies of IL-17-producing CD4\(^+\) T cells within gingival tissue cells from diseased lesions relative to healthy gingival tissue, and for a small number of cases, also paired PBMCs from patients with periodontitis (gating strategy shown in Supporting Information Figure 3). Approximately 12% (median, range 5--18%) of CD4\(^+\) T cells expressed IL-17 in periodontal lesions, which was slightly higher compared to the frequency in healthy gingival tissue (median 9%, range 3--16%, \(p = 0.2\)), and significantly higher than the percentage seen in the blood of patients with periodontitis (median 0.9%, range 0.8--1.3%, \(p = 0.01\)) (Figure 4C). On average the percentages of IL-17\(^+\) cells within the CD8\(^+\) and γδ T cell populations were 1.4±0.2 and 4.1±2.1%, respectively (Supporting Information Figure 3). IL-17 protein was also detectable in GCF from patients with periodontitis at slightly higher levels compared to GCF from healthy subjects (\(p = 0.03\), Figure 4D).

It has been proposed that human IL-17-producing Th cells originate from a CD161\(^+\) CD4\(^+\) T cell precursor [31]. We determined the presence of IL-17 or IFN-γ-expressing cells in
CD161^+ vs. CD161^- CD4^+ T cells (gating strategy shown in Supporting Information Figure

An enrichment of IL-17-expressing cells within CD161^+ CD4^+ T cells was observed when compared to the CD161^- CD4^+ T cells, whereas similar percentages of IFN-γ-expressing T cells were found within CD161^+ and CD161^- cells (Figure 4E). In addition, a slight in the percentage of cells that express both these cytokines was observed in CD161^+ vs. CD161^- CD4^+ T cells.

Recent data indicate that different subsets of Th17 cells may exist producing either IFN-γ or IL-10 [32-34]. We therefore determined the co-expression of inflammatory (IFN-γ, TNF-α) and anti-inflammatory (IL-10) cytokines by IL-17^+ CD4^+ T cells in healthy vs. inflamed gingival tissue. In periodontitis lesions, on average 7±1% of the IL-17^+ CD4^+ T cells co-expressed IFN-γ, 7±1% co-expressed IL-10, and a high proportion co-expressed TNF-α (on average 69±4%) however this was not significantly different from healthy gingival tissue (Figure 4F, gating strategy shown in Supporting Information Figure 5).

**CD4^+ T cells from patients with periodontitis show an enhanced IL-17 response**
Finally, we investigated whether the percentage of IL-17$^+$ CD4$^+$ T cells was increased in peripheral blood from patients with periodontitis relative to periodontally healthy controls (HC). For this purpose we collected PBMCs from patients with PD (n = 15 chronic periodontitis and n = 15 aggressive periodontitis) and HC (n = 13, gender and age matched) (see Supporting Information Table 1). Cells were cryopreserved and then analysed in batches of 5 different samples (consisting of a mix of HC and PD) for ex vivo cytokine expression. No significant difference was found in the percentage of IL-17$^+$ or IFN-$\gamma^+$ cells within CD4$^+$ T cells in PBMCs between patients with periodontitis and healthy controls (Figure 5A, C). However, higher IL-17 protein levels were detected in co-cultures of monocytes and CD4$^+$ T cells from patients with periodontitis, and this was significantly enhanced further upon $P_g$ stimulation (Figure 6B). IFN-$\gamma$ production, in contrast, was not significantly different between healthy controls and patients with PD (Figure 5C, D).

Both IL-17 and IFN-$\gamma$ were produced at increased levels when CD4$^+$ T cells were cultured with monocytes in the presence of $P_g$ or $A_a$ alone (i.e. in the absence of anti-CD3 mAbs). This was observed using cells derived from healthy controls as well as periodontitis patients.
These data highlight the possibility that the IL-17 and IFN-γ production by the CD4+ T cells could be antigen-specific; however we cannot exclude the possibility that this is due to bystander activation caused by innate immune activation due to TLR stimulation. Further work is required to confirm this.

**Discussion**

In this study, we investigated the role of the periodontal pathogens *Pg* and *Aa* in the induction of a Th17/IL-17 response in periodontal disease. Our data show that the periodontal pathogens *Pg* and *Aa* activate monocytes, which can subsequently induce an IL-17/Th17 response in CD4+ T cells in vitro. We demonstrate that the underlying mechanism involves the recognition of *Pg* by TLR2/4 on monocytes leading to an IL-1β/IL-23 dependent induction of IL-17 production by CD4+ T cells. IL-1β and IL-23 have previously been reported to be required to induce the differentiation or generation of human Th17 cells from naïve CD4+ T cells or effector memory CD4+ T cells [31, 35], and our data are consistent with those studies. We also show that IL-17 CD4+ T cells and IL-17 protein are clearly detectable in diseased as well as healthy gingival tissue and GCF. Furthermore, our data suggest that although there is no increase in the frequency of IL-17+ CD4+ T cells in the
peripheral blood of patients with periodontitis, peripheral CD4+ T cells from these patients show enhanced IL-17 production in response to Pg stimulation.

Previous studies have shown that monocyte-derived DCs primed with different Pg strains elicited distinct T cell responses. Specifically it has been shown that certain capsular strains of Pg (W50, HG184) were capable of inducing a Th1/Th17 response when compared with the non-encapsulated strains or other capsular serotypes of Pg [21, 22]. In our study, we used P. gingivalis capsular strain W50 and A. actinomycetemcomitans strain Y4 (serotype b) and found these pathogens induced IL-17 production by CD4+ T cells when co-cultured with monocytes. Aa appears to be more potent than Pg in this in vitro system, as it induced IL-17 production and monocyte activation at lower multiplicity of infection (MOI). These in vitro data are in line with data from a recent mouse study that showed that Pg stimulation induced a TLR2- and IL-1-driven Th17/IL-17 response by splenic CD4+ T cells co-cultured with splenic APCs [36]. Interestingly, in that study Pg-infection induced not only periodontitis but also directly promoted autoimmune experimental arthritis, which correlated with the Pg-induced collagen-specific Th17 response in mice [36].
In addition, a recently published paper suggested that the periodontal bone loss observed in patients with leukocyte adhesion deficiency type I (LAD-I) is associated with an abundant infiltration of IL-17-producing CD3\(^+\) T cells and the excessive production of IL-17 in gingival tissue [37]. The authors further showed that IL-17 overproduction in untreated LFA-1\(^{KO}\) mice (a model of LAD-I periodontitis) was dependent on IL-23 and that local treatment with antibodies to IL-17 or IL-23 in LFA-1\(^{KO}\) mice blocked inflammatory periodontal bone loss. Although, LAD-I-associated periodontitis is a very rare and specific subtype of PD, it is of interest that our in vitro data indicate that patients with PD may also have an exaggerated IL-17 production upon stimulation. Together these data support the notion that the IL-23/IL-17 axis may play an important role in the biology of periodontitis [18, 19, 38].

The ability of periodontal pathogen-stimulated APCs to produce IL-6, IL-1\(\beta\), TNF-\(\alpha\) and IL-23 may contribute to Th17 induction in situ [18]. Indeed, an increased percentage of IL-17 producing cells were detected in diseased gingival tissue. These results are in line with data on IL-17-expressing CD3\(^+\) or CD4\(^+\) T cells obtained using immunohistochemistry [17, 39,
We further detected that these IL-17-producing cells were predominantly CD4+ T cells, and were enriched in the CD161+ fraction of CD4+ T cells. We also observed that IFN-γ-expressing cells were found within CD161+ CD4+ T cells. These data may indicate that some of the IL-17+ cells have become Th1-like as was previously suggested [31], but further work is required to confirm the origin of the Th1 and Th17 cells in gingival tissue.

Although there is evidence that IL-17 production by gingival γδ T cells may play a potential role in tissue damage in experimental periodontitis [15], in our study only very few of the IL-17+ cells were γδ T cells. We previously showed that IL-17+ CD8+ T cells are enriched in the joints of patients with psoriatic arthritis and correlate with clinical parameters of active disease [40]. However, only a low percentage of IL-17+ cells in diseased gingival tissue were found to be CD8+ T cells. These findings suggest that the infiltration of IL-17-producing CD4+ T cells may be important in relation to the periodontal bone destruction in inflamed gingival tissue. It should be noted that we also detected increased levels of IFN-γ and TNF-α expressing cells in diseased gingival tissue, suggesting an inflammatory cell infiltrate and these cells may also contribute to immunopathology in periodontitis.
One possible explanation for the presence of IL-17+ CD4+ T cells in both periodontitis lesions and healthy gingival tissue could be the existence of pathogenic and non-pathogenic Th17 cell subsets. In a mouse model, Th17 cells generated with TGF-β1 and IL-6 produce IL-17 but do not readily induce autoimmune disease without further exposure to IL-23, whilst Th17 generated in the presence of IL-23 or TGF-β3 and IL-6 induced severe EAE [32, 42-44]. A recent study using human T cells showed that different subsets of Th17 cells evolve in response to different types of pathogens [33, 45]. Additionally, IL-17-producing CD4+ T cells can co-express the anti-inflammatory cytokine IL-10 upon modulation of the inflammatory milieu by TNF-blockade [34]. However, our analysis did not reveal a significant difference between patients with periodontitis and periodontally healthy controls in the percentage of gingival IL-17+ CD4+ T cells co-expressing TNF-α, IFN-γ or IL-10.

In conclusion, our data provide evidence that P. gingivalis and A. actinomycetemcomitans can activate monocytes resulting in increased IL-17 production by human CD4+ T cells in vitro, a process that appears enhanced in patients with periodontitis.

**Materials and Methods**

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Patient and healthy control samples

Healthy control samples were obtained from periodontally healthy volunteers. Patients with periodontitis were recruited from the periodontal clinic at Guy’s Hospital (Supporting Information Table 1). Gingival tissue samples from patients with periodontitis were obtained at periodontal surgery or from periodontally healthy control subjects undergoing non-periodontal disease related procedures (e.g. crown lengthening or tooth extraction) in the periodontal department at Guy’s Hospital. Oral and periodontal examination records, age, sex and smoking status were documented on the day of sample collection. Periodontal disease case definitions for moderate to severe chronic periodontitis were used according to those described by Page and Eke, and for aggressive periodontitis according to those proposed by Demmer and Papapanou [48-50], with the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥ 6 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing. All clinical investigations were conducted according to the Declaration of Helsinki principles. Ethics approval for this study was given by the NRES Committee London - City Road & Hampstead Research Ethics Committee.
(12/LO/0376). Written informed consent was received from participants before their inclusion in the study.

**Cell isolation from peripheral blood**

PBMCs were isolated by density gradient centrifugation using lymphocyte separation medium (LSM 1077; PAA Laboratories, Pasching, Austria or Lymphoprep; AXIS-SHIELD, Oslo, Norway). PBMCs were used freshly or cryopreserved within 1 hour of isolation and stored in liquid nitrogen in medium containing 90% fetal bovine serum (lot 030M3399, Sigma-Aldrich, St. Louis, MO, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich). CD14+ monocyte and CD4+ T cell isolation from PBMCs was performed by magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany) via positive and negative selection, respectively. Purity was confirmed by flow cytometry and was consistently found to be > 96% for monocytes and > 95% for CD4+ T cells.

**Cell isolation from gingival tissue**
Gingival tissue collected from surgical procedures was weighed and cut into approx. 1 mm$^3$ fragments and digested using 2 mg/ml collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C for 1 hour. The ratio of tissue to medium was 25 mg to 1 ml. The residual fragments of tissue were dissociated by gentle flushing with a pipette and then filtered through cell strainers of mesh size 70 μm. Disaggregated cell fractions were washed and resuspended in culture medium followed by ex vivo stimulation and staining for cytometry.

**Collection of gingival crevicular fluid (GCF)**

GCF samples were collected from three diseased sites (probing depth ≥ 5 mm) from patients with periodontitis and from three healthy sites (probing depth ≤ 3 mm, no bleeding on probing) from periodontally healthy control subjects. The tooth for sampling was isolated with a cotton wool roll and the supragingival plaque was gently removed with a cotton wool roll before sample acquisition. GCF samples were collected using strips (2 mm x 8 mm) of Durapore® polyvinylidene difluoride (PVDF) hydrophilic membrane filters (pore size of 0.22 μm, GVWP04700, Millipore, Watford, UK) that were gently placed into the periodontal...
crevice until mild resistance was detected, and left in place for 30 seconds. Strips contaminated by blood were discarded. After GCF collection, each strip was placed in an Eppendorf tube containing 150 μl of PBS (Sigma, UK) on ice. The Eppendorf tubes were vortexed for 10 seconds and then left at 4°C for 15 minutes to elute GCF from the strip and vortexed again. The eluted solution was centrifuged at 10,000 rpm for 15 minutes at 4°C before removing the strips, and stored at -80°C until further analysis. Three tubes of eluted solution of healthy sites from a healthy subject or three tubes of eluted solution of diseased sites from a patient with periodontitis were pooled and mixed well prior to assays.

**Ex vivo cytokine staining**

PBMCs and disaggregated gingival tissue cells were stimulated for 3 hours with PMA (50 ng/ml) and ionomycin (750 ng/ml) (both from Sigma-Aldrich) in the presence of GolgiStop (BD, Oxford, UK), according to the manufacturer’s instructions. Extracellular surface staining was performed using PE-Cy7-conjugated anti-CD3 (clone UCHT1), APC-Cy7-conjugated anti-CD14 (clone HCD14), Brilliant Violet 605-conjugated anti-CD19 (clone HIB19) (all from BioLegend, London, UK), PE-CF594-conjugated anti-CD8 (clone
RPA-T8), FITC-conjugated anti-γδ T cell receptor (TCR) (clone 11F2) (both from BD Biosciences, Oxford, UK). The cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin (Sigma) and stained intracellularly with Pacific Blue-conjugated anti-CD4 (clone SK3), Alexa Fluor 488-conjugated anti-IL-10 (clone JES3-9D7), PE-conjugated anti-IL-17A (clone BL168), APC-conjugated anti-TNF-α (clone MAb11), and PerCP-Cy5.5-conjugated anti-IFN-γ (clone 4S.B3) (all from BioLegend). Cells were acquired using a FACSCanto or LSRFortessa (BD Biosciences, Franklin Lakes NJ, USA) and analysed using the FlowJo software (Tree Star, Ashland, OR). Cytometer settings were tracked over time using BD Biosciences Cytometer Setup and Tracking software to monitor cytometer performance. Viable single cells were identified using a LIVE/DEAD® Fixable Blue Dead Cell stain kit (Life technologies, Carlsbad, CA) or based on their FSC-A/FSC-W profile.

**Bacteria**

Heat-killed bacteria were kindly provided by Prof. Mike Curtis (Barts and The London School of Medicine and Dentistry, Queen Mary University of London). *P. gingivalis* (*Pg*)
strain W50 was grown at 37°C in brain heart infusion (BHI) broth supplemented with hemin (1μg/ml) in an anaerobic atmosphere of 80% N₂, 10% H₂, and 10% CO₂ (Don Whitley Scientific) for 48 hours. A. actinomycetemcomitans (Aa) strain Y4 was cultured in BHI medium supplemented with 0.05% cysteine and 0.5% yeast extract and incubated in anaerobic conditions for 48 hours. Cells were harvested by centrifugation at 10,000 g for 15 min at 4°C. Cell pellets were suspended and washed in PBS and then resuspended in PBS to an optical density of 1.0 at 600 nm. The bacteria were then heat-killed at 65°C for 60 minutes, washed and resuspended in PBS at a final density of 5×10⁹ CFU/ml. Non viability was confirmed by plating aliquots and incubating as described above.

Cell culture

CD4⁺ T cells (0.5×10⁶ cells) and CD14⁺ monocytes were co-cultured at a 1:1 ratio with αCD3 mAbs (Okt-3, Janssen-Cilag, Buckinghamshire, UK) in culture medium in a 24-well plate in the presence or absence of heat-killed Pg or Aa at the indicated MOI (multiplicity of infection) for different time points. E. coli LPS (100 ng/ml, Sigma) was used as a positive control. In monocyte only cultures, cells (0.5×10⁶/ml) were stimulated with Pg or Aa at the indicated
MOI for 20 hours. Neutralising mAbs to IL-1β (clone 8516, mIgG1), IL-6 (clone 1936, mIgG2b), TNF-α (clone 6401, mIgG1) (all from R&D Systems Europe Limited, Abingdon, UK), IL-23p19 (clone HNU2319; eBiosciences, San Diego, CA), or isotype controls (mIgG1, clone 11711, mIgG2a, clone 20102, mIgG2b, clone 20116 and hIgG1 Fc; R&D Systems Europe Limited) were used at 10 μg/ml. Anti-TLR-2 (clone TL2.1) and TLR-4 (clone HTA125) mAbs (BioLegend) were used at 5 μg/ml.

Analysis of monocyte phenotype

CD14+ monocyte phenotype was assessed after 20 hours of culture with bacteria, by staining with APC-Cy7-conjugated anti-CD14 (clone HCD14), APC-conjugated anti-CD54 (clone HCD54), Pacific Blue-conjugated anti-CD86 (clone IT2.2) (all from BioLegend), PE-conjugated anti-CD40 (clone LOB7/6, AbD Serotec, Kidlington, UK), PerCP-Cy5.5-conjugated anti-HLA-DR (clone G46-6, BD Biosciences), FITC-conjugated anti-TLR2 (clone TL2.1), PE-conjugated anti-TLR4 (clone HTA125) (both from eBiosciences).

Cytokine detection
Culture supernatants were collected and stored at -80°C until further use. The levels of IL-23 (eBioscience), IL-17, IL-1β, IL-6, IFN-γ, IL-12p70 and TNF-α (all from BioLegend) were detected using ELISA according to the manufacturers’ protocols. IL-17A levels in GCF were detected by Luminex assay (Bio-Plex Pro™ Human Th17 Cytokine Assays, Bio-Rad Laboratories, CA, USA).

**Statistical analysis**

Values are expressed as mean with SEM or as median with interquartile range (IQR). Data were tested for normality using D’Agostino & Pearson omnibus normality testing and where not normally distributed data were log10 transformed prior to analysis (which provided normally distributed results). Comparisons between patients and healthy controls were made using one-way analysis of variance followed by Bonferroni’s multiple comparison test for parametric data. Data were analysed using Prism version 5 (GraphPad Software Inc, La Jolla, USA). For all tests, p values of less than 0.05 were considered significant.

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**Supplementary Figure 1. Gating strategy to identify IL-17, IFNγ or TNFα producing cells ingingival tissue.** Gingival tissue cells were isolated from an inflamed gingival tissue
sample from a patient with periodontitis. Samples were digested with collagenase. Cells were stimulated \textit{ex vivo} with PMA and ionomycin for 3 hours prior to intracellular cytokine staining. The following gating strategy was employed: first, the live lymphocyte population was gated (A), and then doublets were excluded based on FSC-W vs. FSC-A (B). Total IL-17+ or IFNγ+ or TNFα+ cells were gated using the cell gates shown (C-E), which were based on negative control stains (FMO) (F-H).

**Supplementary Figure 2.** Gating strategy to identify IL-17, IFNγ or TNFα producing CD4+, CD8+ or γδ T-cells in gingival tissue. Gingival tissue cells were isolated from an inflamed gingival tissue sample from a patient with periodontitis. Samples were digested with collagenase. Cells were stimulated \textit{ex vivo} with PMA and ionomycin for 3 hours prior to intracellular cytokine staining. A gate was set on cells that stained positive for the cytokines IL-17 (A), IFNγ (B) or TNFα (C), and subsequently the percentages of CD3+, CD3+CD4+, CD3+CD8+ and CD3+γδ+ cells within these gates were determined. Representative gating strategy and typical dot plots for the CD3+CD4+, CD3+CD8+ or γδ T-cells within the cytokine-positive cells are shown.

**Supplementary Figure 3.** Quantification of IL-17+, IFNγ+ or TNFα+ cells within CD4+, CD8+ or γδ T-cells in gingival tissue. Gingival tissue cells were isolated from inflamed gingival tissue samples from patients with periodontitis (n=3-8). Samples were digested with collagenase. Cells were stimulated \textit{ex vivo} with PMA and ionomycin for 3 hours prior to intracellular cytokine staining. (A) Representative gating strategy and (B-D) cumulative data showing the percentages of IL-17+, IFNγ+ or TNFα+ cells within CD4+ T-cells (B), CD8+ T-cells (C) or γδ T-cells (D).

**Supplementary Figure 4.** Quantification of IL-17+ and IFNγ+ cells within CD4+CD161+ T-cells in gingival tissue. Gingival tissue cells were isolated from inflamed gingival tissue samples from patients with periodontitis (n=4). Samples were digested with collagenase. Cells were stimulated \textit{ex vivo} with PMA and ionomycin for 3 hours prior to intracellular cytokine staining. Representative gating strategy to identify CD161+ vs. CD161- CD4+ T-cells and the percentages of IL-17+ and IFNγ+ cells within these populations.

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Conflict of interest

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References


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Figure legends

Figure 1. Heat-killed P.g and A.a stimulate IL-17 production in CD4+ T cell/monocyte co-cultures. (A-D) CD4+ T cells (0.5×10^6 cells) and CD14+ monocytes from PBMCs of healthy donors were co-cultured at a 1:1 ratio in the presence of soluble anti-CD3 mAbs without (Med) or with (A, B) heat-killed P.g or (C, D) heat-killed A.a (A, C) at the indicated
MOI (multiplicity of infection) or (B, D) for different time periods. *E. coli* LPS (100 ng/ml) was used as positive control. IL-17 production in culture supernatants was measured by ELISA after 3 days (A, C, n = 6 different donors) or 1 to 7 days of culture (B, D, n = 8-9). Data are shown as the mean ± SEM. Data were analysed by one-way (A, C) or two-way (B, D) repeated measures ANOVA. Individual groups were compared to control (Med) values using the Bonferroni’s multiple comparison test, *P<0.05, **P<0.01, ***P<0.001. (E-H) Monocyte/CD4+ T cell co-cultures were stimulated without (Med) or with (E, G) *P. gingivalis* (MOI = 100) or (F, H) *A. actinomycetemcomitans* (MOI = 10) for 3 days. The production of (E, F) IL-17 and (G, H) IFN-γ in supernatants was measured by ELISA. Each symbol represents an individual donor (n = 12--22). Data were analysed by Wilcoxon matched-pairs signed rank test. **P<0.01, ***P<0.001.
Figure 2. Periodontal pathogens activate CD14⁺ monocytes. (A, C) PB CD14⁺ monocytes from healthy control subjects were treated for 20 hours with medium vs. (A) \( P_g \) (MOI = 100) or (C) \( A_a \) (MOI = 10). Surface expression of the indicated markers was determined by flow cytometry. Data are shown as the median ± IQR (n = 6 different donors). Log-transformed data were analysed by paired t test. *\( P < 0.05 \). (B, D) Production of IL-1β, TNF-α, IL-6 and IL-23 by monocytes following 20 hours stimulation with medium vs. (B) \( P_g \) (MOI = 100, n =
8) or (D) Aa (MOI = 10, n = 8) was investigated by ELISA. Data are shown as the mean ± SEM. Log transformed data were analysed by paired t test, *P<0.05, **P<0.01.

Figure 3. Mechanism underlying IL-17 induction by Pg-activated CD14+ monocytes. (A) CD4+ T cells (0.5×10^6 cells) and CD14+ monocytes from PBMCs of healthy donors were co-cultured for three days at a 1:1 ratio in the presence of soluble anti-CD3 mAbs without (Med) or with Pg (MOI = 100) in the absence or presence of blocking antibodies to TLR2 and TLR4 or appropriate isotype control Abs (5 μg/ml). IL-17 production in culture
supernatants was measured by ELISA. (B) CD14⁺ monocytes (0.5×10⁶ cells) from the blood of healthy control subjects were incubated with medium or heat-killed P. aeruginosa (MOI = 100), in the absence or presence of blocking antibodies to TLR2 and TLR4, or appropriate isotype control Abs. Production of IL-1β, TNF-α, IL-6 and IL-23 in culture supernatants was investigated by ELISA. (C, D) CD4⁺ T cells (0.5×10⁶ cells) and CD14⁺ monocytes from PBMCs of healthy donors were co-cultured for three days at a 1:1 ratio in the presence of soluble anti-CD3 mAbs without (Med) or with P. aeruginosa (MOI = 100) in the absence or presence of neutralising antibodies to IL-1β, TNF-α, IL-6 and/or IL-23 or the appropriate isotype control Abs. IL-17 production in culture supernatants was measured by ELISA. Each symbol represents an individual donor (n = 6). Data were analysed by Wilcoxon matched-pairs signed rank test, * P<0.05.
Figure 4. Presence and quantification of IL-17+ CD4+ T cells in gingival tissue. Gingival tissue cells were isolated from inflamed gingival tissue samples from patients with periodontitis (PD lesion, n = 12) or from healthy gingival tissue samples from healthy donors (Healthy, n = 6). Samples were digested with collagenase. Cells were stimulated ex vivo with PMA and ionomycin for 3 hours prior to intracellular cytokine staining and flow cytometry. (A) Cumulative data showing the frequencies of IL-17+ cells, IFN-γ+ cells and TNF-α+ cells within FSC/SSC-gated cells. Each symbol represents an individual donor sample and data are shown as scatter plots with median ± IQR, analysed by unpaired t test of log-transformed
data, **P<0.01; (B) Pie diagrams showing the average percentages of CD3⁺ CD4⁺ T cells, CD3⁺ CD8⁺ T cells, γδ T cells or CD3⁻ cells within the IL-17⁺ cells, IFN-γ⁺ cells and TNF-α⁺ cells in gingival tissue cells from patients with periodontitis (mean of n = 3-12). (C) Cumulative data showing the percentages of CD4⁺ T cells expressing IL-17 within gingival tissue from healthy controls (HC GT, n = 6) vs. patients with periodontitis (PD GT, n = 12) vs. PBMCs from patients with periodontitis (PD PBMCs, n = 3). Open circles indicate paired samples. Each symbol represents an individual donor sample and data are shown as median ± IQR, analysed by unpaired t test of log-transformed data, *P<0.05. (D) IL-17 protein levels in GCF from patients with PD (n = 15) vs. healthy controls (n = 13) were measured by Luminex assay. Minimal detection limit was 3.16 pg/ml. Data shown as mean ± SEM, analysed by unpaired t test, *P<0.05. (E) Cumulative data showing the percentages of IL-17⁺ and IFN-γ⁺ cells within CD161⁺ or CD161⁻ CD4⁺ T cells in diseased gingival tissue from patients with periodontitis (n = 4). Each symbol represents an individual donor, lines show the mean. (F) Cumulative data showing the percentages of IL-17⁺ CD4⁺ T cells that co-express IFN-γ, TNF-α or IL-10 in gingival tissue from patients with periodontitis (n = 12)
vs. healthy controls (n = 4). Each symbol represents an individual donor, lines show the mean ± SEM.

Figure 5. Peripheral CD4+ T cells from patients with periodontitis show an enhanced IL-17 response upon stimulation. (A, C) PBMCs were isolated from patients with periodontitis (PD, n = 30) or from periodontally healthy controls (HC, n = 13). Cells were stimulated ex vivo with PMA and ionomycin for 3 hours prior to intracellular staining. Data show the percentage of (A) IL-17+ or (C) IFN-γ+ cells within CD4+ T cells. Each symbol...
represents an individual donor, lines show the median ± IQR. Log-transformed data were
analysed by unpaired t test. (B, D) CD4⁺ T cells (0.5×10⁶ cells) and CD14⁺ monocytes were
isolated from PBMCs of periodontally healthy donors (n = 13) and periodontitis patients (n =
11), and co-cultured at a 1:1 ratio in the presence of soluble anti-CD3 mAbs with or without
heat-killed Pg (MOI = 100) for 7 days. Production of (B) IL-17 or (D) IFN-γ production in
culture supernatants was measured by ELISA. Each symbol represents an individual donor.
Log-transformed data were analysed by paired and unpaired t test, *P<0.05, **P<0.01, ***
P<0.001.
Periodontal pathogens Porphyromonas gingivalis (Pg) and Aggregatibacter actinomycetemcomitans (Aa) activate human monocytes to produce IL-1β, IL-23, IL-6 and TNF-α, leading to enhanced IL-17 production by CD4+ T cells in vitro. Our data suggest that the effect on IL-17 production is enhanced in T cells from patients with periodontitis.