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IL-17 family cytokines in periodontitis

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Abstract:	<p>Objective: IL-17A is implicated periodontitis pathogenesis. The roles of IL-17B to IL-17F are unknown. This study aimed to determine clinical associations between IL-17 family cytokines and periodontitis and to investigate the biological significance of IL-17A and IL-17E using in vitro model systems.</p> <p>Materials and Methods: Serum, saliva and gingival crevicular fluid (GCF) levels of IL-17 family cytokines were measured by ELISA. Oral keratinocytes were stimulated with a P. gingivalis biofilm, or IL-17A, in the presence and absence of IL-17E and the expression of IL-8 and CXCL5 were investigated by ELISA and real-time-PCR. NF-κB phosphorylation in similar experiments was also measured using a cell-based ELISA.</p> <p>Results: Serum, saliva and GCF IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters of attachment loss, pocket depth and bleeding on probing. Serum IL-17E levels were lower in periodontitis patients and the serum IL-17A:IL-17E ratio correlated positively with clinical parameters. In vitro, IL-17E inhibited P. gingivalis and IL-17A induced expression of chemokines by reducing phosphorylation of the NF-κB p65 subunit.</p> <p>Conclusions: Serum IL-17A:IL-17E may be a marker of disease severity. IL-17E may have opposing roles to IL-17A in periodontitis pathogenesis. IL-17E can negatively regulate IL-17A and perio-pathogen induced expression of chemokines.</p>

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IL-17 family cytokines in periodontitis

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Abstract

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Materials and Methods: Serum, saliva and gingival crevicular fluid (GCF) levels of IL-17 family cytokines were measured by ELISA. Oral keratinocytes were stimulated with a *P. gingivalis* biofilm, or IL-17A, in the presence and absence of IL-17E and the expression of IL-8 and CXCL5 were investigated by ELISA and real-time-PCR. NF- κ B phosphorylation in similar experiments was also measured using a cell-based ELISA.

Results: Serum, saliva and GCF IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters of attachment loss, pocket depth and bleeding on probing. Serum IL-17E levels were lower in periodontitis patients and the serum IL-17A:IL-17E ratio correlated positively with clinical parameters. *In vitro*, IL-17E inhibited *P. gingivalis* and IL-17A induced expression of chemokines by reducing phosphorylation of the NF- κ B p65 subunit.

Conclusions: Serum IL-17A:IL-17E may be a marker of disease severity. IL-17E may have opposing roles to IL-17A in periodontitis pathogenesis. IL-17E can negatively regulate IL-17A and perio-pathogen induced expression of chemokines.

Keywords: IL-17, IL-25, IL-17E, IL-17A, Periodontitis, IL-8

1. Introduction

The IL-17 family of cytokines consists of six members (IL-17A - IL-17F) [1]. IL-17A has been associated with periodontitis pathogenesis [2-6]. *In vivo* models suggest that IL-17A plays a protective role in periodontitis. Indeed, IL-17RA deficient mice show exacerbated bone loss in a *Porphyromonas gingivalis* induced model of periodontal disease [7]. However, an excessive IL-17A response promotes inflammatory alveolar bone loss in an ageing mouse model [8]. IL-17A signalling activates the receptor activator of nuclear factor kappa-B (NF- κ B), which in turn induces expression of a variety of pro-inflammatory mediators [9]. Furthermore, IL-17A can act in synergy with TNF- α and IL-1 β and augment the expression of pro-inflammatory mediators from keratinocytes and fibroblasts [10-11]. Moreover, IL-17A can modulate bone remodelling through induction of RANKL both *in vivo* and *in vitro* [12-13].

Like IL-17A, IL-17F can be secreted as a homodimer. However, a heterodimeric species consisting of a disulphide linked IL-17A and IL-17F molecule can also be formed [14]. Similarly to IL-17A, both the IL-17F homodimer and IL-17A/F heterodimer have pro-inflammatory properties [15]. Elevated levels of IL-17F and IL-17A/F have been associated with chronic inflammatory diseases such as rheumatoid arthritis [13]. The biology of IL-17B, IL-17C and IL-17D are relatively poorly understood at present. Levels of IL-17B, IL-17C and IL-17D are elevated in inflammation driven pathologies, such as rheumatoid arthritis, and *in vivo* IL-17B, IL-17C and IL-17D can induce pro-inflammatory cytokine release from a variety of epithelial and myeloid cells [16]. Therefore, IL-17B, IL-17C, IL-17D, IL-17F and IL-17A/F appear to drive the pathogenesis of inflammatory diseases, but at present, their role in periodontitis pathogenesis remains unclear.

IL-17E has been described as a ‘double edged sword’ and has been shown to down-regulate localised destructive inflammation and promote Th2 driven pathologies [17], playing a major role in the clearance of parasitic infections [18] and the pathogenesis of allergic disease [19]. IL-17E induces activation and differentiation of Th2 cells and regulates their recruitment to sites of inflammation [20]. IL-17E also regulates Th9 cells [21] and activates multi-potent progenitor type 2 (MPP^{type2}) cells, innate type 2 helper (Ih2) cells, natural helper cells (NHCs) and neuocytes [22]. In contrast, IL-17E down-regulates Th1/Th17 and IL-17A responses [23-25] and can directly inhibit toll-like receptor ligand driven expression of pro-inflammatory cytokines via p38 MAP kinase-driven SOCS-3 activation in human blood monocytes and intestinal CD14⁺ cells [26-27]. Therefore, it is interesting to speculate that IL-17E may play a role in down regulating potentially damaging localized inflammatory responses within the periodontium and protect against tissue destruction and alveolar bone loss.

The aim of this study was to investigate the relationships between all IL-17 family cytokines and periodontitis, by investigating serum, saliva and GCF levels of IL-17A - IL-17F and the IL-17A/F heterodimer in patients with periodontitis and periodontally healthy controls. In addition, we sought to investigate whether IL-17A and IL-17E might have opposing roles in periodontal innate immunity. Therefore, the ability of IL-17E to inhibit IL-17A and *P. gingivalis* induced expression of the neutrophil chemoattractant chemokines (IL-8 and CXCL5), via differential activation of the NF-κB transcription factor, was investigated.

2. Materials and Methods

2.1 Ethics and sample collection

Samples were selected from independent studies conducted at Glasgow and Newcastle and were from patients with periodontitis or healthy controls that were systemically healthy non smokers. The sample collection and ethical approvals for the Glasgow sample cohort were as previously described [28]. The sample collection and ethical approvals for the Newcastle cohort are as previously described [29-31]. All samples were collected by similar methods in the two centres except for saliva. Therefore, saliva data was analysed separately.

2.2 Enzyme-linked immunosorbent assays (ELISA)

IL-17A – IL-17F and IL-17A/F were assayed using commercially available ELISA kits, according to the manufacturer's instructions. IL-17D was assayed using an optimised ELISA developed with 2 µg/ml rabbit anti-human IL-17D capture antibody (Peprotech, UK) and 0.5 µg/ml biotinylated anti-human IL-17D detection antibody (Peprotech, UK). CXCL5 (ENA-78) and CXCL8 (IL-8) concentrations were investigated by ELISA (CXCL5/ENA-78 DuoSet, R&D Systems, UK and IL-8 Cytoset, Invitrogen, UK). The limits of detection were determined as two mean standard deviations higher than the mean baseline from 6 replicate standard curves: IL-17A = 1.9 pg/ml, IL-17B = 7.8 pg/ml, IL-17C = 7.8 pg/ml, IL-17D = 9.8 pg/ml, IL-17E = 1.9 pg/ml, IL-17F = 1.9 pg/ml, IL-17A/F = 2.5 pg/ml, IL-8 = 1.6 pg/ml and CXCL5 = 3.9 pg/ml.

2.3 *Porphyromonas gingivalis* biofilms

P. gingivalis ATCC 33277 was cultured under anaerobic conditions at 37 °C in Schaedler anaerobe broth (Oxoid, UK) for 2 days. The bacteria were then washed by centrifugation in PBS and standardised to 1×10^7 cells/ml in artificial saliva (AS) [32]. 500 µl of *P. gingivalis*

suspension was cultured on Thermanox[®] plastic coverslips (NUNC, UK) at 37 °C in an anaerobic environment for 4 days, with AS replaced daily. After incubation, the non-adherent cells were removed and the biofilms were kept at -80 °C prior to use.

2.4 *In vitro* experiments

OKF6/TERT-2 oral keratinocytes were a gift from the Rheinwald laboratory (Brigham and Women's Hospital, Boston). OKF6/TERT-2 cells were cultured as previously described [33]. *P. gingivalis* ATCC 33277 biofilms were used in co-culture experiments with epithelial cells as described previously [34], with varying concentrations of IL-17E (Peprotech, UK). Un-stimulated cells and cells stimulated with IL-17E alone were used as controls.

The effect of IL-17E on IL-17A induced IL-8 expression was determined by stimulating OKF6/TERT-2 cells concomitantly with IL-17A (Peprotech, UK) alone or in combination with IL-17E (Peprotech, UK) for 24 h. In addition, cells were pre-incubated for 30 min with IL-17E, prior to stimulation with IL-17A. Un-stimulated cells and cells stimulated with IL-17A and IL-17E alone were used as controls.

2.5 *Real time PCR analysis*

mRNA was isolated using the RNeasy[®] Kit (Qiagen, UK), and reverse transcribed using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystem, UK), according to the manufacturer's instructions. IL-8 and CXCL5 mRNA expression was analysed using SYBR[®] green Real Time PCR with the following primers, *GAPDH*: Fwd: CAAGGCTGAGAACGGAAG and Rev: GGTGGTGAAGACGCCAGT, *IL-8*: Fwd: CAGAGACAGCAGAGCACACAA and Rev: TTAGCACTCCTTGGCAAAAC and *CXCL5*: Fwd: CCCTGGGTTCAGAGACCTCCA and Rev:

1 CCAGAAAATTTTGGACGGTGGAAACA. Relative expression of IL-8 and CXCL5
2 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method [35].
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6 *2.6 Fast Activated Cell-based ELISA (FACE™) NF-κB p65 Profiler*

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8 Phosphorylation profiles of the NF-κB p65 subunit was investigated using the FACE™ NF-
9 κB p65 Profiler (Active Motif, UK) in accordance with manufacturer's instructions. The % of
10 the total NF-κB p65 subunit phosphorylated at serine 468 or serine 536 was calculated in
11 accordance with the manufacturer's instructions.
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21 *2.7 Statistical analyses*

22 All statistical analyses were carried out using SPSS (Version 18). Serum and GCF samples
23 from Glasgow and Newcastle were originally analysed separately and similar results
24 observed. To increase statistical power, data from the 2 patient cohorts were combined; with
25 the exception of the data from analysis of saliva samples.
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36 Statistical power exceeded 80 % when $n \geq 29$ utilizing a fixed effect size of 1 and the
37 assumption of normally distributed data. Q-Q plot analysis showed the data was not normally
38 distributed. Therefore group sizes were increased to ≥ 34 . For the majority of analyses,
39 sufficient statistical power was achieved. However, due to limitations in sample volume,
40 some analyses were underpowered and therefore described as exploratory in nature.
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50 The Mann-Whitney-U test and Bonferroni correction was used to determine significant
51 differences between health and disease. Due to a significant age difference between the two
52 groups (Table 1) adjustments were performed using a weighted t-test analysis of the natural
53 log (Ln) transformed data.
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1 Bivariate correlations were performed using Spearman rho serum and GCF samples.
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3 However, due to the limited number of saliva samples from the Newcastle cohort only the
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5 Glasgow saliva samples were analysed in this respect. A Pearson correlation of Ln
6
7 transformed data was used to adjust for differences in age. Serum IL-17A:IL-17E ratios were
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9 subjected to Ln transformations to facilitate graphical representation.
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14 Statistical analysis of chemokine ELISA data was performed by Ln transformation followed
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16 by an ANOVA with a *post hoc* Bonferroni correction. Real time PCR data was analysed by
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18 performing a Levenes's test on the Ln transformed $2^{-\Delta CT}$ values followed by an independent
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20 *t*-test to compare two means [35].
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26 Fast Activated Cell-based ELISA (FACETM) NF-κB p65 Profiler data was transformed and
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28 analysed with a Holm-Bonferroni corrected t-test.
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3. Results

3.1 Clinical parameters

77 healthy volunteers (62 Glasgow; 15 Newcastle) and 97 patients with periodontitis (71 Glasgow; 26 Newcastle) were enrolled in the study. The median age of the healthy subjects in the Glasgow study was significantly lower than periodontitis patients (Table 1A), but patients and controls were of similar age in the Newcastle study (Table 1B). Commensurate with periodontal health, the healthy subjects in both studies had significantly lower median % of sites that bled on probing (BOP), lower median clinical probing depth (CPD) and lower clinical attachment loss (CAL) compared to patients with periodontitis (Table 1A and B). Table 1C shows that in the combined cohort the median age, the median BOP, the median CPD and the median CAL were all significantly lower for healthy volunteers than periodontitis patients.

3.2 Serum, gingival crevicular fluid and saliva levels of IL-17 family cytokines

Circulating median concentration levels of IL-17A and IL-17A/F were greater in patients with periodontitis compared to healthy volunteers (Table 2A). There were significantly lower circulating levels of IL-17E in periodontitis patients compared to healthy volunteers. Weighted analyses of transformed data confirmed that none of these differences were due to differences in age of participants. There were no significant differences in circulating median concentrations of IL-17B, C, or D between healthy volunteers and periodontitis patients; irrespective of correction for age.

Due to limitations of sample volume, GCF analysis was restricted to IL-17A, IL-17E, IL-17F and IL-17A/F. The median total amounts of IL-17A, IL-17F and IL-17A/F in GCF was elevated in patients with periodontitis (Table 2B). However, there was no significant difference in amounts of IL-17E. Weighted analysis of transformed data confirmed that these

findings were not due to differences in age of the participants. The findings for IL-17F and IL-17A/F can only be interpreted as exploratory due to insufficient power.

Saliva samples from Glasgow and Newcastle were analysed in isolation due to differences in collection methodology (Table 2C and Table 2D). Only IL-17A, IL-17E, IL-17F and IL-17A/F were measured due to limitations in sample volume. In the Glasgow cohort salivary levels of IL-17A, IL-17E, IL-17F and the IL-17A/F heterodimer were greater in periodontitis patients than healthy controls. Weighted analysis of transformed data confirmed these findings were not due to age differences. Analysis of the Newcastle samples also revealed elevated salivary levels of IL-17A and IL-17A/F in periodontitis patients compared to healthy controls. In contrast, there were no significant differences in levels of IL-17E and IL-17F. These findings can only be interpreted as exploratory due to insufficient power.

The IL-17A:IL-17E ratio was calculated for serum, GCF and saliva of each participant (Table 2E). The IL-17A:IL-17E ratio was significantly higher in serum of periodontitis patients than healthy controls ($p<0.0001$), but not in GCF or saliva. This was confirmed not to be influenced by differences in the age of the participants using a weighted analysis.

3.3 Associations between IL-17 family cytokines, clinical parameters and demographics

Table 3 shows correlations between serum, saliva (Glasgow only) and GCF levels of IL-17A, IL-17E, IL-17F and IL-17A/F and clinical parameters as well as age. Serum concentrations of IL-17A demonstrated a significant positive correlation with age, PD, CAL and BOP. In contrast, serum concentrations of IL-17E showed a significant negative correlation with PD and CAL, but not with age or BOP. Partial bivariate correlation analysis demonstrated that

after correction for age, significantly stronger negative correlations were seen for IL-17E with PD ($R=-0.275$, $p=0.0021$) and CAL ($R=-0.317$, $p=0.0007$). Circulating levels of IL-17E negatively correlated with IL-17A ($\rho=-0.194$, $p=0.041$). Again, adjustment for age resulted in a stronger correlation ($R=-0.580$, $p<0.0001$). Serum levels of IL-17A/F showed significant positive correlations with age, PD, CAL and BOP, both before and after correction for age.

GCF levels of IL-17A correlated significantly with age, PD, CAL and BOP. In addition, GCF levels of IL-17E showed a significant positive correlation with PD, CAL and BOP, but not with age. Furthermore, GCF levels of IL-17F and IL-17A/F correlated with age, PD, CAL and BOP. Partial correlation analysis confirmed that these correlations were not influenced by age.

Due to the limited number of saliva samples from the Newcastle cohort, correlation analysis was only performed on the Glasgow samples. Salivary levels of IL-17A significantly correlated with age, PD, and CAL, but not with BOP. In addition, salivary levels of IL-17E showed significant positive correlations with age, PD, CAL and BOP. Salivary IL-17F concentrations also correlated significantly with age, PD and CAL, but not with BOP and salivary levels of IL-17A/F only correlated significantly with age. Partial bivariate correlation analysis confirmed that these correlations were not influenced by age.

3.4 Correlations between serum IL-17A/IL-17E ratio and clinical parameters

Circulating levels of IL-A negatively correlated with levels of IL-17E ($R=-0.323$, $p=0.0006$). The serum IL-17A:IL-17E ratio was significantly elevated in patients with periodontitis compared to healthy volunteers (Table 2E), suggesting the ratio may be a marker of disease.

1 The serum IL-17A:IL-17E exhibited moderate/strong correlations with CAL, PD and a weak
2 correlation with BOP. Partial correlation analyses confirmed these findings were not
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4 influenced by the subject's age.
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10 *3.5 IL-17E inhibits the IL-17A induced release of IL-8 from oral keratinocytes*

11 Stimulation of OKF6/TERT-2 cells with IL-17A alone for 24 h induced significant release of
12 IL-8 compared to unstimulated control cells ($P<0.01$, Figure 2A). In contrast, stimulation
13 with IL-17E alone had no effect. There was a dose-dependent decrease in IL-17A-induced
14 IL-8 release from OKF6/TERT-2 cells when stimulated concomitantly with varying
15 concentrations of IL-17E, which was significant only with 400 ng/ml IL-17E ($p<0.01$).
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17 Thirty minutes of pre-incubation with IL-17E resulted in greater inhibition of IL-17A-
18 induced IL-8 release. Once again, there was a dose-dependent decrease in IL-17A-induced
19 IL-8 release from OKF6/TERT-2 cells. However, on this occasion the inhibition was
20 significant with 10, 50, 100, 200 and 400 ng/ml IL-17E (Figure 2B). The inhibitory effect of
21 IL-17E was mediated at the transcriptional level. Stimulation of OKF6/TERT-2 cells with 10
22 ng/ml IL-17A alone induced a significant 7-fold increase in IL-8 mRNA expression
23 ($p<0.01$, Figure 2C). Furthermore, 30 minute pre-stimulation with 10 and 50 ng/ml IL-17E
24 reduced the IL-17A induced up-regulation of IL-8 mRNA expression. However, this
25 reduction was only significant with 50 ng/ml IL-17E ($p<0.01$, Figure 2C).
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51 *3.6 IL-17E inhibits the IL-17A induced activation of NF κ B in epithelial cells*

52 Exposure of OKF6/TERT-2 cells to 10 ng/ml IL-17A or 50 ng/ml IL-17E alone (or in
53 combination) had no significant effect on total NF- κ B p65 subunit levels compared to
54 unstimulated control cells (data not shown).
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1 Stimulation of OKF6/TERT-2 cells with 10 ng/ml IL-17A caused significantly elevated
2 phosphorylation of the NF- κ B p65 subunit at serine 468 and 536 ($p<0.05$) compared to
3 unstimulated control cells. Addition of 50 ng/ml IL-17E to cultures 30 minutes prior to
4 addition of IL-17A significantly decreased IL-17A-induced NF- κ B P65 serine 468 and 536
5 phosphorylation ($p<0.05$, Figure 3A and B).
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11 12 13 14 15 16 *3.7 IL-17E inhibits the Porphyromonas gingivalis induced release of chemokines from oral* 17 18 *keratinocytes* 19 20

21 Stimulation of OKF6/TERT-2 cells with a live *P. gingivalis* biofilm for 4 h and 24 h induced
22 a significant increase in IL-8 mRNA expression ($p<0.05$) compared to unstimulated control
23 cells, which was inhibited by 50 ng/ml IL-17E ($p<0.05$, Figure 4A). These changes in mRNA
24 expression were mirrored by changes in protein expression. *P. gingivalis* significantly
25 elevated release of IL-8 after 4 h and 24 h ($p<0.05$), and this was significantly inhibited by 50
26 ng/ml IL-17E at 24 h ($p<0.05$, Figure 4B).
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38 Stimulation of OKF6-TERT2 cells with a live *P. gingivalis* biofilm for 4 h and 24h induced a
39 significant increase in CXCL5 mRNA expression ($P<0.05$) compared to unstimulated control
40 cells, which was inhibited by 50 ng/ml IL-17E ($P<0.05$, Figure 4C). These changes in mRNA
41 expression were mirrored by changes in protein expression. The *P. gingivalis*-induced
42 CXCL5 release was inhibited by 50 ng/ml IL-17E at 4h and 24 h ($P=<0.05$) (Figure 4D).
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53 None of the observed effects were related to cell viability as determined by a lactate
54 dehydrogenase (LDH) activity assay (data not shown).
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4. Discussion

This study further advances understanding of IL-17 family cytokines in periodontitis, with the novel observation of a significant increase in serum levels of the IL-17A/F heterodimer in periodontitis patients which correlated with clinical parameters. In agreement with previous studies these findings confirm that serum, saliva, and GCF levels of IL-17A are elevated in periodontitis patients and correlate with clinical parameters of periodontitis and age [2-6]. In addition, serum levels of the IL-17A/F heterodimer positively correlated with serum levels of both IL-17A and IL-17F. There were no significant differences in serum IL-17F, IL-17B, IL-17C and IL-17D levels between periodontitis patients and healthy controls. Periodontitis patients presented with decreased serum levels of IL-17E, which negatively correlated with PD and CAL and serum IL-17A. The negative correlation between serum levels of IL-17A and IL-17E is particularly interesting given the proposed opposing immunological functions of these cytokines. Indeed, the findings presented suggest that the circulating IL-17A:IL-17E ratio may be a marker of disease as it showed moderate-good correlations with CAL and PD.

This study has demonstrated further novel findings with regards to GCF and salivary levels of IL-17A, IL-17E, IL-17F and IL-17A/F and associations between levels of these individual cytokines as well as clinical parameters of periodontitis. In addition, the studies show that relationships between these cytokines and clinical parameters differ depending on the bodily fluids investigated. Indeed, in contrast to the serum analysis, the exploratory investigations revealed that the GCF and salivary IL-17A:IL-17E ratio was not significantly increased in periodontitis patients. This was possibly because GCF and salivary levels of IL-17E were in fact elevated in patients with periodontitis. Differences in levels of IL-17 family cytokines in serum, saliva and GCF could possibly be explained by the anatomical location from which

these biological fluids are derived [36]. However, this is a matter of conjecture until we have a complete picture of IL-17 family cytokine biology.

GCF levels of IL-17A in patients with periodontitis [2] and serum levels of IL-17A in patients with aggressive periodontitis [3] have been shown to be reduced after non-surgical therapies. In addition, there is also evidence of Th17 cells in the periodontium [37-38]. IL-17A promotes recruitment of neutrophils and monocytes by inducing chemokine expression [39], commensurate with the data in our current study demonstrating that IL-17A induced oral keratinocytes to express IL-8. This was in contrast to IL-17E which alone had no effect on IL-8 expression. However, IL-17E was found to inhibit the IL-17A-induced expression of IL-8 by oral keratinocytes. This inhibition was more pronounced if IL-17E was added to cells 30 minutes prior to IL-17A. The notable inhibition of IL-17A induced IL-8 following pre-incubation with IL-17E suggests that IL-17E induces intracellular signalling events that mediate IL-17A induced IL-8 transcription. Hence, we therefore investigated NF- κ B. IL-17A was shown to promote IL-8 expression via intracellular signalling pathways leading to the phosphorylation of the NF- κ B p65 subunit at serine 536 and serine 468. Phosphorylation of serine 536 of the NF- κ B p65 subunit has been demonstrated to be important in transcription of the IL-8 gene [40], while phosphorylation at serine 468 has been shown to have an important role for NF- κ B ubiquitination and degradation [41]. However, despite their opposing functions, phosphorylation at serine 536 and 468 occurs simultaneously [42]. In this study, IL-17E inhibited the IL-17A induced phosphorylation events which promote NF- κ B p65 subunit activation. This suggests that IL-17E inhibition of IL-8 expression is regulated at the transcriptional level by intracellular signalling pathways which inhibit activation of the NF- κ B transcription factor. However, further studies are required to delineate which pathways are responsible.

IL-17E has been shown to regulate the innate immune responses of bacterial LPS stimulated myeloid cells [27]. Our studies show that a similar phenomenon occurs in oral keratinocytes. *P. gingivalis* up-regulated the expression of IL-8 and CXCL5 in accordance with previous studies [43-44]. However, IL-17E inhibited the *P. gingivalis* induced expression of both these chemokines and this inhibition was evident at the mRNA level. Increased expression of IL-17E occurs only in later stages of disease in a collagen-induced arthritis mouse model [69], which may explain the paradoxical findings between the serum and salivary and GCF levels of IL-17E. Compared with serum, GCF and to some extent saliva may provide a more representative picture of the events occurring within diseased periodontal tissue. Therefore based on the findings in this manuscript, and the current literature, it could be argued that increased expression of IL-17E in periodontal tissue occurs as an attempt to down-regulate the damaging localised periodontal inflammatory response.

In conclusion, these data confirm that IL-17 family cytokines play a role in periodontitis. Interestingly, the data suggests that IL-17A and IL-17E may have opposing functions in disease pathogenesis and that the serum IL-17A:IL-17E ratio may be a marker of disease severity. Furthermore, these data suggest that IL-17E down-regulates both IL-17A and *P. gingivalis*-induced expression of chemokines by oral keratinocytes. The cellular sources and exact signalling mechanisms of most IL-17 family cytokines in the periodontium remain to be characterised hence further research is required to fully delineate the biological functions of these cytokines and their role in periodontitis.

Figure legends

Fig. 1

Correlations between the serum IL-17A:IL-17E ratio and clinical and demographic parameters. Serum IL-17A:IL-17E ratios were subjected to natural log transformations to facilitate graphical representation of the correlations between (A) Clinical attachment loss (CAL), (B) Probing depth, (C) bleeding on probing (BOP) and (D) age. The Pearson correlation coefficient was determined (R). All were statistically significant correlations ($p < 0.010$).

Fig. 2

The effect of IL-17E on IL-17A induced IL-8 expression by OKF6/TERT-2 cells. (A) Mean $\pm$ standard deviation IL-8 release from OKF6/TERT-2 cells stimulated concomitantly with combinations of IL-17A and IL-17E (see x axis for concentrations) ($n=3$). Statistical analysis was performed by log transforming the data followed by an ANOVA with a *post hoc* Bonferroni correction ($**p < 0.01$). (B) Mean $\pm$ standard deviation IL-8 release from OKF6/TERT-2 cells pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations) ($n=3$). Statistical analysis was performed by log transforming the data followed by an ANOVA with a *post hoc* Bonferroni correction ($*p < 0.05$, $**p < 0.01$). (C) Fold induction change ($\pm$ standard deviation) in IL-8 mRNA expression by OKF6/TERT-2 cells pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations) ($n=3$). Statistical analysis of real time PCR data was performed by a Levenes's test of homoscedasticity on the natural log transformed $2^{-\Delta CT}$ values followed by an independent *t*-test to compare two means ($**p < 0.01$). $\pm$ =statistical significance in

comparison to un-stimulated control, # = statistical significance in comparison to cells stimulated with 10 ng/ml IL-17A only.

Fig. 3

The effect of IL-17E on IL-17A induced phosphorylation of the NF- κ B p65 subunit at serine 468 and serine 536 by OKF6/TERT-2 cells. (A) Mean ($\pm$ standard deviation) angular transformed % of NF- κ B p65 subunit phosphorylated at serine 468 pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations) (n=3). (B) Mean ($\pm$ standard deviation) angular transformed % of NF- κ B p65 subunit phosphorylated at serine 536 pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations) (n=3). Statistical analysis was performed by ANOVA and a *post hoc* Holm-Bonferroni (H-B) corrected least significant difference test (* $p < 0.05$ /H-B). $\pm$ = statistical significance in comparison to un-stimulated control, # = statistical significance in comparison to cells stimulated with 10 ng/ml IL-17A only.

Fig. 4

The effect of IL-17E on *P. gingivalis* biofilm induced IL-8 expression by OKF6/TERT-2 cells. The data shows the effect of *P. gingivalis* alone, IL-17E alone or IL-17E (50 ng/ml; added 30 minutes prior) in combination with a *P. gingivalis* biofilm on the expression of IL-8 at the mRNA (A) and protein level (B) and CXCL5 at the mRNA (C) and protein (D) level. Changes in mRNA expression were determined by real time PCR and protein release by ELISA. For the real time data mRNA levels were normalised against GAPDH (housekeeping gene) and fold induction calculated by the $2^{-\Delta\Delta CT}$ method. Each bar represents the mean ($\pm$ standard deviation). For the ELISA data, each bar represents the mean ($\pm$ standard deviation). All data is derived from duplicate wells of four independent experiments performed on

1 separate days (n=4). *P < 0.05. Ns = not significant. ± = statistical significance in comparison
2 to un-stimulated control, # = statistical significance in comparison to cells stimulated with *P.*
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4 *gingivalis* biofilm only.
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Figure 1

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1. Demographic and clinical data for the sample cohorts

A. Glasgow	Healthy (n=62; 44% M)	Periodontitis (n=71; 49% M)
AGE	34 (26 – 44) 36 ± 11	47 (41 – 53) 47 ± 8
% BOP	2.7 (0.0 - 9.0) 8.2 ± 11.9	36.3 (23.5 -44.7) 35.3 ± 20.7
CPD	1.34 (1.00 - 1.69) 1.54 ± 0.50	2.83 (2.69 - 3.11) 2.92 ± 0.63
CAL	< 2mm^a	3.45 (3.03 - 3.94) 3.66 ± 0.89
B. Newcastle	Healthy (n=15; 69% M)	Periodontitis (n=26; 31% M)
AGE	47 (42 – 54) 49 ± 8	48 (46 – 54) 50 ± 6
% BOP	0.6 (0.0 – 2.1) 1.1 ± 1.1	39.9 (29.2 – 51.9) 43.0 ± 20.6
CPD	1.48 (1.21 - 1.69) 1.58 ± 0.12	2.87 (2.69 - 3.27) 3.67 ± 1.10
CAL	< 2mm^a	3.33 (3.03 - 3.94) 3.12 ± 0.77
C. Combined	Healthy (n=77; 56.5% M)	Periodontitis (n=97; 40.0% M)
AGE	38 (27 – 49) 38 ± 12	47 (42 – 53) 47 ± 8
% BOP	2.7 (0.0 - 9.0) 6.7 ± 11.0	36.0 (25.0 - 48.8) 38.8 ± 20.8
CPD	1.48 (1.21 - 1.69) 1.51 ± 0.48	2.87 (2.69 - 3.27) 3.10 ± 0.83
CAL	< 2mm^a	3.33 (3.03 - 3.94) 3.53 ± 0.89

BOP = Bleeding on probing

CPD = Clinical probing depth

CAL = Clinical attachment loss (^acould not accurately be determined in the healthy group)

Significant differences between health and periodontitis groups shown in bold (p<0.01)

Median values shown with inter-quartile range in brackets and mean ± standard deviation underneath

Table 2. IL-17 family cytokine levels and the IL-17A:IL-17E ratio in serum, gingival crevicular fluid and saliva

A				
Serum (pg/ml)	Healthy	n=	Periodontitis	n=
IL-17A	15.5 (5.3 - 30.1)*	77	47.5 (38.7 - 98.0)*	97
IL-17B	38.8 (30.8 - 48.1)	40	33.8 (18.2 - 59.3)	55
IL-17C	40.9 (2.9 - 49.8)	40	31.6 (12.0 - 51.7)	55
IL-17D	22.1 (20.1 - 30.6)	38	23.8 (21.0 - 28.8)	41
IL-17E	35.3 (23.1 - 44.0)*	77	17.2 (15.9 - 50.3)*	97
IL-17F	14.3 (4.0 - 32.2)	77	14.2 (7.2 - 20.0)	97
IL-17A/F	8.0 (0.0 - 26.0)*	77	31.4 (24.8 - 53.7)*	97
B				
GCF (pg)	Healthy	n=	Periodontitis	n=
IL-17A	44.4 (5.0 - 117.2)*	37	94.9 (76.6 - 119.6)*	41
IL-17E	53.5 (2.0 - 85.9)	37	70.6 (42.2 - 128.8)	41
IL-17F	3.9 (1.9 - 7.8)*	21	6.4 (5.8 - 12.5)*	13
IL-17A/F	5.5 (4.9 - 15.0)*	21	19.0 (15.0 - 35.0)*	13
C				
Saliva (pg/ml) Glasgow	Healthy	n=	Periodontitis	n=
IL-17A	9.8 (8.0 - 15.9) *	24	17.8 (10.7 - 28.4)*	38
IL-17E	5.3 (3.9 - 17.5) *	24	51.7 (25.1 - 128.5)*	38
IL-17F	3.9 (1.9 - 7.8)*	24	11.2 (6.6 - 19.3)*	38
IL-17A/F	12.6 (4.4 - 15.1) *	24	29.3 (24.4 - 47.8)*	38
D				
Saliva (pg/ml) Newcastle	Healthy	n=	Periodontitis	n=
IL-17A	16.9 (10.2 - 25.2) *	13	48.3 (21.7 - 130.4)*	26
IL-17E	46.8 (38.9 - 92.9)	13	57.5 (30.0 - 100.1)	26
IL-17F	10.0 (8.5 - 13.6)	13	9.5 (8.0 - 16.3)	26
IL-17A/F	31.1 (23.5 - 37.8)*	13	46.8 (38.9 - 92.9) *	26
E				
IL-17A:IL-17E	Healthy	n=	Periodontitis	n=
Serum	0.48 (0.10 - 0.98)*	77	2.62 (0.82 - 4.47)*	97
GCF	0.50 (0.21 - 2.43)	37	0.59 (0.16 - 1.49)	41
Saliva	0.75 (0.30 - 1.28)	37	0.82 (0.50 - 1.07)	64

*Significant differences ($p < 0.050$) between health and periodontitis groups shown in bold
Median values shown with inter-quartile range in brackets

Table 3. Correlations between clinical and demographic parameters and levels of IL-17A, IL-17E, IL-17F and IL-17A/F in serum, gingival crevicular fluid and saliva

A					
Serum		AGE	PD	CAL	%BOP
IL-17A	rho=	0.338	0.429	0.464	0.331
	p=	0.00001	0.00001	0.00001	0.00001
IL-17E	rho=	0.031	-0.265	-0.291	-0.060
	p=	0.68444	0.00029	0.00010	0.43266
IL-17F	rho=	-0.080	-0.025	-0.002	0.024
	p=	0.30314	0.74575	0.97826	0.75304
IL-17A/F	rho=	0.598	0.544	0.436	0.375
	p=	0.00001	0.00001	0.00001	0.00001

B					
GCF		AGE	PD	CAL	%BOP
IL-17A	rho=	0.484	0.611	0.543	0.273
	p=	0.00001	0.00001	0.00001	0.01620
IL-17E	rho=	0.217	0.415	0.463	0.355
	p=	0.05853	0.00017	0.00002	0.00153
IL-17F	rho=	0.481	0.449	0.469	0.349
	p=	0.00397	0.00777	0.00517	0.04325
IL-17A/F	rho=	0.531	0.435	0.444	0.368
	p=	0.00125	0.01005	0.00845	0.03228

C					
Saliva (Glasgow only)		AGE	PD	CAL	%BOP
IL-17A	rho=	0.385	0.585	0.520	0.275
	p=	0.0239	0.00001	0.00193	0.11485
IL-17E	rho=	0.5999	0.673	0.761	0.480
	p=	0.00019	0.00001	0.00001	0.00407
IL-17F	rho=	0.405	0.462	0.404	0.2536
	p=	0.01855	0.01145	0.0199	0.16671
IL-17A/F	rho=	0.485	0.199	0.212	0.050
	p=	0.00509	0.55480	0.45691	0.85645

Significant correlations shown in bold (p<0.050)

Bivariate correlations performed using Spearmans rho