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## Peripheral T helper cell profiles during management of periodontitis

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## RUNNING TITLE

Peripheral Th cells & periodontitis

## CONFLICT OF INTEREST AND FUNDING INFORMATION

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## ABSTRACT

**Aim:** Periodontitis has been associated with other systemic diseases with underlying inflammation responsible for the shared link. This study evaluated longitudinal variation in peripheral T helper cells in periodontitis patients undergoing management over one year.

**Methods:** Periodontal parameters and peripheral blood mononuclear cells (PBMCs) were collected from 54 periodontitis patients at baseline, 3-, 6- and 12-months post-treatment and 40 healthy controls. IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-17<sup>+</sup>, Foxp3<sup>+</sup> and their double positive expression was identified in CD4<sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup> cells using flow cytometry. PBMCs were incubated with *P. gingivalis* and IFN- $\gamma$ , IL-4, IL-17 and IL-10 in cell-supernatant was measured by ELISA. Cells and cytokines were also assessed based on clinical response to treatment where good (<10% of sites), moderate (10-20%) and poor (>20%) treatment outcome (TxO) groups had probing depths of  $\geq 5$ mm at study conclusion.

**Results:** IFN- $\gamma$ <sup>+</sup> cells were lower at baseline, 3- and 6-months compared to health, whereas Foxp3<sup>+</sup> cells were increased at 12-months compared to all preceding timepoints and health. The good TxO group showed treatment-related variation in IFN- $\gamma$ <sup>+</sup> and Foxp3<sup>+</sup> cells, whereas the poor TxO group did not. IFN- $\gamma$  and IL-17 cytokine expression in cell-supernatants was significantly lower at baseline compared to health, and IFN- $\gamma$  and IL-10 showed treatment-related decrease.

**Conclusion:** This study suggests that IFN- $\gamma$ <sup>+</sup> and Foxp3<sup>+</sup> cells may have a role in the systemic compartment in periodontitis. Periodontal management has local and systemic effects and thus, assessment and management of periodontitis should form an integral part of overall systemic health.

**Keywords:** T-Lymphocytes, Helper-Induced; T-Lymphocytes, Regulatory; Cytokines; Periodontitis; Periodontal Debridement

## CLINICAL RELEVANCE

**Scientific rationale:** Periodontitis is linked to other chronic diseases such as diabetes and cardiovascular diseases. This link is thought to be due to the leaking of bacteria and inflammatory products which affects areas distant to the mouth. Ultimately, the host immune response determines disease aetiology and clinical outcome in periodontitis. T helper (Th) cells are known to

play a role in disease pathogenesis. It remains unclear as to what effect treatment has on peripheral Th cells during disease management longitudinally.

**Principal findings:** IFN- $\gamma$ <sup>+</sup> cells were lower in periodontitis compared to health. Foxp3<sup>+</sup> cells were higher at 12-months post-treatment. The good treatment outcome group showed treatment-related changes in IFN- $\gamma$ <sup>+</sup> and Foxp3<sup>+</sup> expressing cells, whereas the poor treatment outcome group did not.

**Practical implications:** Our results provide a broad understanding of T cells in peripheral circulation and establish for the first time the relative roles of IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-17<sup>+</sup> and Foxp3<sup>+</sup> cells up to 12-months during management of periodontitis. Treatment has an effect on some T cell subsets in peripheral blood and management of periodontitis may have beneficial effects not only in the oral cavity but also for overall systemic health.

## INTRODUCTION

Periodontitis is a polymicrobial immune-inflammatory disease affecting the supporting structures of a tooth. Although localised to the oral cavity, mounting evidence points to a role for periodontitis as a risk factor for several systemic diseases including atherosclerotic cardiovascular diseases (Dietrich et al. 2013) and type 2 diabetes (Borgnakke et al. 2013). This association is thought to be related to systemic dissemination of periodontal bacteria or inflammatory mediators and co-localisation of risk factors such as increasing age, stress and smoking behaviours (Bui et al. 2019; Konkel et al. 2019), and is corroborated by animal models (Blasco-Baque et al. 2017; Jain et al. 2003). In periodontitis, the chronic non-resolving inflammation and dysbiotic microbiota leads to ulceration of the highly vascularised oral epithelial barrier with greater access to systemic circulation which may lead to adverse effects at distal tissue sites or subversion and/or amplification of the systemic inflammatory response (Konkel et al. 2019).

The mechanisms by which the host develops periodontitis remains unclear. However, disease pathogenesis is at least, in part, mediated by the actions of T cells and cytokines. Upon antigenic stimulation, naïve CD4 T helper (Th) cells activate, differentiate and proliferate into distinct effector cells characterised by the production of a signature cytokine where Th1, Th2, Th17 and T regulatory (Treg) cells produce interferon (IFN)- $\gamma$ , interleukin (IL)-4, IL-17 and IL-10, respectively. Both protective and pathogenic roles for these Th cells and their cytokines are described in periodontitis (Hajishengallis and Korostoff 2017). This functional heterogeneity serves to target cells to necessary sites and define the class of immune and tissue responses appropriate for a particular pathogen.

*Porphyromonas gingivalis* may be described as a 'keystone pathogen' in periodontitis, where it is thought to be an essential component of plaque biofilm altering the local immune microenvironment allowing subsequent colonisation and overgrowth of pathobionts leading to disease (Hajishengallis et al. 2011). *P. gingivalis* interaction with immune cells may direct the response towards a particular Th cell lineage and effector function. The presence of IFN- $\gamma$  in the microenvironment skews towards a Th1 phenotype, and inhibits Th2 (Mosmann et al. 1986) and Th17 (Harrington et al. 2005) cell differentiation. Likewise, the presence of other signature cytokines may induce polarisation towards a particular lineage and regulate differentiation into others (Cooney et al. 2011; Grünig et al. 1997; Toh et al. 2009).

Altogether, there is a consensus that local microbial dysbiosis and ensuing immune response in periodontitis may have distant systemic effects (Chapple and Genco 2013; Tonetti and Van Dyke 2013). Previous literature investigating Th cells in peripheral blood mononuclear cells (PBMCs) is conflicting with studies reporting higher Th1 (Chen et al. 2016; Schmidt et al. 2014), Th2 (Aoyagi et al. 1995; Lima et al. 2011), Th17 (Chen et al. 2016; Luo et al. 2014; Schmidt et al. 2014) and Treg (Sabarish et al. 2016) cells in periodontitis or no significant differences (Cheng et al. 2018; Gemmell and Seymour 1998; Lalla et al. 2007; Okada et al. 2017) or treatment-related reduction (Zhao et al. 2011). Therefore, this study aimed to investigate the longitudinal variation of IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells and their double-positive expression in CD4 $^+$  and TCR $\alpha\beta^+$  cells in PBMCs during management of periodontitis up to 12-months post-treatment. Furthermore, IFN- $\gamma$ , IL-4, IL-17 and IL-10 production from PBMCs in response to *P. gingivalis* was also evaluated. Finally, differences based on response to treatment were evaluated. A greater understanding of T cell profiles will improve our knowledge of the aetio-pathogenesis of periodontitis and lead to targeted treatment modalities.

## METHODS

### Study population

The Human Ethics Sub-Committee, The University of Melbourne (1339812.3), and the Human Ethics Research Committee, Dental Health Services Victoria (279) approved the study. Sample size calculations were based on previously published work by the same group (Byrne et al. 2009). Informed written consent was gained from each subject at the commencement of the study. Data collection was executed between 2015-2017. The study design and recruitment process are displayed in appendix figure 1.

Periodontitis subjects ( $n=54$ ) were recruited from the specialist periodontics clinic at The Royal Dental Hospital of Melbourne and Melbourne Dental Clinic, The University of Melbourne. Inclusion criteria for the periodontitis group were at least two non-adjacent sites per quadrant exhibiting probing depths (PD)  $\geq 5$ mm, excluding the third molars (Darby et al. 2001). Subjects were  $>21$  years, had a minimum of 16 teeth (excluding third molars) and were systemically healthy with no periodontal treatment or antibiotic use within the preceding six months. Exclusion criteria included pregnancy or lactation and medical conditions affecting the progression of periodontitis (e.g. diabetes) or requiring pre-medication prior to treatment.

For the control group ( $n=40$ ), the inclusion criteria were a gender and age ( $\pm 5$  years) match for the periodontitis subject with no PD  $>4$ mm or percentage of sites with bleeding on probing (%BOP)  $>30\%$ . The remaining inclusion and exclusion criteria were the same as for the periodontitis group.

The periodontal parameters, mean PD and %BOP, and blood were collected from periodontitis subjects at baseline before treatment, and at 3-, 6- and 12-months post-treatment, and once from healthy controls. PD and BOP were recorded at six sites around each tooth using a William's probe (Hu-Friedy Mfg. Co., Chicago, USA). PD was measured to the nearest millimeter from the base of the gingival sulcus to the free gingival margin. BOP was assessed visually following probing to the base of the pocket up to 30 seconds after probing.

Periodontal treatment involved patient education about periodontitis and oral hygiene instruction (OHI) prior to non-surgical quadrant scaling and root debridement usually over a period of 4 weeks. They were re-evaluated at 3-, 6-, 9- and 12-months. At re-evaluation, clinical parameter were charted, the outcome of treatment evaluated, patients given OHI as needed, and residual pockets re-debrided or surgery performed ( $n=5$ ) as necessary. The treatment goal was no PD  $\geq 4$ mm and %BOP  $>30\%$ .

At the end of one year or when periodontitis patients exited the study, they were assigned to a treatment outcome (TxO) group where the good TxO group had  $<10\%$ , moderate TxO group had between 10-20%, and poor TxO group had  $>20\%$  of sites with PD  $\geq 5$ mm. Periodontitis subjects ( $n=8$ ) who only contributed to baseline were not assigned a TxO group.

## Flow cytometry for T cell phenotyping

Peripheral venous blood was collected in potassium-EDTA tubes (BD Biosciences, NSW, Australia) and PBMCs were isolated by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, NSW, Australia) following manufacturer's instructions. PBMCs were used straightaway or cryopreserved for later analysis.

PBMCs ( $1 \times 10^6$  cells) were diluted in 1mL cRPMI (RPMI-1640, 10% v/v heat-inactivated FBS, 100U/mL Penicillin-Streptomycin, 2mM L-glutamine) and stimulated with PMA (5ng/mL) and ionomycin ( $2 \mu\text{M}$ ) for 6 hours at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  with brefeldin A ( $20 \mu\text{g/mL}$ ) added after 2 hours. After stimulation, PBMCs were washed twice (8mL PBS, 800RCF, 5 minutes,  $20^\circ\text{C}$ ) and resuspended to  $1 \times 10^6$  cells/mL in PBS. Fixable viability stain BV510 ( $1 \mu\text{L}$ ) was added and the cells were incubated at  $37^\circ\text{C}$  for 5 minutes protected from light, after which they were washed once (2mL azide-free FACS wash (2% w/v BSA, 2mM EDTA in PBS), 800RCF, 5 minutes,  $20^\circ\text{C}$ ). PBMCs were resuspended in  $100 \mu\text{L}$  azide-free FACS wash and intracellular cytokine staining was performed using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific, SA, Australia) following the manufacturer's instructions with pre-diluted antibodies against CD25-BV650 ( $4 \mu\text{L}$ , clone-M-A251), TCR $\alpha\beta$ -BV785 ( $4 \mu\text{L}$ , clone-T10B9.1A-31), CD4-APCCy7 ( $4 \mu\text{L}$ , clone-RPA-T4), IL-17-AF488 ( $15 \mu\text{L}$ , clone-N49-653), IFN- $\gamma$ -PerCPCy5.5 ( $4 \mu\text{L}$ , clone-B27), Foxp3-AF647 ( $15 \mu\text{L}$ , clone-259D/C7) and IL-4-PE ( $20 \mu\text{L}$ , clone-8D4-8). Samples were acquired with BD LSRFortessa™ X-20 (BD Bioscience) and analysed using FlowJo™ Software (v10, Tree Star Inc.). All antibodies were purchased from BD Bioscience and volumes used were optimised based on manufacture's recommendations. The gating strategy is displayed in figure 1.

## Cytokine detection

Fresh PBMCs ( $1 \times 10^6$  cells) were either incubated alone, with  $5 \mu\text{g/mL}$  *P. gingivalis* W50 whole-cell lysate or  $5 \mu\text{g/mL}$  concanavalin A (ConA) (Sigma-Aldrich) for 48 hours at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in 2mL cRPMI. *P. gingivalis* was obtained from the Oral Health CRC, Melbourne Dental School, The University of Melbourne and was grown and processed as previously described (Lam et al. 2015). The aspirated supernatant was centrifuged at 800RCF, 5 minutes,  $20^\circ\text{C}$ , aliquoted and stored at  $-80^\circ\text{C}$  until analysis. Samples were analysed in duplicate and pre-matched ELISA kits were used for IFN- $\gamma$  (BD Bioscience), IL-4 (BD Bioscience), IL-17 (Thermofisher Scientific) and IL-10 (BD Bioscience) quantification following the manufacturer's instructions.

## Statistical analysis

Independent samples *t*-tests with unequal variances were used for comparison between health and periodontitis at each timepoint. Linear mixed models (LMMs) were used to determine changes with treatment in the mean of the outcome variable over timepoint and TxO group in periodontitis. The base model for variation over timepoint included timepoint, age and gender as fixed effects and subject as random effect. The base model for variations over TxO group included TxO group, timepoint, interaction between TxO group and timepoint, age and gender as fixed effects and subject as random effect. To assess the effect of periodontal management, mean PD and %BOP were added as fixed effects to the base model. Pairwise comparisons of estimated marginal means were used to assess significant differences. Visual inspection of residual plots did not show any obvious deviations from normality.

Spearman's correlations were used for associations to the periodontal parameters at each timepoint. Paired samples *t*-tests were used for comparison between cell phenotypes and cytokine production with the various stimulation methods within each timepoint.

The level of significance was set at  $p \leq 0.05$  for all variable. All statistical analyses were performed in SPSS (v23, IBM SPSS Statistics for Windows) and graphs prepared using GraphPad Prism (v5, GraphPad Software).

## RESULTS

### Study cohort

The cohort demographics and periodontal parameters are displayed in table 1. Mean PD and %BOP were significantly higher at baseline compared to health, and decreased at subsequent timepoints post-treatment compared to baseline. When separated into TxO groups, mean PD was significantly different at baseline and 12-months between all three TxO groups, with the poor group showing higher mean PD followed by moderate and good TxO groups. At 3- and 6-months, mean PD in the poor TxO group was significantly higher compared to good and moderate groups. There were no differences in %BOP between the TxO groups at baseline. However, %BOP was significantly higher in the poor compared to the good TxO group at all timepoints post-treatment.

## IFN- $\gamma$ , IL-4, IL-17 and Foxp3 expression in PBMCs

In both healthy and periodontitis groups, IFN- $\gamma$ <sup>+</sup> cells were the dominant cell type whereas IL-17<sup>+</sup> cells were the minority. When separated based on TCR $\alpha\beta$ <sup>+</sup> and CD4<sup>+</sup> expression, the majority of IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> cells were TCR $\alpha\beta$ <sup>+</sup>CD4<sup>-</sup> followed by TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> cells, whereas the majority of IL-17<sup>+</sup> and Foxp3<sup>+</sup> cells were TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> cells (figure 2).

Total IFN- $\gamma$ <sup>+</sup> cells (figure 3A) were significantly lower at baseline, 3- and 6-months in periodontitis compared to health. When separated into TxO groups, within the good TxO group, IFN- $\gamma$ <sup>+</sup> cells were significantly lower at 3-, 6- and 12-months post-treatment compared to health, and decreased significantly at 3- and 12-months compared to baseline. In the moderate and poor TxO groups, IFN- $\gamma$ <sup>+</sup> cells were significantly lower at baseline compared to health, however, no changes were observed post-treatment.

Total Foxp3<sup>+</sup> cells (figure 3B) were significantly higher at 12-months post-treatment compared to health and all preceding timepoints in periodontitis. When separated into TxO groups, Foxp3<sup>+</sup> cells were significantly increased at 12-months compared to all timepoints in periodontitis within the good TxO group, and significantly increased at 12-months compared to 3- and 6-months in the moderate TxO group. However, no treatment-related changes were observed in the poor TxO group.

No definitive patterns were found for total IL-4<sup>+</sup> and IL-17<sup>+</sup> cells, combinations of CD4<sup>+</sup> or TCR $\alpha\beta$ <sup>+</sup> expression in IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-17<sup>+</sup> and Foxp3<sup>+</sup> cells or double positive cytokine expression in TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> cells and the data is presented in appendix tables 1 and 2.

## Cytokine production in PBMC-supernatant upon *P. gingivalis* stimulation

Stimulation with ConA elicited the greatest amount of cytokine production, followed by *P. gingivalis* and cells alone. Upon stimulation with *P. gingivalis*, IL-10 was the largest cytokine produced followed by IFN- $\gamma$  (appendix figures 2, 3).

IFN- $\gamma$ , IL-4, IL-17 and IL-10 production with various stimulation methods are displayed in figure 4. Upon stimulation with *P. gingivalis*, IFN- $\gamma$  was lower at all timepoints, IL-4 was lower at 3-, 6- and 12-months, and IL-17 was lower at baseline and 3-months in comparison to health. With treatment,

IFN- $\gamma$  was decreased at 3- and 6-months and IL-4 was decreased at all timepoints compared to baseline, whereas no treatment-related changes were observed for IL-17 and IL-10.

The data for IFN- $\gamma$ , IL-4, IL-17 and IL-10 production in PBMC-supernatant when separated based on TxO groups are presented in appendix figures 4-7.

## Associations with periodontal parameters

T cell phenotypes and cytokine production with various stimuli were correlated with mean PD and %BOP and are displayed in tables 2 and 3.

When periodontal parameters were added as fixed effects to the base LMMs for T cell phenotypes, %BOP was a significant predictor for TCR $\alpha\beta^+$ CD4 $^+$ IFN- $\gamma^+$  (estimate of fixed effect ( $b$ ) = 0.13; standard error (SE) = 0.05 for timepoint, SE=0.06 for TxO), TCR $\alpha\beta^+$ CD4 $^+$ Foxp3 $^+$ IL-17 $^+$  ( $b$ =0.08; SE=0.02) and TCR $\alpha\beta^+$ CD4 $^+$ CD25 $^+$ Foxp3 $^+$  ( $b$ =0.08; SE=0.03) cells in the models for both timepoint and TxO. A 1% increase in BOP was associated with 0.13% increase in TCR $\alpha\beta^+$ CD4 $^+$ IFN- $\gamma^+$  cells, and 0.08% increase in both TCR $\alpha\beta^+$ CD4 $^+$ Foxp3 $^+$ IL-17 $^+$  and TCR $\alpha\beta^+$ CD4 $^+$ CD25 $^+$ Foxp3 $^+$  cells with both timepoint and treatment outcome. Mean PD ( $b$ =-0.79, SE=0.37) and %BOP ( $b$ =0.02, SE=0.01) were both significant predictors for TCR $\alpha\beta^+$ CD4 $^+$ IL-4 $^+$ Foxp3 $^+$  cells in the base model for treatment outcome. A 1mm increase in mean PD was associated with 0.79% decrease, and a 1% increase in BOP was associated with 0.02% increase in TCR $\alpha\beta^+$ CD4 $^+$ IL-4 $^+$ Foxp3 $^+$  cells. Likewise, for the base LMMs for *P. gingivalis* stimulation, mean PD was a significant predictor for IL-17 production ( $b$ =7.82, SE=3.00) and %BOP was a significant predictor for IFN- $\gamma$  production ( $b$ =0.42, SE=0.19) in the model for timepoint.

## DISCUSSION

Many studies have proposed a role for Th1, Th2, Th17 and Treg cells in the pathogenesis of periodontitis based on IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  or IL-10 $^+$  expression (Chen et al. 2016; Luo et al. 2014). However, these studies require careful interpretation as the expression of these cytokines is not restricted to CD4 $^+$ TCR $\alpha\beta^+$  cells in PBMCs. This study found that the majority of Foxp3 $^+$  and IL-17 $^+$  cells were TCR $\alpha\beta^+$ CD4 $^+$ , i.e. true T helper cells, whereas the majority of IFN- $\gamma^+$  and IL-4 $^+$  cells were TCR $\alpha\beta^+$ CD4 $^-$  followed by TCR $\alpha\beta^+$ CD4 $^+$  cells. Likely, these TCR $\alpha\beta^+$ CD4 $^-$  cells are CD8 $^+$  T cells, CD4 $^+$ TCR $\alpha\beta^-$  cells are NK and NKT cells, and TCR $\alpha\beta^-$ CD4 $^-$  cells are  $\gamma\delta$  T cells, macrophages, basophils or B cells in PBMCs. Interestingly, TCR $\alpha\beta^-$ CD4 $^-$  cells were not the smallest subset expressing IFN- $\gamma$  and

IL-4 and thus, other non-canonical cytokine producing cells may also contribute to peripheral immune dysregulation in periodontitis. In line with this, TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells were significantly positively correlated with health. Previous studies that investigated other cellular sources of cytokines have reported that IFN- $\gamma$  mRNA was equally frequently detected in both CD4<sup>+</sup> and CD8<sup>+</sup> gingival mononuclear cells (GMCs) in periodontitis patients (Takeichi et al. 2000). Likewise, GMCs extracted from periodontitis and healthy patients showed no significant differences in IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> expression in CD4<sup>+</sup> and CD8<sup>+</sup> cells or IL-10<sup>+</sup> in CD4<sup>+</sup> cells, however, IL-10<sup>+</sup> in CD8<sup>+</sup> cells was significantly lower in periodontitis compared to health (Gemmell and Seymour 1998).

IFN- $\gamma$ <sup>+</sup> cells were significantly lower at baseline, 3- and 6-months post-treatment compared to health which is contrary to several studies in peripheral blood that reported higher CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in periodontitis compared to health (Chen et al. 2016; Schmidt et al. 2014) or reported no significant differences (Cheng et al. 2018; Lima et al. 2011; Okada et al. 2017). Fewer IFN- $\gamma$ <sup>+</sup> expressing cells at baseline was mirrored by lower IFN- $\gamma$  production upon stimulation with *P. gingivalis*. One other study reported lower IFN- $\gamma$  in early-onset periodontitis compared to chronic periodontitis and health in PBMC-supernatant upon stimulation with phytohaemagglutinin (Sigusch et al. 1998). This depressed IFN- $\gamma$  response may be a function of compartmentalisation of immune system. The periodontal sulcus perpetually harbours microbes and consequently, there is always sub-clinical inflammation in the periodontal connective tissue. Thus, compartmentalisation within the local and systemic immune component is an essential survival strategy. Indeed, a previous study that explored cytokine mRNA expression in gingival tissues and PBMCs reported higher IFN- $\gamma$  in PBMCs compared to gingival tissues and higher IL-10 in gingival tissues compared to peripheral blood (Yamazaki et al. 1997). The depressed IFN- $\gamma$  response in periodontitis might then indicate a hyper-responsive suppression of normal immune function. Thus, paradoxically, although there were lower IFN- $\gamma$ <sup>+</sup> expressing cells in periodontitis in the present study, variation with treatment was observed in the good TxO group, whereas no changes with treatment were observed in IFN- $\gamma$ <sup>+</sup> and Foxp3<sup>+</sup> cells in the poor TxO group. This decrease with treatment may represent a reduction in the overall inflammatory burden representing good health outcomes. Accordingly, mean PD and %BOP were significantly positively correlated with TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells at baseline. Compartmentalisation of the immune response has also been shown for other disease states such as sarcoidosis (Hudspith et al. 1987) and tuberculosis (Guglielmetti et al. 2013).

Treg cells are involved in the resolution of inflammation as well as maintaining homeostatic control of immune responses. Their principal suppressive role is mainly via inhibition of APC maturation and effector cell differentiation, and secretion of inhibitory cytokines, such as IL-10, TGF- $\beta$  and IL-35

(Sakaguchi et al. 2010). It has also been shown that during disease resolution, T cells change their transcriptional profile and acquire regulatory properties in the presence of TGF- $\beta$  (Gagliani et al. 2015). Thus, the increase in Foxp3<sup>+</sup> cells at 12-months seen in this study may be associated with the conversion of inflammatory cells into regulatory cells and the resolution of inflammation. The late increase in Foxp3<sup>+</sup> cells at 12-months rather than early increase corresponding to treatment may reflect Treg cell role in periodontal tissue repair and remodelling which may last for a year or more after treatment (Gurtner et al. 2008; Waerhaug 1978; Wilson et al. 2008).

There were neither any significant differences in total IL-17<sup>+</sup> or IL-4<sup>+</sup> cell proportions nor when separated into CD4<sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup> subsets. During data collection with flow cytometry, the mean number IL-17<sup>+</sup> and IL-4<sup>+</sup> cells acquired within the cytokine gate were 1,900 and 4,500 cells respectively, which is well above the threshold of 100 cells for rare populations (Roederer 2008). This corroborates other cross-sectional studies that reported no significant differences in CD4<sup>+</sup>IL-17<sup>+</sup>, CD8<sup>+</sup>IL-17<sup>+</sup> or TCR $\gamma\delta$ <sup>+</sup>IL-17<sup>+</sup> cells between health and periodontitis (Cheng et al. 2018; Okada et al. 2017). Nonetheless, this does not exclude a role for Th2 (Aoyagi et al. 1995) and Th17 cells (Cardoso et al. 2009) at the gingival interface.

ConA stimulation of PBMCs elicited greater cytokine production compared to PBMCs alone in line with previous studies (Berker et al. 2013; Fujihashi et al. 1993; Gonçalves et al. 2013; McFarlane et al. 1990). Upon *P. gingivalis* stimulation, IL-10 was the most abundant cytokine produced with 3.5-fold and 7-fold higher mean concentration than IFN- $\gamma$  at health and baseline, respectively. IL-10 has many anti-inflammatory functions including downregulating MHC class II and co-stimulatory molecule expression on APCs impairing their antigen-presenting capacity (de Waal Malefyt et al. 1991; Ding et al. 1993; Willems et al. 1994), and negatively regulating Th1, Th2 and Th17 cells (Coomes et al. 2017; O'Garra and Vieira 2007) while sustaining Treg cell production (Murai et al. 2009). Thus, it follows that incubation with periodontal pathogens would elicit large amounts of IL-10 production irrespective of health or disease. This study, like others (Borch et al. 2010; de Heens et al. 2009; Goncalves et al. 2010), found no differences in IL-10 between periodontitis and health, however, others reported higher production in periodontitis supporting an anti-inflammatory role for IL-10 (Gonçalves et al. 2013; Trindade et al. 2012). *P. gingivalis* stimulation only elicited low amounts of IL-4 (timepoint total mean: 2.93pg/mL; range 0.00-18.82pg/mL) and IL-17 (timepoint total mean: 7.18pg/mL; range 0.00-155.54pg/mL). Moreover, at 6-months, there was no statistically significant difference in IL-17 produced with and without *P. gingivalis* stimulation. Low levels of IL-4 and IL-17 with antigens and not common mitogens are also seen in other studies (Kurtzhals et al. 1992; Lenarczyk et al. 2000). Apart from IL-10 mediated suppression, other potential reasons for low

IL-4 and IL-17 production include short half-life after secretion, rapid degradation of the expressed cytokine or possible sequestration of the cytokine in culture by receptor on other leukocytes. Moreover, activation of  $\alpha\beta$  T cells,  $\gamma\delta$  cells or NK cells may not be directly mediated by bacterial products, and co-stimulatory molecules like CD28, effective antigen processing by APCs, and the strength and duration of TCR signalling may all affect efficient T cell activation (Champaiboon et al. 2000; Gemmell and Seymour 2004).

In this study, we can only speculate  $CD4^+TCR\alpha\beta^-$ ,  $TCR\alpha\beta^+CD4^-$  or  $CD4^-TCR\alpha\beta^-$  cell lineages and further studies with specific markers that identify other cellular sources in PBMCs would provide a broader understanding of the host immune response in periodontitis. The T helper cell family has also expanded to include Th9, Th22 and T follicular helper cells and longitudinal clinical studies in this area are also needed. T cells typically demonstrate low to undetectable amounts of spontaneous cytokine expression requiring *in vitro* activation using antigenic, mitogenic or pharmacologic (PMA/ionomycin) stimuli. Previous studies have reported CD4 downregulation upon stimulation with PMA/ionomycin (O'Neil-Andersen and Lawrence 2002), and higher IFN- $\gamma$  and IL-17 secretion in  $CD4^+$  T cell clones with PMA/ionomycin stimulation compared to anti-CD3/CD28 (Olsen and Sollid 2013). Likewise in PBMC-supernatants, this study did not identify which specific cells contributed to the cytokine milieu. Kobayashi *et al.*, 2000 (Kobayashi et al. 2000) have reported 30-fold higher IFN- $\gamma$  production by PBMCs compared to purified T cells when stimulated with *Aggregatibacter actinomycetemcomitans*, suggesting that T cells may not be the major cytokine contributors. This study used whole-cell lysate which represents the entire cohort of virulence factors that could elicit an immune response. Previous literature has reported differential cytokine responses with different bacterial species (Kobayashi et al. 2000), live and dead bacteria (Scheres et al. 2010), and various preparation methods such as formalin-killed whole-cells and sonicates (Aoyagi et al. 1995) or virulence factors (Trindade et al. 2012). Mean PD and %BOP were the sole clinical periodontal parameters used in this study as the treatment goal was shallow pockets that do not bleed when probed. Other parameters such as clinical attachment levels and radiographic bone loss may provide a more dynamic picture of the periodontal status and peripheral immune response of the study cohort. Lastly, the initial study parameters were set in 2013 well before the definition of health was established in the current periodontal classification. Thus, the healthy control group in this study includes health and localised gingivitis cases and the results should be interpreted in light of this (Cheng et al. 2018).

Th cells play a key role in periodontitis immunopathology and the contribution of Th cells to this process remains unresolved. This study is the first to report longitudinal variation in four canonical

cytokines for Th1, Th2, Th17 and Treg cells in response to *P. gingivalis* challenge in PBMCs. In cell-supernatant, IFN- $\gamma$  and IL-17 was significantly lower at baseline compared to health, and treatment resulted in a reduction in IFN- $\gamma$  and IL-4 compared to baseline. Moreover, this study found that TCR $\alpha\beta^+$ CD4 $^+$  and TCR $\alpha\beta^+$ CD4 $^-$  cells were the major sources of IFN- $\gamma^+$  and IL-4 $^+$  expression in PBMCs while TCR $\alpha\beta^+$ CD4 $^+$  cells were the major sources of Foxp3 $^+$  and IL-17 $^+$  cells. The good TxO group showed a treatment-related reduction in IFN- $\gamma^+$ , whereas no changes in IFN- $\gamma^+$  or Foxp3 $^+$  cells were observed in the poor TxO group. Collectively, this study corroborates the presence of distinct Th cell profiles in peripheral blood during management of periodontitis and highlights the complexity of systemic immune responses at sites distant to the local bacterial challenge.

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## TABLE AND FIGURE LEGENDS

### Table 1. Cohort demographics and periodontal parameters.

† significant difference to health

‡ significant difference to baseline

§ significant difference to 3-months post-treatment

£ – significant difference between good and moderate treatment outcome groups at same timepoint

¢ – significant difference between good and poor treatment outcome groups at same timepoint

¥ – significant difference between moderate and poor treatment outcome groups at same timepoint

Periodontitis subjects ( $n = 8$ ) who only contributed to baseline were not assigned to a TxO group.

%BOP – percentage of sites exhibiting bleeding on probing, mm – millimetres, PD – probing depth,

SD – standard deviation, TxO – treatment outcome

### Table 2. Correlations between cell phenotypes and periodontal parameters.

Spearman's correlation coefficients ( $\rho$ ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells divided based on CD4 $^+$ TCR $\alpha\beta^-$ , TCR $\alpha\beta^+$ CD4 $^+$ , TCR $\alpha\beta^+$ CD4 $^-$ , CR $\alpha\beta^-$ CD4 $^-$  expression as well as Foxp3 $^+$ , IFN- $\gamma^+$ , IL-4 $^+$  and IL-17 $^+$  double positive TCR $\alpha\beta^+$ CD4 $^+$  cells in health and at baseline, 3-, 6- and 12- months in periodontitis. Significant correlations ( $p \leq 0.05$ ) are represented in bold.

### Table 3. Correlations between cytokine production in PBMC-supernatant upon various stimulation methods and periodontal parameters.

Spearman's correlation coefficients ( $\rho$ ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and IFN- $\gamma$ , IL-4, IL-17 and IL-10 cytokine expression from PBMCs without any stimulation and upon *P. gingivalis* and Concanavalin A (ConA) stimulation in health and at baseline, 3-, 6- and 12- months in periodontitis. Significant correlations ( $p \leq 0.05$ ) are represented in bold.

### Figure 1. Gating strategy for phenotyping IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$ and Foxp3 $^+$ expressing T cells.

Lymphocytes were identified based on forward scatter area (FSC-A) and side scatter area (SSC-A). Live cells were identified based on the exclusion of viability stain. The lymphocyte population was better identified based on FSC-A and SSC-A, and doublets were excluded using FSC-A and forward

scatter height (FSC-H) followed by side scatter height (SSC-H) and SSC-A. IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells in the total population were identified and divided based on CD4 $^+$ TCR $\alpha\beta^+$ , CD4 $^+$ TCR $\alpha\beta^-$ , TCR $\alpha\beta^+$ CD4 $^-$  or CD4 $^-$ TCR $\alpha\beta^-$  expression. CD4 $^+$ TCR $\alpha\beta^+$  cells were further divided into IFN $\gamma^+$ IL-4 $^+$ , IFN $\gamma^+$ Foxp3 $^+$ , IL-4 $^+$ Foxp3 $^+$ , IL-4 $^+$ IL-17 $^+$ , IFN $\gamma^+$ IL-17 $^+$ , Foxp3 $^+$ IL-17 $^+$  and CD25 $^+$ Foxp3 $^+$  cells.

**Figure 2. IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells in PBMCs and their CD4 and TCR $\alpha\beta$  expression.**

Clustered bar charts showing mean and SD for IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells as a percentage of PBMCs (A) and CD4 $^+$ TCR $\alpha\beta^+$ , TCR $\alpha\beta^+$ CD4 $^+$ , TCR $\alpha\beta^+$ CD4 $^-$  and TCR $\alpha\beta^-$ CD4 $^-$  expression in IFN- $\gamma^+$ , IL-4 $^+$ , IL-17 $^+$  and Foxp3 $^+$  cells (B) in health and periodontitis at baseline, 3-, 6- and 12-months. \* above the bars represent significant differences ( $p \leq 0.05$ ) to other cell types within each timepoint. † next to the title represents significant differences ( $p \leq 0.05$ ) to other cell types within each group at all timepoints. Analysis of significance was calculated using paired samples *t*-test within each timepoint.

**Figure 3. IFN- $\gamma^+$  and Foxp3 $^+$  cells in PBMCs.**

IFN- $\gamma^+$  (A) cells and Foxp3 $^+$  (B) cells as a percentage of PBMCs and separated based on treatment outcome (TxO) groups as good, moderate and poor in health and periodontitis at baseline, 3-, 6-, and 12-months. Line graphs display the mean and SD and show differences between the three TxO groups; symbols above the plots represent  $p \leq 0.05$  between \* good – moderate, † moderate – poor, ‡ good – poor TxO groups. Box and whisker plots display the median, interquartile ranges and 10<sup>th</sup> – 90<sup>th</sup> percentiles and show differences within timepoint or the three TxO groups; ◻ above the plots represent  $p \leq 0.05$ . Line graphs display the mean and SD and show differences between the three TxO groups. Analysis of significance was calculated using independent samples *t*-test for differences between health and each timepoint in periodontitis, and linear mixed models for variation with timepoint and treatment outcome in periodontitis.

**Figure 4. IFN- $\gamma$ , IL-4, IL-17 and IL-10 cytokine production in PBMC-supernatant with various stimulation methods.**

IFN- $\gamma$  (A), IL-4 (B), IL-17 (C) and IL-10 (D) in pg/mL produced by PBMCs without any stimulation, and upon *P. gingivalis* and concanavalin (ConA) stimulation in health and at baseline, 3-, 6-, 12-months in periodontitis. Box and whisker plots represent the median, interquartile ranges and 10<sup>th</sup> – 90<sup>th</sup> percentiles and show differences within timepoint; ◻ above the plots represent  $p \leq 0.05$ . Analysis

of significance was calculated using independent samples *t*-test for differences between health and each timepoint in periodontitis, and linear mixed models for variation with timepoint in periodontitis.

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**Table 1. Cohort demographics and periodontal parameters.**

	Health	Periodontitis			
	(n = 40)	Baseline	3-months	6-months	12-months
Gender (n, % male)	14 (35%)	20 (37%) (n = 54)			
Age at baseline (years, mean ± SD)	49.30 ± 10.62	53.28 ± 11.44 (n = 54)			
<b>Periodontal parameters</b>					
Mean PD (mm, mean ± SD)	2.80 ± 0.28	3.72 ± 0.74† (n = 54)	3.01 ± 0.51†‡ (n = 46)	2.92 ± 0.55†‡ (n = 44)	2.80 ± 0.44†‡§ (n = 37)
%BOP (% mean ± SD)	10.86 ± 7.72	51.61 ± 25.57† (n = 54)	28.35 ± 15.17†‡ (n = 46)	25.82 ± 18.84†‡ (n = 44)	24.66 ± 18.05†‡ (n = 37)
<b>Periodontal parameters separated based on TxO groups</b>					
<b>Mean PD (mm, mean ± SD)</b>					
Good TxO group	2.80 ± 0.28	3.22 ± 0.38†£¢ (n = 18)	2.66 ± 0.29†‡¢ (n = 18)	2.49 ± 0.23†‡¢ (n = 18)	2.41 ± 0.20†‡£¢ (n = 14)
Moderate TxO group	2.80 ± 0.28	3.84 ± 0.76†¥ (n = 13)	2.99 ± 0.30†‡¥ (n = 13)	2.96 ± 0.33†‡¥ (n = 12)	2.83 ± 0.21†‡¥ (n = 12)
Poor TxO group	2.80 ± 0.28	4.22 ± 0.77† (n = 15)	3.53 ± 0.50†‡ (n = 15)	3.48 ± 0.45†‡ (n = 14)	3.26 ± 0.36†‡ (n = 11)
<b>%BOP (% mean ± SD)</b>					
Good TxO group	10.86 ± 7.72	47.95 ± 22.94† (n = 18)	20.29 ± 11.85†‡¢ (n = 18)	17.86 ± 11.37†‡¢ (n = 18)	16.67 ± 16.55†¢ (n = 14)
Moderate TxO group	10.86 ± 7.72	53.17 ± 31.22† (n = 13)	30.95 ± 15.79†‡ (n = 13)	27.26 ± 20.40† (n = 12)	26.62 ± 15.87†‡ (n = 12)
Poor TxO group	10.86 ± 7.72	53.62 ± 24.61† (n = 15)	38.92 ± 12.92† (n = 15)	38.27 ± 22.67†‡ (n = 14)	36.11 ± 18.18† (n = 11)

† significant difference to health

‡ significant difference to baseline

§ significant difference to 3-months post-treatment

£ – significant difference between good and moderate treatment outcome groups at same timepoint

¢ – significant difference between good and poor treatment outcome groups at same timepoint

¥ – significant difference between moderate and poor treatment outcome groups at same timepoint

Periodontitis subjects (n = 8) who only contributed to baseline were not assigned to a TxO group.

%BOP – percentage of sites exhibiting bleeding on probing, mm – millimetres, PD – probing depth,  
SD – standard deviation, TxO – treatment outcome

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**Table 2. Correlations between cell phenotypes and periodontal parameters.**

Cell phenotypes	Health		Periodontitis							
	Mean PD	%BOP	Baseline		3-months		6-months		12-months	
			Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP
<b>IFN-<math>\gamma</math> cells<sup>+</sup></b> (% of PBMC)	0.011	-0.007	-0.012	0.037	0.148	0.336	<b>0.410</b>	<b>0.571</b>	<b>0.533</b>	0.357
<b>CD4<sup>+</sup>TCR<math>\alpha\beta</math><sup>-</sup></b> (% IFN- $\gamma$ <sup>+</sup> cells)	0.246	-0.032	-0.104	-0.125	-0.108	<b>-0.349</b>	0.045	0.053	-0.022	0.025
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>+</sup></b> (% IFN- $\gamma$ <sup>+</sup> cells)	-0.006	-0.070	-0.232	<b>-0.401</b>	-0.051	<b>-0.445</b>	<b>-0.366</b>	-0.297	0.126	-0.231
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>-</sup></b> (% IFN- $\gamma$ <sup>+</sup> cells)	-0.125	0.053	<b>0.319</b>	<b>0.558</b>	0.054	<b>0.383</b>	0.126	0.151	0.053	0.157
<b>TCR<math>\alpha\beta</math><sup>-</sup>CD4<sup>-</sup></b> (% IFN- $\gamma$ <sup>+</sup> cells)	0.183	0.098	-0.105	-0.189	0.019	0.141	0.254	0.266	-0.068	0.212
<b>IL-4<sup>+</sup> cells</b> (% of PBMC)	-0.005	0.028	-0.034	0.012	<b>0.311</b>	0.140	-0.041	-0.088	0.054	0.195
<b>CD4<sup>+</sup>TCR<math>\alpha\beta</math><sup>-</sup></b> (% IL-4 <sup>+</sup> cells)	0.065	-0.030	0.187	0.019	-0.057	<b>-0.400</b>	-0.157	-0.115	-0.121	-0.136
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>+</sup></b> (% IL-4 <sup>+</sup> cells)	0.083	0.052	-0.016	-0.026	-0.194	-0.181	0.021	-0.085	0.051	-0.212
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>-</sup></b> (% IL-4 <sup>+</sup> cells)	-0.068	0.064	0.006	0.028	0.183	0.239	0.001	0.132	0.055	0.228
<b>TCR<math>\alpha\beta</math><sup>-</sup>CD4<sup>-</sup></b> (% IL-4 <sup>+</sup> cells)	0.099	-0.071	0.0035	-0.008	-0.048	-0.179	-0.203	-0.175	0.008	0.144
<b>IL-17<sup>+</sup> cells</b> (% of PBMC)	0.014	0.153	0.069	-0.119	0.214	0.271	<b>0.353</b>	<b>0.369</b>	<b>0.417</b>	0.156
<b>CD4<sup>+</sup>TCR<math>\alpha\beta</math><sup>-</sup></b> (% IL-17 <sup>+</sup> cells)	0.026	-0.058	-0.107	-0.043	-0.227	<b>-0.367</b>	-0.158	-0.078	-0.080	0.062
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>+</sup></b> (% IL-17 <sup>+</sup> cells)	-0.025	-0.118	0.074	-0.077	0.147	0.331	0.244	0.300	0.017	-0.174
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>-</sup></b> (% IL-17 <sup>+</sup> cells)	0.067	0.223	0.013	0.080	-0.133	-0.264	-0.206	-0.229	0.015	0.206
<b>TCR<math>\alpha\beta</math><sup>-</sup>CD4<sup>-</sup></b> (% IL-17 <sup>+</sup> cells)	0.123	0.168	-0.064	0.004	-0.031	-0.108	-0.341	<b>-0.440</b>	-0.017	0.184
<b>Foxp3<sup>+</sup> cells</b> (% of PBMC)	-0.054	-0.068	-0.022	-0.127	0.183	0.073	0.071	<b>0.372</b>	-0.037	0.120
<b>CD4<sup>+</sup>TCR<math>\alpha\beta</math><sup>-</sup></b> (% Foxp3 <sup>+</sup> cells)	-0.027	-0.105	0.043	0.031	-0.272	<b>-0.401</b>	-0.056	-0.036	-0.024	0.067
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>+</sup></b> (% Foxp3 <sup>+</sup> cells)	-0.220	-0.005	-0.147	-0.278	0.059	0.332	0.262	0.227	-0.057	-0.140
<b>TCR<math>\alpha\beta</math><sup>+</sup>CD4<sup>-</sup></b> (% Foxp3 <sup>+</sup> cells)	<b>0.383</b>	0.159	0.094	0.215	-0.031	-0.262	-0.267	-0.030	0.049	0.093
<b>TCR<math>\alpha\beta</math><sup>-</sup>CD4<sup>-</sup></b> (% Foxp3 <sup>+</sup> cells)	0.010	-0.025	-0.027	0.102	-0.017	-0.184	-0.114	-0.117	-0.011	0.047

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(% Foxp3<sup>+</sup> cells)

<b>IFN<math>\gamma</math><sup>+</sup>IL-4<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	-0.024	0.033	0.103	0.275	-0.002	0.001	0.072	-0.017	-0.083	0.152
<b>IFN<math>\gamma</math><sup>+</sup>Foxp3<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	-0.109	-0.282	0.018	0.160	0.111	0.120	0.153	0.223	-0.316	-0.224
<b>IL-4<sup>+</sup>Foxp3<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	-0.248	-0.182	0.129	<b>0.379</b>	-0.104	-0.152	0.062	0.161	-0.161	-0.063
<b>IL-4<sup>+</sup>IL-17<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	0.098	0.326	-0.117	-0.048	0.080	0.066	-0.050	-0.169	-0.066	0.104
<b>IFN<math>\gamma</math><sup>+</sup>IL-17<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	0.179	0.170	-0.125	-0.298	0.009	-0.064	0.126	0.224	0.253	0.027
<b>Foxp3<sup>+</sup>IL-17<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	-0.054	-0.320	-0.061	0.237	0.112	0.049	-0.089	0.309	-0.031	0.216
<b>Foxp3<sup>+</sup>CD25<sup>+</sup></b> (% TCR $\alpha\beta$ <sup>+</sup> CD4 <sup>+</sup> cells)	0.015	0.009	0.081	0.268	-0.037	0.112	0.111	0.050	0.042	0.075

Spearman's correlation coefficients ( $\rho$ ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-17<sup>+</sup> and Foxp3<sup>+</sup> cells divided based on CD4<sup>+</sup>TCR $\alpha\beta$ <sup>-</sup>, TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>, TCR $\alpha\beta$ <sup>+</sup>CD4<sup>-</sup>, TCR $\alpha\beta$ <sup>-</sup>CD4<sup>-</sup> expression as well as Foxp3<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup> and IL-17<sup>+</sup> double positive TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> cells in health and at baseline, 3-, 6- and 12-months in periodontitis. Significant correlations ( $p \leq 0.05$ ) are represented in bold.

**Table 3. Correlations between cytokine production in PBMC-supernatant upon various stimulation methods and periodontal parameters.**

Cytokines	Health		Periodontitis							
			Baseline		3-months		6-months		12-months	
	Mean	%BOP	Mean	%BOP	Mean	%BOP	Mean	%BOP	Mean	%BOP
	PD		PD	PD	PD	PD	PD	PD	PD	PD
IFN- $\gamma$ - cells alone	0.222	0.081	0.034	-0.100	0.302	0.305	<b>0.542</b>	<b>0.493</b>	-0.116	-0.216
IFN- $\gamma$ - <i>P. gingivalis</i> stimulation	0.021	-0.010	0.106	0.158	-0.004	0.302	0.305	<b>0.442</b>	0.030	-0.115
IFN- $\gamma$ - ConA stimulation	0.053	-0.141	-0.090	-0.075	-0.297	-0.284	-0.106	0.183	0.192	0.191
IL- 4 - cells alone	0.124	-0.141	-0.062	0.080	-0.181	-0.243	0.027	-0.144	<b>-0.356</b>	<b>-0.412</b>
IL- 4 - <i>P. gingivalis</i> stimulation	0.225	-0.079	-0.104	0.077	-0.253	-0.171	0.091	-0.082	-0.367	<b>-0.451</b>
IL- 4 - ConA stimulation	0.024	<b>-0.339</b>	-0.035	-0.105	<b>0.345</b>	0.290	0.499	0.167	0.119	0.041
IL-17 - cells alone	0.077	-0.001	-0.041	-0.290	<b>0.316</b>	0.206	0.171	0.135	-0.149	-0.328
IL-17 - <i>P. gingivalis</i> stimulation	0.031	0.043	0.272	0.099	0.177	<b>0.425</b>	<b>0.578</b>	<b>0.490</b>	-0.109	-0.292
IL-17 - ConA stimulation	-0.128	-0.103	<b>0.329</b>	0.021	0.296	<b>0.499</b>	<b>0.441</b>	0.337	0.287	0.266
IL- 10 - cells alone	-0.066	0.272	0.223	0.122	0.244	-0.142	-0.124	-0.047	-0.065	-0.210
IL- 10 - <i>P. gingivalis</i> stimulation	-0.112	-0.171	0.065	0.194	0.088	-0.062	0.004	-0.101	0.338	<b>0.429</b>
IL- 10 - ConA stimulation	-0.052	-0.140	0.131	-0.077	0.176	0.326	0.311	0.364	-0.033	-0.198

Spearman's correlation coefficients ( $\rho$ ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and IFN- $\gamma$ , IL-4, IL-17 and IL-10 cytokine expression from PBMCs without any stimulation and upon *P. gingivalis* and Concanavalin A (ConA) stimulation in health and at baseline, 3-, 6- and 12- months in periodontitis. Significant correlations ( $p \leq 0.05$ ) are represented in bold.

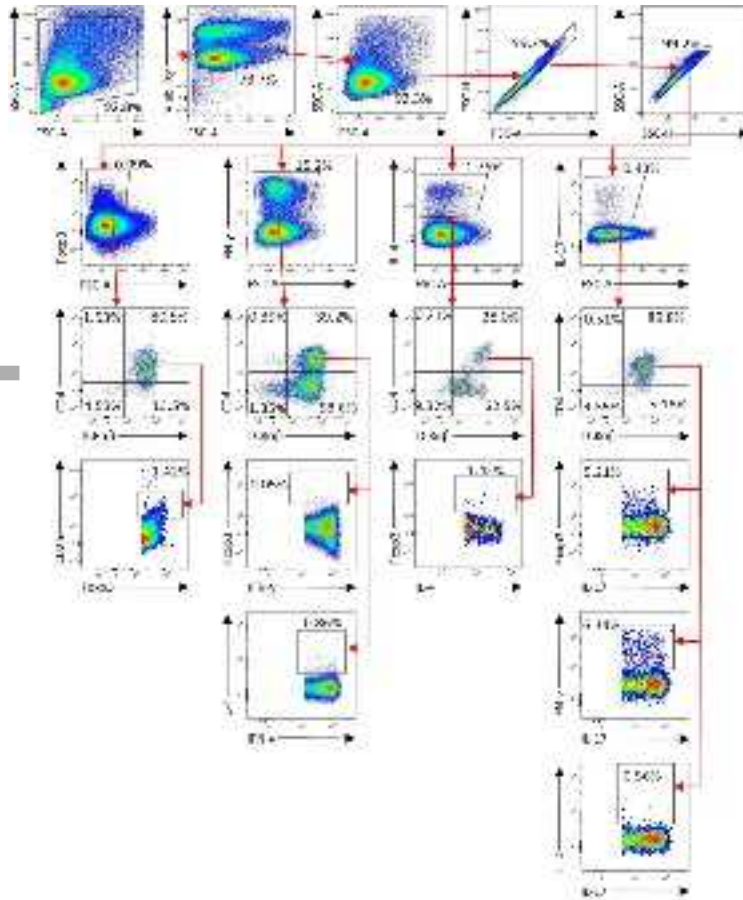


Fig. 1  
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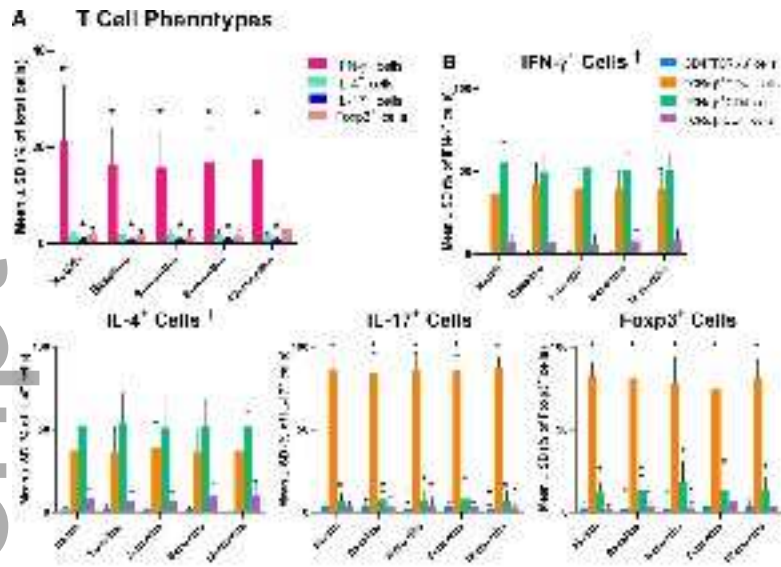


Fig. 2

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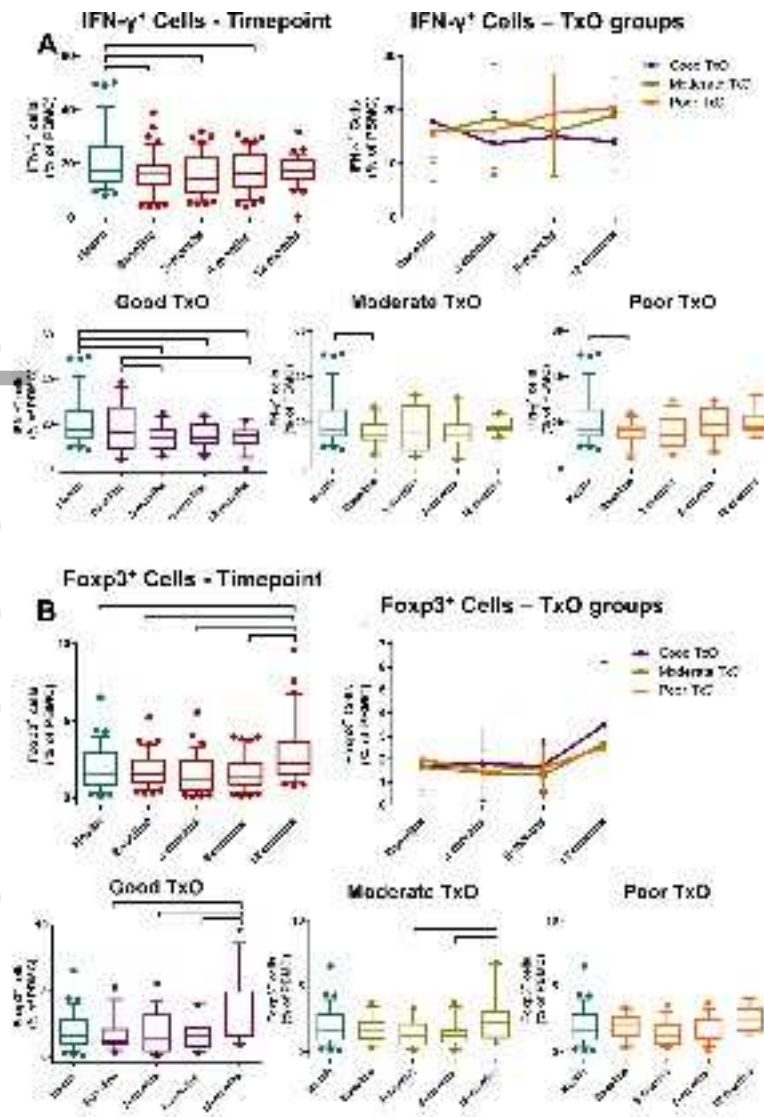


Fig. 3

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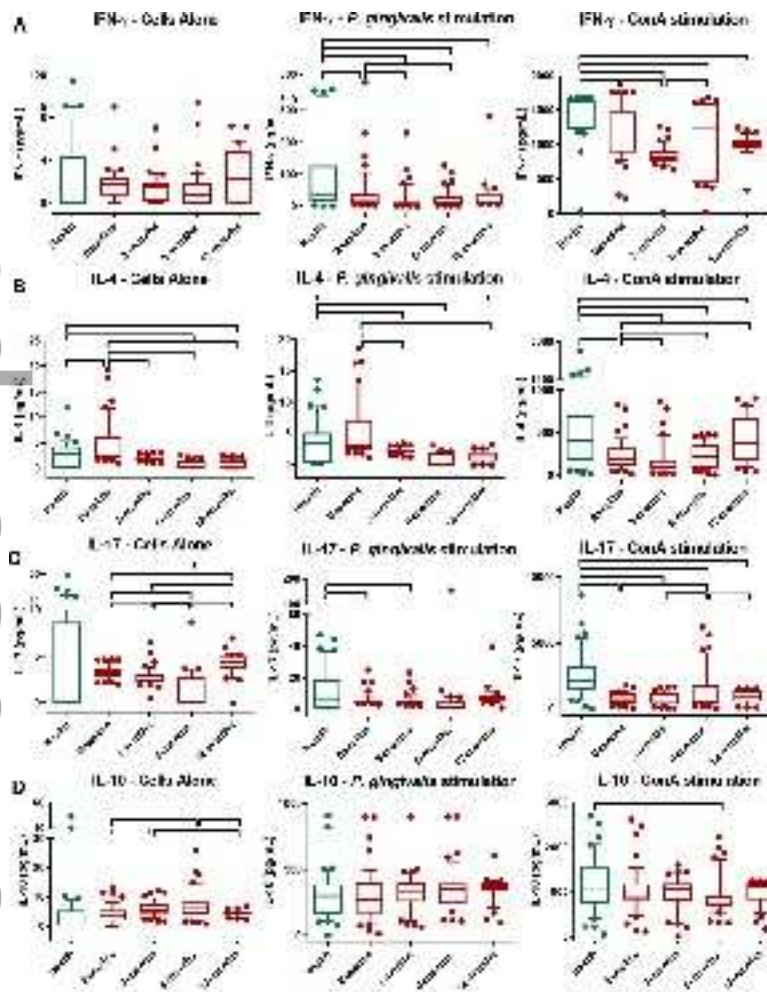


Fig. 4

jcpe\_13389\_f4.tif