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Peripheral memory T-cell profile is modified in patients undergoing periodontal management

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RUNNING TITLE

Memory T-cells & Periodontitis

CONFLICT OF INTEREST AND FUNDING INFORMATION

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ABSTRACT

Aims: T-cells are known to have a role in periodontitis, however, the effect of periodontal therapy on peripheral memory T-cells is unclear. This study evaluated variation in peripheral memory T-cells and red complex bacteria in sub-gingival plaque in patients undergoing periodontal management.

Methods: Peripheral blood mononuclear cells and sub-gingival plaque were collected from 54 periodontitis patients at baseline, 3-, 6- and 12-months post-therapy and 40 healthy controls. Periodontitis patients were divided into treatment outcome (TxO) groups based on prevalence of sites with probing depth ≥5mm as good (<10% of sites), moderate (10-20%) or poor (>20%) at study conclusion. Naïve (T_N – $CCR7^+CD45RA^+$), central memory (T_{CM} – $CCR7^+CD45RA^-$), effector memory (T_{EM} – $CCR7^-CD45RA^-$) and effector memory T-cells re-expressing CD45RA (T_{EMRA} – $CCR7^-CD45RA^+$) were phenotyped using flow cytometry in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ T-cells and red complex bacteria were quantified using qPCR.

Results: At baseline, periodontitis subjects had significantly greater mean probing depths and *P. gingivalis* proportions, lower T_N but higher $CD4^+$ T_{CM}, $CD8^+$ T_{CM}, $CD4^+CD8^+$ T_{EM}, and $CD4^-CD8^-$ T_{EM} cell proportions compared to health. Periodontal therapy decreased mean probing depths, *P. gingivalis* proportions, T_{EM} and $CD4^+$ and $CD8^+$ T_{CM} cells, but increased T_N and $CD4^+$ and $CD8^+$ T_{EMRA} cells. The T-cell profile in the good TxO group showed therapy-related changes in $CD4^+$ T_{EM}, and $CD8^+$ T_N and T_{EM} cells, whereas, no changes were observed in the poor TxO group.

Conclusion: Management and the reduction in red complex bacteria were associated with changes in peripheral memory T-cells in periodontitis.

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Keywords: T-lymphocytes, immunologic memory, periodontitis, disease management, host-pathogen interactions

CLINICAL RELEVANCE

Scientific rationale: Immunological memory an important feature of adaptive immunity and the peripheral memory T-cell profile in periodontitis is not well characterised in response to periodontal management. This study examined the effect of periodontal therapy including scaling and root debridement and, if indicated, additional access flap surgery on four memory T-cell phenotypes in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cells over one year in periodontitis patients.

Principal findings: Naïve T-cells increased, whereas effector memory and $CD4^+$ and $CD8^+$ central memory T-cells decreased with therapy. $CD4^+$ effector memory and $CD8^+$ naïve and effector memory T-cells showed changes with therapy in the good treatment outcome group but not in the poor.

Practical implications: Our findings suggest that conventional periodontal management does have an effect on peripheral memory T-cells in periodontitis. Moreover, this effect is only seen in the good treatment outcome group in some memory cells subsets suggesting that those who have more sites with deeper probing depths at the end of management do not show an underlying change in immune response. Understanding the peripheral memory T-cell response in the pathogenesis of periodontitis may lead to better disease management and identification of novel T-cell based diagnostics and therapies that clinicians can use to monitor management and provide on-going strategies to improve patients' periodontal health.

INTRODUCTION

Periodontitis is a multifactorial disease associated with a dysbiotic biofilm and a chronic dysregulated immune-inflammatory response. T-cells are a major subset of adaptive immunity that play a central role in periodontitis (Fujihashi et al. 1996). One distinguishing feature of adaptive immunity is immunological memory which enables a rapid and efficient response to previously encountered pathogens or antigens (Farber et al. 2016).

The surface expression of cellular markers restricts migration through specific tissues and facilitates functional phenotyping of memory cells. Based on the expression of CCR7 and CD45RA, memory T-cells can be phenotyped as naïve (T_N – $CCR7^+CD45RA^+$), effector memory T-cells re-expressing

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CD45RA (T_{EMRA} – $CCR7^{-}CD45RA^{+}$), central memory (T_{CM} – $CCR7^{+}CD45RA^{-}$) and effector memory (T_{EM} – $CCR7^{-}CD45RA^{-}$) T-cells (Sallusto et al. 1999). Antigen-inexperienced T_N cells recirculate between secondary lymphoid organs and blood. They are long-lived but display a restricted cytokine profile. Upon activation, T_N cells undergo expansion and differentiate into effector cells with potent pathogen-eliminating functions. T_{CM} cells preferentially home to secondary lymphoid organs. They are characterized by low effector function, but have high proliferative capacity and reduced need for co-stimulation compared to T_N cells. Upon antigenic stimulation, T_{CM} cells are converted to T_{EM} cells which preferentially home to peripheral sites and display rapid effector function, but have poor proliferative capacity. Most T_{EM} cells are $CD45RA^{-}$, however, a subset re-express CD45RA and are termed T_{EMRA} cells. T_{EMRA} cells are more terminally differentiated compared to T_{CM} and T_{EM} cells (Harari et al. 2004) with poor proliferative potential, but high cytotoxicity and sensitivity to apoptosis (Geginat et al. 2003; Hamann et al. 1997).

Memory T-cell profiles in peripheral blood in response to therapy are not well established in periodontitis. Previously, $CD4^{+}CD45RA^{+}$ naïve (Afar et al. 1992) and $CD4^{+}CD45RO^{+}$ antigen-experienced memory cells (Afar et al. 1992; Aoyagi et al. 1995) were reported to be higher in periodontitis, whereas others reported that neither $CD4^{+}CD45RA^{+}$ naïve (Seymour et al. 1997) nor $CD4^{+}CD45RO^{+}$ memory cells (Cheng et al. 2018) were significantly different in chronic or aggressive periodontitis compared to health. Longitudinal studies have reported significantly increased $CD4^{+}CD45RA^{+}$ naïve cells post-treatment (Kimura et al. 1991).

The immune-inflammatory response in periodontitis may be subverted by keystone pathogens and over-activated by pathobionts in a synergistic polymicrobial community. *Porphyromonas gingivalis* represents a keystone pathogen in periodontitis causing microbial and immune dysbiosis through its virulence factors and ability to subvert the host's immune response (Hajishengallis et al. 2011). *P. gingivalis* is often closely localised with *Treponema denticola* and *Tannerella forsythia*, together termed the red complex bacteria, which have been reported to be strongly associated with clinical parameters of periodontitis (Socransky et al. 1998).

There is mounting evidence that local microbial dysbiosis and ensuing immune response in periodontitis may have distant systemic effects (Chapple and Genco 2013; Tonetti and Van Dyke 2013). This may be meditated by the leakage of microbial and inflammatory products into systemic circulation via the ulcerated and highly vascularised oral epithelial barrier in diseased states (Konkel et al. 2019). The effect of periodontal management and memory T-cells in peripheral blood needs further investigation. The primary aim of this study was to evaluate management-related variation

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of peripheral T_N, T_{EMRA}, T_{CM} and T_{EM} cells in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ populations and red complex bacteria proportions in sub-gingival plaque. The secondary aim was to investigate whether there were differences based on response to management. An understanding of the memory T-cell response to therapy could provide insight into the underlying host response in periodontitis and will aid in better disease management strategies.

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MATERIALS AND METHODS

Study protocol

The Human Ethics Sub-Committee, The University of Melbourne (1339812.3) and 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) and were calculated for a parent study on T helper cells which considered a significant decrease of 2.25% in $IL\text{-}17^+$ cells (Zhao et al. 2011). At α=0.05% and power of 0.85%, 38 patients were required in periodontitis arm which was rounded up to 40. To account for attrition rate of 20%, 50 subjects were required in the periodontitis group. Informed written consent was gained from each participant at study commencement. Data collection took place between 2015-2017. The study design and recruitment process are displayed in appendix figure 1 and conforms to the STROBE guidelines.

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. The inclusion criteria for periodontitis subjects were at least two non-adjacent sites per quadrant exhibiting probing depths (PD) ≥5mm, excluding the third molars (Darby et al. 2001). Subjects were >21 years, had a minimum of 16 teeth (excluding the third molars) and were systemically healthy with no periodontal therapy or antibiotic use within the preceding 6 months (appendix tables 1, 2). Exclusion criteria included pregnancy or lactation and medical conditions affecting the progression of periodontitis (e.g. diabetes) or requiring pre-medication prior to management.

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

The periodontal parameters, mean PD and %BOP, sub-gingival plaque and blood were collected from periodontitis subjects at baseline before therapy and at 3-, 6- and 12-months post-therapy. PD and

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BOP were recorded at six sites around each tooth using William's probe (Hu-Friedy Mfg. Co., Chicago, USA). PD was rounded up 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 sulcus and recorded as present or absent up to 30 seconds after probing. Smoking status was defined as current smoker or non-smoker at study commencement.

Periodontal management involved patient education and oral hygiene instructions (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 therapy evaluated, patients given OHI as needed, and residual pockets re-debrided or access flap surgery performed (*n*=5) as necessary. The management goal was PD <4mm 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 ≥5mm.

Memory T-cell phenotyping

Peripheral blood was collected in potassium-EDTA tubes (BD Biosciences, NSW, Australia) and peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, NSW, Australia) following manufacture's instruction. Fresh PBMCs ($1x10^6$ cells/100µL) were incubated for 30 minutes at 4^0C with pre-diluted antibodies against CD45-APC (1µL, clone – HI30), CD4-APCCy7 (1µL, clone – RPA-T4), CD8-AF488 (5µL, clone – RPA-T8), TCRαβ-BV785 (2µL, clone – T10B9.1A-31), CD45RA-PECy7 (0.5µL, clone – L48) and CCR7-PE (10µL, clone – 150503). Antibodies were purchased from BD Bioscience and volumes used were optimised based on manufacture's recommendation. After incubation, PBMCs were washed twice with 2mL FACS wash (2% w/v BSA, 2mM EDTA, 0.02% v/v sodium azide in PBS) by centrifugation at 800RCF, 5 minutes, 4^0C and resuspended in 200µL FACS wash for acquisition on BD LSRFortessa™ X-20. Unstained and single antibody-stained PBMCs were used as controls for each sample. Data analysis was performed using FlowJo (v10, Tree Star Inc., OR, USA).

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Sub-gingival plaque collection and quantification of red complex bacteria

Sub-gingival plaque from the same site was used for analysis in the periodontitis subject. Site selection criterion for the periodontitis subject was a tooth with PD ≥5mm and for the control subject, a tooth with PD ≤3mm. The exclusion criteria for both groups were mobility ≥grade 2, pulp or periapical diseases and teeth subject to occlusal trauma or inadequate prosthetic restorations.

For plaque collection, the respective tooth surface was isolated with cotton rolls, gently air-dried and supra-gingival plaque removed with a sterile curette. A sterile Gracey curette (Hu-Friedy) was inserted as deep as possible into the gingival sulcus without applying pressure to the tooth surface. The plaque sample was collected with a single vertical stroke and immediately placed into 300µL TE buffer (10mM tris(hyrdroxymethy)-methylamine chloride, 1mM EDTA (pH 7.4)) and stored at -80^{0}C until processing.

P. gingivalis (F – AGGCAGCTTGCCATACTGCG; R – ACTGTTAGCAACTACCGATGT), *T. denticola* (F – TAATACCGAATGTGCTCATTTACAT; R – TCAAAGAAGCATTCCCTCTTCTTCTTA), *T. forsythia* (F – AAAACAGGGGTTCCGCATGG; R – TTCACCGCGGACTTAACAGC) and total bacteria (F – GATTAGATACCC TGGTAGTCCAC; R – CCCGGGAACGTATTCACCG) were quantified by qPCR (appendix material and methods). Values are reported as a proportion of total bacteria.

Statistical analysis

Independent samples *t*-tests with unequal variances were used for comparisons between health and periodontitis at each timepoint. Linear mixed models (LMMs) were used to determine changes in the mean of the outcome variable over timepoint and TxO group in periodontitis. The base model for variations 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. Pairwise comparisons of estimated marginal means were used to assess significant differences.

Independent samples *t*-test with unequal variance were used to assess differences between smokers and non-smokers at each timepoint. The interaction of smoking and timepoint, and smoking and TxO group were also added as fixed effects to the base LMMs.

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Paired samples *t*-tests were used for comparison of red complex bacteria and memory T-cells within each timepoint. Spearman's correlations were used for associations with the periodontal parameters, memory T-cells and red complex bacteria at each timepoint.

The significance level was set at $p \leq 0.05$ for all variables. All statistical analyses were performed in SPSS (v23, IBM Corp., NY, USA) and graphs prepared using GraphPad Prism (v5, GraphPad Software, CA, USA).

RESULTS

Periodontal parameters

The cohort demographics and periodontal parameter are displayed in table 1. No significant differences were observed in gender, age and smoking status between health and baseline, however, the healthy cohort had significantly more teeth. Of the 54 subjects at baseline, 6 achieved the management goal of no PD ≥4mm and %BOP >30%.

Mean PD and %BOP were significantly higher in periodontitis compared to health, and decreased significantly at subsequent timepoints compared to baseline.

Periodontitis subjects (*n*=8) who only contributed to baseline were not assigned to a TxO group. Mean PD was significantly different at baseline and 12-months between the three TxO groups, with the poor TxO group showing higher mean PD followed by moderate and good groups. %BOP was not significantly different between the TxO groups at baseline, however, %BOP was significantly higher in the poor compared to the good TxO group post-therapy.

Smokers displayed significantly less %BOP (mean difference (*d*) = 22.35%) at baseline and deeper mean PD (*d*=0.40mm) at 3-months compared to non-smokers. At baseline in the moderate TxO group, mean PD (*d*=0.75mm) and %BOP (*d*=43.72%) were significantly lower in smokers compared to non-smokers. The interaction between smoking and timepoint was a predictor for %BOP at baseline at *p*=0.056 (estimate of fixed effect (*b*)=29.03, standard error (SE)=2.91), where smokers had 29.03% less %BOP compared to non-smokers. The interaction between smoking and TxO group was also a significant predictor for %BOP in the good (*b*=-32.54, SE=12.99) and moderate (*b*=-33.07, SE=10.59) TxO groups where BOP increased as the TxO group worsened from good to moderate and smokers had significantly less %BOP in the good and moderate TxO groups compared to non-smokers.

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Memory T-cells

The gating strategy to phenotype memory T-cells is displayed in figure 1. T-cell populations and their memory subsets in relation to each other are displayed in appendix figure 2. $CD4^+CD8^+$ memory cells exhibited a similar profile to $CD4^+$ cells which had a small T_{EMRA} and large T_{CM} cell proportions, whereas $CD4^-CD8^-$ memory cells were similar to $CD8^+$ cells which had a large T_{EMRA} and small T_{CM} cell proportions.

$CD4^+$ T_N, $CD4^+CD8^+$ T_N, $CD4^-CD8^-$ T_N and $CD4^+CD8^+$ T_{EMRA} cells were lower, whereas total $CD4^+CD8^+$, $CD4^+$ T_{CM}, $CD8^+$ T_{CM}, $CD4^+CD8^+$ T_{EM} and $CD4^-CD8^-$ T_{EM} cells were higher at baseline compared to health (figure 2). Overall, periodontal therapy resulted in an increase in T_N cells in all four subsets and T_{EMRA} cells in $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ cell subsets, whereas T_{EM} cells in all four subsets, and $CD4^+$ and $CD8^+$ T_{CM} cells decreased.

Memory T-cells separated based on TxO groups are displayed in figure 3 and appendix figures 3–6. $CD8^+$ T_N cells were significantly increased post-therapy in the good group, but showed no therapy-related changes in the poor TxO group. Likewise, $CD4^+$ and $CD8^+$ T_{EM} cells displayed a significant therapy-related reduction in the good group, but no differences were observed in the poor TxO group.

Smokers had significantly more $CD8^+$ T_N (*d*=12.32%) and fewer $CD8^+$ T_{EMRA} (*d*=11.61%) cells at baseline, and more $CD4^+$ T_N (*d*=11.90%) and fewer $CD4^+$ T_{EMRA} (*d*=0.65%), $CD4^+$ T_{CM} (*d*=6.05%), $CD4^+$ T_{EM} (*d*=5.14%) and $CD4^+CD8^+$ T_{CM} (*d*=9.67%) cells at 6-months compared to non-smokers. The interaction between smoking and timepoint was a significant predictor for $CD8^+$ T_{EMRA} cells at 3- (*b*=8.00, SE=4.00) and 6-months (*b*=10.59, SE=4.03) where they were significantly higher in smokers compared to non-smokers.

Red complex bacteria

At baseline, *P. gingivalis* proportions were significantly higher than *T. denticola* and *T. forsythia* (appendix figure 7). *P. gingivalis* and *T. denticola* were significantly higher at baseline compared to health and decreased post-therapy (table 2). All three bacteria were recovered from a significantly greater number of sites at all timepoints in periodontitis compared to health, however, no difference were observed in bacterial recovery in the periodontitis group post-therapy.

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When separated into TxO groups (figure 4), *P. gingivalis* was significantly decreased at 3- and 12-months post-therapy compared to baseline within all three TxO groups, however, only the poor TxO group had a significantly higher proportion of *P. gingivalis* at baseline compared to health.

Associations between periodontal parameters, memory T-cells and red complex bacteria

Periodontal parameters were correlated with each other (table 1), memory T-cells (table 3) and red complex bacteria (table 2). Memory T-cells were also correlated with red complex bacteria (appendix table 6).

When %BOP was added to the base LMM as a fixed effect, it was a significant predictor for mean PD (*b*=0.01, SE= 0.00(1)) with both timepoint and TxO group. Likewise, mean PD was a significant predictor for %BOP with timepoint (*b*=29.03, SE=2.91) and TxO group (*b*=30.74, SE=3.19).

When mean PD and %BOP were added to the base LMMs, both mean PD and %BOP were significant predictors for $CD8^+$ T_{CM} and $CD8^+$ T_{EM} cells in the model for timepoint. For a 1mm increase in mean PD, $CD8^+$ T_{CM} cells (*b*=2.65, SE=1.25) increased by 2.65% and $CD8^+$ T_{EM} cells (*b*=-5.66, SE=2.82) decreased by 5.66%. Likewise, for a 1% increase in %BOP, $CD8^+$ T_{CM} cells (*b*=-0.08, SE=0.03) decreased by 0.08% and $CD8^+$ T_{EM} cells (*b*=0.21, SE=0.06) increased by 0.21%. Upon separation into TxO groups, mean PD was a significant predictor for $CD8^+$ T_N (*b*=-0.15, SE=0.07) and $CD8^+$ T_{EM} (*b*=-9.16, SE=3.09) cells, and %BOP for $CD8^+$ T_{CM} (*b*=-0.08, SE=0.03) and $CD8^+$ T_{EM} (*b*=0.22, SE=0.06) cells. Mean PD was a significant predictor for *T. forsythia* with both timepoint (*b*=0.29, SE=0.14) and TxO (*b*=0.40, SE=0.19).

When all red complex bacteria were included as fixed effects in the base LMMs, *P. gingivalis* was a significant predictor for $CD8^+$ T_{CM} (*b*=-0.05, SE=0.03) and $CD4^-CD8^-$ T_{EM} (*b*=-0.17, SE=0.08) cells for timepoint.

DISCUSSION

The main goal of periodontal therapy is to establish clinically healthy periodontal conditions. Given that these are manifested as shallow pockets that do not bleed when probed, mean PD and %BOP were used as clinical parameters of periodontitis in this study. Periodontal therapy is an effective

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means for disease management. However, response to treatment varies considerably between patients and treatment sites making the assessment of overall response in a patient methodologically problematic (Badersten et al. 1981; 1984; Lindhe et al. 1984; Pihlstrom et al. 1983). Thus, in addition to assessing mean PD and %BOP as a measure of response to management, this study also used the prevalence of sites that exhibited PD ≥5mm as a more clinically relevant measure of assessing overall treatment outcome. This was based on a previous study that described poor responders as having >10% of sites with PD >4mm and %BOP ≥20% after one year of active treatment (Holmlund et al. 2017). However, unlike the study by Holmlund *et al.*, %BOP was not used to define TxO groups in this study as mean %BOP at 12-months exceeded ≥20% and %BOP was significantly associated with mean PD with both Spearman's correlations and LMMs. Moreover, a moderate TxO group with 10-20% of sites with PD ≥5mm was also included as it was felt that this group was masking the effects of the poor TxO group. Accordingly, mean PD was significantly different between the three TxO groups at baseline and 12-months. Previous studies have also reported other ways of evaluating response to periodontal therapy (Badersten et al. 1985; Haffajee et al. 1997; Hughes et al. 2006; Sexton et al. 2011).

The proportion of T_N cells in all four T-cells subsets was lower at baseline compared to health (although not significant in $CD8^+$ cells, $CD8^+$ T_N cells were 6.8% lower at baseline). This suggests that within circulating PBMCs, there are significantly more T-cells of all subsets that are of a memory phenotype in periodontitis patients compared to healthy controls. At 12-months post-therapy, T_N cells were significantly increased in all four T-cell populations. This increase in T_N cells was also reflected when separated into TxO groups, where the greatest increase from baseline to 12-months was evident in the good TxO group for $CD4^+$ and $CD8^+$ cells. This may be due to active differentiation of T_N cells into effector and/or memory cells by periodontal antigens before therapy at baseline. Periodontal therapy reduces bacterial load and this may decrease active differentiation of T_N cells restoring levels to that seen in health. This is coincident with the findings that the greatest reduction in mean PD and *P. gingivalis* proportions occurred between baseline and 3-months and T_N cell levels were restored to those similar in health in $CD4^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cells at 3-months post-therapy. However, as this study did not evaluate the specificity of T-cells these results should be interpreted with caution and future studies investigating periodontopathogen-specific memory T-cells are needed.

T_{CM} and T_{EM} cells have complimentary functions where T_{EM} cells homing to peripheral tissues mount a rapid initial response to check the replication and spread of invading pathogens, whereas T_{CM} cells mount a robust response and can undergo rapid proliferation and differentiation (Sallusto et al.

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2004). Thus, it follows that these rapidly proliferating $CD4^+$ and $CD8^+$ T_{CM} cells were significantly higher at baseline possibly in response to the systemic dissemination of periodontal antigens. The different migration capabilities of T_{CM} and T_{EM} cells may also account for this differences where $CD4^+$ and $CD8^+$ T_{CM} cells and not $CD4^+$ and $CD8^+$ T_{EM} cell were higher at baseline. T_{EM} cells may be increased at barrier sites, and indeed a study in healthy gingival tissues found higher $CD8^+$ T_{EM} cells compared to $CD8^+$ T_{CM} cells (Dutzan et al. 2016). The functional differences in $CD4^+$ T helper cells, which orchestrate the immune response, and $CD8^+$ T cytotoxic cells, which have more direct effector functions, may also affect T_{CM} and T_{EM} cell distribution in peripheral blood. Accordingly, there were higher proportions of $CD4^+$ T_{CM} and lower T_{EM} cells, and higher $CD8^+$ T_{EMRA} and T_{EM} and lower T_{CM} cells in peripheral circulation. In this study, $CD8^+$ T_{CM} cells were negatively associated with *P. gingivalis,* and $CD8^+$ cells may play an important adjuvant role to that of the dominant $CD4^+$ response. Interestingly, a small proportion of $CD4^+CD8^+$ and $CD4^-CD8^-$ T_{EM} cells were significantly higher at baseline and decreased post-therapy and may represent a population with more immediate effector function (Weiss et al. 1998). In contrast, a study found similar proportions of $CD4^+$ and $CD8^+$ T_{CM} and T_{EM} cells in gingival tissues in periodontitis and health (Mahanonda et al. 2018). Moreover, peripheral blood only contains 2-3% of total T-cell pool (Di Rosa and Pabst 2005), and may not be representative of memory phenotypes found at sites of disease activity. This preferential localization is to be expected, as it is more likely that microbes are encountered at barrier sites. In addition, as this study phenotyped memory T-cells based on the expression receptors which restricts entry into specific tissues such as the secondary lymphoid organs (CCR7), this may not necessarily translate to periodontal tissue infiltration.

Bacterial quantification in this study found that while the prevalence of red complex bacteria generally decreased with therapy, the detection rate remained unchanged. High detection rates and persistence following management is in agreement with previous studies (Ehmke et al. 2005) and indicates that the mere presence of virulent bacteria may not be indicative of a pathological clinical situation. This is in line with the currently held concepts of “polymicrobial synergy and dysbiosis” (Hajishengallis et al. 2012) and the newer “inflammation-mediated polymicrobial-emergence and dysbiotic-exacerbation (IMPEDE)” (Van Dyke et al. 2020) in the pathogenesis of periodontitis where the shift to a dysbiotic microflora occurs largely due to excessive and non-resolving inflammation leading to pocket formation and favourable bacterial growth conditions. The trend for decreasing proportions of *P. gingivalis* following therapy was consistent over time, except for an increase at 6-months. This was due to high proportions measured in four patients (83.60% – moderate TxO, 44.02% – poor TxO, 31.46% – good TxO and 24.67% – poor TxO), which increased the group mean with wide standard deviations. Separating periodontitis based on clinical responses showed higher *P.*

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gingivalis proportions at baseline only in the poor TxO group. The poor TxO group displayed higher mean PD at baseline and deeper pockets may serve as a more conducive environment for effective bacterial growth. Deeper pockets pose difficulties with gaining access and thus are harder to manage efficaciously. Previous studies have observed that pockets with deeper PD have increased plaque and calculus still remaining after instrumentation (Adriaens and Adriaens 2004).

Smoking did not have an effect on red complex bacteria in this study. However, in general, smokers had higher T_N and lower T_{EM} and T_{EMRA} cell proportions compared to non-smokers. Higher $CD4^+CD45RA^+$ naïve and lower $CD4^+CD45RO^+$ memory cells in heavy smokers compared to non-smokers were reported in a previous study in healthy adults (Tanigawa et al. 1998). The reason for this unknown, but smoking may impair the functional potential of $CD4^+$ cells leading to decreased generation of effector memory populations (Tejero et al. 2018) and increased persistence of naïve cells (Tanigawa et al. 1998). In contrast, other studies have observed higher $CD4^+CD45RA^+$ naïve and $CD4^+CD45RO^+$ memory cells (Schaberg et al. 1997; Vardavas et al. 2010) or no differences in memory T-cells in smokers compared to non-smokers (Chavance et al. 1993). Due to the limited sample size in this study, the effects of smoking on memory T-cells should be interpreted cautiously and warrants further investigation.

Previous literature has used other cell surface marker expression such as CD28, CD29, CD31, CD44, CD45RO, CD62L and CD69 to phenotype memory T-cells (van den Broek et al. 2018). It is unclear at this stage if the combination of markers used identify naïve and memory cell populations are consistent between studies. The roles of T_{CM} and T_{EM} cells may be fluid and their precise effector roles need investigation. Some research has been undertaken into skin and gut homing receptors for memory T-cells (Mora and von Andrian 2006), however, gingival homing receptors are yet to be elucidated. The longitudinal nature of the study partly accounts for temporal biological variation, such as underlying chronic medical conditions or medication use (appendix tables 1, 2), and provides information on the short and medium-term effects of periodontal therapy. However, while the sample size at baseline exceeded the number of subjects required to have sufficiently acceptable statistical power, there was a reduction in sample size (31.5%) at 12-months due to dropouts. Furthermore, separation into TxO groups essentially divided the sample size at each timepoint by one-third. This may increase type II error and thus, future studies with larger sample size acquired from multiple locations would increase the reliability of these results. T-cell memory is an evolving field and recently tissue-resident (Gebhardt et al. 2009), stem cell (Gattinoni et al. 2009), peripheral (Gerlach et al. 2016) and virtual (Marusina et al. 2017) memory T-cells have also been identified. Moreover, innate cells such as natural killer cells and macrophages are also thought to have

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immunological memory (Pradeu and Du Pasquier 2018). These different memory cell subsets may have distinct roles in chronic inflammation and further studies are required to elucidate their role in periodontitis.

To our knowledge, this is the first study that phenotypes longitudinal variation in peripheral T_N, T_{EMRA}, T_{CM} and T_{EM} cells in periodontitis based on CCR7 and CD45RA expression. This study showed that conventional periodontal therapy does impact peripheral memory T-cells, and in some cell subsets such as $CD8^+$ T_N and $CD4^+$ and $CD8^+$ T_{EM} cells this variation with treatment may be dependent on the response to treatment. Overall, these findings provide new insight into the changing phenotype of memory T-cells in response to therapy in periodontitis.

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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

Significant Spearman's correlations are represented in bold

Periodontitis subjects (*n* = 8, 14.8%) who only contributed to baseline were not assigned to a TxO group.

%BOP, percentage of sites with bleeding on probing; mm, millimetres; PD, probing depth; SD, standard deviation; TxO, treatment outcome; ρ, Spearman's correlation coefficient

Table 2. Variation in red complex bacteria and correlations with periodontal parameters by timepoint.

† significant difference to health

‡ significant difference to baseline

§ significant difference to 3-months post-treatment

Significant Spearman's correlations are represented in bold

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%BOP, percentage of sites with bleeding on probing; PD, probing depth; SD, standard deviation; ρ, Spearman's correlation coefficient

Table 3. Correlations between periodontal parameters and memory T-cells by timepoint.

Spearman's correlation coefficients (ρ) between the periodontal parameters, mean probing depth (PD) and percentage of sites with bleeding on probing (%BOP), and naïve (T_N), effector memory T-cells re-expressing CD45RA (T_{EMRA}), central memory (T_{CM}), effector memory (T_{EM}) T-cells in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cell subsets 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 memory T-cells.

Lymphocytes were identified based on forward scatter area (FSC-A) and side scatter area (SSC-A). The pan-lymphocyte marker CD45 was used to further isolate lymphocytes. Doublets were excluded using FSC-A and forward scatter height (FSC-H) followed by side scatter width (SSC-W) and SSC-A. T-cells were identified using $TCR\alpha\beta^+$, and were further divided into $CD4^+$, $CD8^+$, $CD4^+CD8^+$ double positive and $CD4^-CD8^-$ double negative cells. Subsequently, $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cells were identified as naïve T-cells (T_N – $CCR7^+CD45RA^+$), central memory T-cells (T_{CM} – $CCR7^+CD45RA^-$), effector memory T-cells re-expressing CD45RA (T_{EMRA} – $CCR7^-CD45RA^+$) and effector memory T-cells (T_{EM} – $CCR7^-CD45RA^-$).

Figure 2. T_N, T_{EMRA}, T_{CM} and T_{EM} cell subsets in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cell populations by timepoint.

$CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cells (as a percentage of $TCR\alpha\beta^+$ cells) **(A)** phenotyped into naïve (T_N – $CCR7^+CD45RA^+$), effector memory T-cells re-expressing CD45RA (T_{EMRA} – $CCR7^-CD45RA^+$), central memory (T_{CM} – $CCR7^+CD45RA^-$) and effector memory (T_{EM} – $CCR7^-CD45RA^-$) T-cells (as a percentage of $TCR\alpha\beta^+$ and $CD4^+$, $CD8^+$, $CD4^+CD8^+$ or $CD4^-CD8^-$ cells) **(B)** in health and periodontitis at baseline, 3-, 6- and 12-months. Box and whisker plots display the median, interquartile range and 10^{th} – 90^{th} percentile; ⌐ 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.

Figure 3. Treatment outcome groups for $CD4^+$ T_N, $CD4^+$ T_{EM}, $CD8^+$ T_N and $CD8^+$ T_{EM} cells.

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$CD4^+$ naïve (T_N) **(A)**, $CD4^+$ effector memory (T_{EM}) **(B)**, $CD8^+$ T_N **(C)** and $CD8^+$ T_{EM} **(D)** T-cells as a percentage of $TCR\alpha\beta^+$ and $CD4^+$ or $CD8^+$ cells 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^{th} – 90^{th} percentiles and show differences within the three TxO groups; ⌐¬ 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 and treatment outcome in periodontitis.

Figure 4. Treatment outcome groups for red complex bacteria.

P. gingivalis **(A)**, *T. denticola* **(B)** and *T. forsythia* **(C)** (as a percentage of total bacteria) 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^{th} – 90^{th} percentiles and show differences within the three TxO groups; ⌐¬ 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 and treatment outcome in periodontitis.

AUTHOR CONTRIBUTIONS

Nidhi Medara: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Funding acquisition, Writing - original draft. Jason C. Lenzo: Conceptualization, Methodology, Funding acquisition, Supervision, Writing - review & editing. Katrina A. Walsh: Conceptualization, Methodology, Supervision, Writing - review & editing. James A. Holden: Methodology, Writing - review & editing. Eric C. Reynolds: Conceptualization, Funding acquisition, Writing - review & editing. Ivan B. Darby: Conceptualization, Methodology, Supervision, Funding acquisition, Writing - review & editing. Neil M. O'Brien-Simpson: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

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Table 1. Cohort demographics and periodontal parameters.

	Health	Periodontitis			
	(*n* = 40)	Baseline (*n* = 54)	3-months (*n* = 46)	6-months (*n* = 44)	12-months (*n* = 37)
Cohort demographics					
Gender (*n*, % male)	14 (35.0%)	20 (37.0%)	19 (43.2%)	18 (42.9%)	15 (46.9%)
Age at baseline (years, mean ± SD)	49.30 ± 10.62	53.28 ± 11.44	53.72 ± 11.18	53.70 ± 11.66	54.53 ± 9.70
Smokers (*n*, %smokers)	6 (15.0%)	15 (27.8%)	12 (26.1%)	10 (22.7%)	7 (18.9%)
Number of teeth (mean ± SD)	26.88 ± 1.40	24.43 ± 2.93†	23.70 ± 3.81†‡	24.03 ± 3.83†	23.44 ± 3.67†
Periodontal parameters separated based on timepoint					
Mean PD (mm, mean ± SD)	2.80 ± 0.28	3.72 ± 0.74†	3.01 ± 0.51†‡	2.92 ± 0.55†‡	2.80 ± 0.44†‡§
%BOP (%, mean ± SD)	10.86 ± 7.72	51.61 ± 25.57†	28.35 ± 15.17†‡	25.82 ± 18.84†‡	24.66 ± 18.05†‡
% of sites with PD ≥ 5mm (%, mean ± SD)	0.00 ± 0.00	30.41 ± 20.49†	11.90 ± 11.37†‡	10.39 ± 10.36†‡	7.54 ± 7.54†‡
Periodontal parameters separated based on TxO groups					
Mean PD (mm, mean ± SD)					
Good TxO group	2.80 ± 0.28	3.22 ± 0.38†£¢ (*n* = 18, 33.3%)	2.66 ± 0.29†‡¢ (*n* = 18, 39.1%)	2.49 ± 0.23†‡¢ (*n* = 18, 40.9%)	2.41 ± 0.20†‡£¢ (*n* = 14, 37.8%)
Moderate TxO group	2.80 ± 0.28	3.84 ± 0.76†¥ (*n* = 13, 24.1%)	2.99 ± 0.30†‡¥ (*n* = 13, 28.3%)	2.96 ± 0.33†‡¥ (*n* = 12, 27.3%)	2.83 ± 0.21†‡¥ (*n* = 12, 32.4%)
Poor TxO group	2.80 ± 0.28	4.22 ± 0.77† (*n* = 15, 27.8%)	3.53 ± 0.50†‡ (*n* = 15, 32.6%)	3.48 ± 0.45†‡ (*n* = 14, 31.8%)	3.26 ± 0.36†‡ (*n* = 11, 29.7%)
%BOP (%, mean ± SD)					
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)
% of sites with PD ≥ 5mm (%, mean ± SD)					
Good TxO group	0.00 ± 0.00	18.64 ± 9.68†£¢	5.54 ± 4.53†‡	3.58 ± 2.59†‡	2.30 ± 2.14†‡

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		(*n* = 18)	(*n* = 18)	(*n* = 18)	(*n* = 14)
Moderate TxO group	0.00 ± 0.00	32.09 ± 21.36†	10.24 ± 4.89†‡	10.12 ± 5.98†‡	7.69 ± 2.92†‡
		(*n* = 13)	(*n* = 13)	(*n* = 12)	(*n* = 12)
Poor TxO group	0.00 ± 0.00	45.10 ± 24.16†	21.50 ± 14.92†‡	19.67 ± 11.43†‡	14.08 ± 9.70†‡
		(*n* = 15)	(*n* = 15)	(*n* = 14)	(*n* = 11)

Spearman's correlations between mean PD and %BOP by timepoint (ρ)

Mean PD and %BOP	**0.517**	**0.540**	**0.645**	**0.623**	**0.646**

† 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

Significant Spearman's correlations are represented in bold

Periodontitis subjects (*n* = 8, 14.8%) who only contributed to baseline were not assigned to a TxO group.

%BOP, percentage of sites with bleeding on probing; mm, millimetres; PD, probing depth; SD, standard deviation; TxO, treatment outcome; ρ, Spearman's correlation coefficient

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Table 2. Variation in red complex bacteria and correlations with periodontal parameters by timepoint.

	Health (*n* = 40)	Periodontitis			
		Baseline (*n* = 54)	3-months (*n* = 46)	6-months (*n* = 44)	12-months (*n* = 37)
Percentage of red complex bacteria as a proportion of total bacteria (mean ± SD)					
P. gingivalis	0.09 ± 0.57	7.81 ± 18.65†	1.71 ± 6.75‡	4.38 ± 15.03	0.25 ± 0.90‡
T. denticola	0.20 ± 0.87	0.75 ± 1.77†	0.67 ± 2.16	0.35 ± 1.15‡	0.34 ± 1.01
T. forsythia	0.21 ± 1.16	0.40 ± 0.67	0.63 ± 1.88	0.27 ± 0.83§	0.29 ± 0.77
Percentage of sites red complex bacteria were recovered from (% detected)					
P. gingivalis	12.5	66.7†	66.7†	64.8†	68.5†
T. denticola	47.5	87.0†	74.1†	88.9†	77.8†
T. forsythia	32.5	90.7†	81.5†	83.3†	77.8†
Spearman's correlations between red complex bacteria and mean PD (ρ)					
P. gingivalis	-0.016	**0.299**	**0.357**	0.263	0.050
T. denticola	**0.329**	0.154	**0.341**	0.223	0.116
T. forsythia	**0.398**	**0.306**	**0.356**	**0.355**	0.113
Spearman's correlations between red complex bacteria and %BOP (ρ)					
P. gingivalis	0.130	0.225	**0.342**	0.269	0.035
T. denticola	**0.319**	0.233	**0.363**	0.042	0.099
T. forsythia	**0.380**	0.185	0.286	0.341	0.149

† significant difference to health

‡ significant difference to baseline

§ significant difference to 3-months post-treatment

Significant Spearman's correlations are represented in bold

%BOP, percentage of sites with bleeding on probing; PD, probing depth; SD, standard deviation; ρ, Spearman's correlation coefficient

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Table 3. Correlations between periodontal parameters and memory T-cells by timepoint.

Memory T-cells	Health (*n* = 40)		Periodontitis							
			Baseline (*n* = 54)		3-months (*n* = 46)		6-months (*n* = 44)		12-months (*n* = 37)	
	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP
$CD4^+$ cells	0.033	0.129	-0.004	-0.151	-0.077	**-0.360**	-0.348	**-0.487**	-0.140	0.049
$CD4^+$ T_N cells	0.022	-0.112	0.199	0.066	-0.017	-0.075	-0.124	0.000	-0.134	-0.161
$CD4^+$ T_{EMRA} cells	-0.147	**-0.360**	0.122	0.212	-0.224	-0.155	-0.141	-0.068	-0.237	-0.079
$CD4^+$ T_{CM} cells	-0.047	0.211	-0.169	-0.055	0.053	0.151	0.085	0.069	-0.081	-0.097
$CD4^+$ T_{EM} cells	-0.044	-0.103	0.126	0.113	0.058	0.159	0.276	0.186	0.302	0.247
$CD8^+$ cells	-0.042	-0.019	-0.059	0.051	0.027	0.324	0.156	**0.387**	0.214	0.043
$CD8^+$ T_N cells	-0.008	0.109	-0.002	-0.268	-0.075	-0.134	-0.140	-0.040	-0.218	-0.293
$CD8^+$ T_{EMRA} cells	0.198	-0.052	0.099	**0.329**	-0.106	-0.101	-0.005	-0.085	0.069	0.018
$CD8^+$ T_{CM} cells	-0.009	0.055	-0.134	-0.147	0.058	-0.059	0.064	-0.040	-0.189	-0.051
$CD8^+$ T_{EM} cells	-0.225	-0.131	0.033	0.145	0.223	**0.369**	0.284	0.254	0.211	**0.525**
$CD4^+CD8^+$ cells	-0.191	-0.270	-0.148	-0.150	-0.117	0.125	0.018	0.287	0.022	0.026
$CD4^+CD8^+$ T_N cells	-0.017	**-0.401**	**0.324**	0.110	0.034	0.231	0.209	0.328	0.009	-0.064
$CD4^+CD8^+$ T_{EMRA} cells	0.173	-0.091	0.078	0.276	-0.054	0.142	0.079	0.140	-0.250	-0.206
$CD4^+CD8^+$ T_{CM} cells	0.058	0.191	-0.118	-0.025	-0.284	**-0.505**	**-0.358**	**-0.575**	-0.269	-0.202
$CD4^+CD8^+$ T_{EM} cells	-0.115	-0.149	0.095	0.125	0.305	**0.408**	0.183	0.260	0.322	0.317
$CD4^-CD8^-$ cells	-0.043	0.014	0.074	-0.035	0.014	0.225	0.264	0.371	0.152	**0.421**
$CD4^-CD8^-$ T_N cells	0.070	-0.122	-0.060	-0.214	-0.152	**-0.363**	-0.057	-0.131	0.013	-0.117
$CD4^-CD8^-$ T_{EMRA} cells	0.083	-0.164	-0.161	**-0.321**	-0.164	-0.280	-0.306	**-0.601**	0.046	0.131
$CD4^-CD8^-$ T_{CM} cells	0.119	0.101	0.105	0.136	0.052	**0.397**	0.202	0.320	-0.054	-0.097
$CD4^-CD8^-T_{EM}$ cells	-0.252	0.154	0.115	0.283	0.126	0.318	0.098	0.331	0.057	0.115

Spearman's correlation coefficients (ρ) between the periodontal parameters, mean probing depth (PD) and percentage of sites with bleeding on probing (%BOP), and naïve (T_N), effector memory T-cells re-expressing CD45RA (T_{EMRA}), central memory (T_{CM}), effector memory (T_{EM}) T-cells in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ cell subsets in health and at baseline, 3-, 6- and 12-months in periodontitis. Significant correlations ($p \leq 0.05$) are represented in bold.

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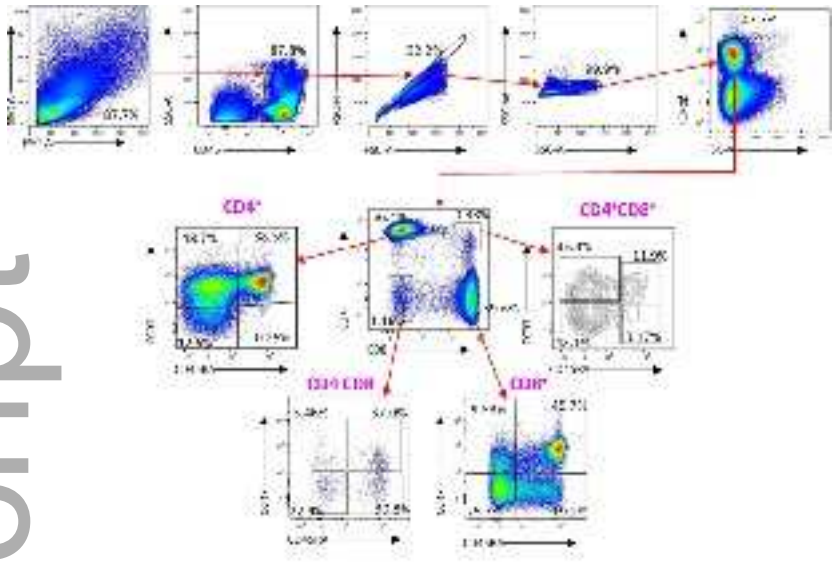


Fig. 1

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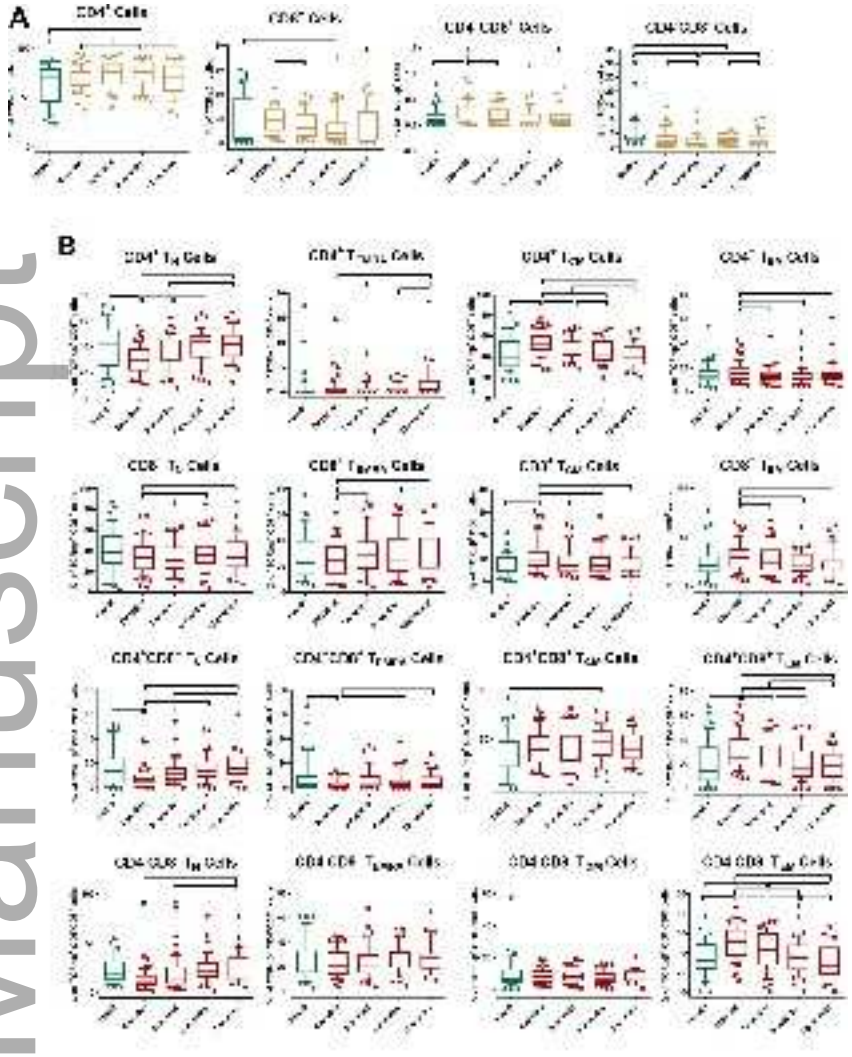


Fig. 2

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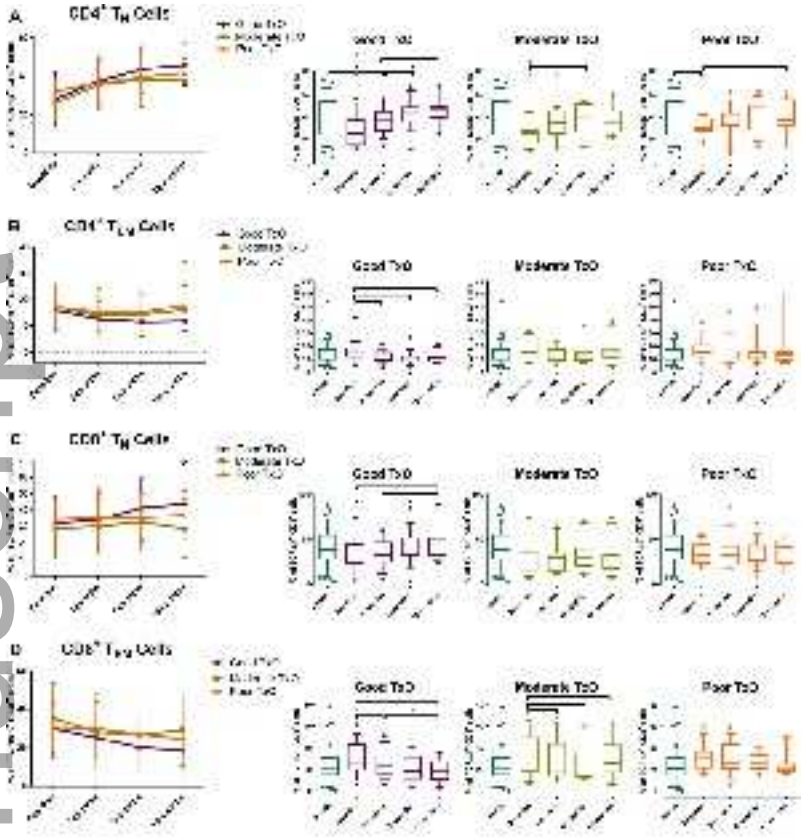


Fig. 3

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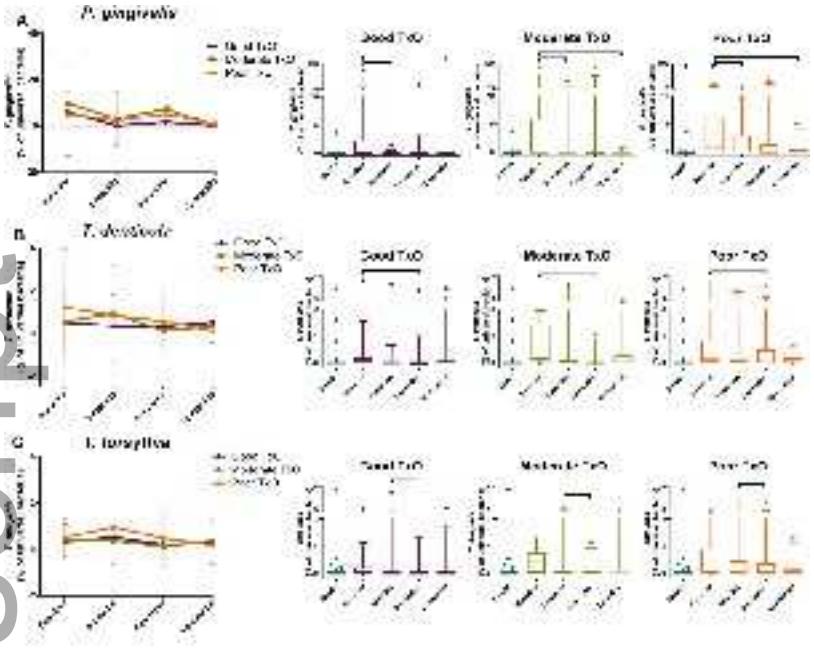


Fig. 4

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