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DOI:

[10.1111/cei.13205](https://doi.org/10.1111/cei.13205)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Cheng, W.-C., Saleh, F., Karim, B. A., Hughes, F. J., & Taams, L. S. (2018). Comparative analysis of immune cell subsets in peripheral blood from patients with periodontal disease and healthy controls. *Clinical & Experimental Immunology*, 194(3), 380-390. <https://doi.org/10.1111/cei.13205>

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Article type : Original Article

Comparative analysis of immune cell subsets in peripheral blood from patients with periodontal disease and healthy controls

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Running Head: Immune cell subsets in periodontitis

Keywords: Periodontal disease, peripheral blood mononuclear cells, T helper 17 cells, Tregs

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cei.13205

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Summary

Periodontitis is a chronic inflammatory disease caused by the colonisation of teeth by the bacterial plaque biofilm and the resultant host immune responses in adjacent periodontal tissues. Disease severity can vary dramatically between patients with periodontitis, with some subjects displaying inflammation without bony destruction (gingivitis), whilst others experience chronic progressive or rapidly aggressive gingival connective tissue damage and bone loss. To determine whether peripheral immune dysregulation is associated with periodontitis, we performed extensive analysis of immune cell subsets in peripheral blood from patients with chronic or aggressive periodontitis vs. periodontally healthy control subjects. Peripheral blood mononuclear cells (PBMC) from patients with chronic periodontitis or aggressive periodontitis and from periodontally healthy controls were analysed by 8-10 colour flow cytometry for the frequencies of various lymphocyte subsets, including IL-17, IFN γ , TNF α and IL-10 producing cells, and the frequencies and phenotype of monocytes. Cytokine levels in serum from the different groups were determined by

Luminex assay. We found no significant differences in the frequencies of major immune cell populations (CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, CD4⁺CD45RO⁺CD25⁺CD127^{low} regulatory T cells (Tregs), CD19⁺ B cells, CD14⁺ monocytes) or of cytokine producing T cells, or in the phenotype of CD14⁺ monocytes in peripheral blood from these patient cohorts. Additionally, no significant differences were observed in serum levels of prototypical inflammatory cytokines. These results suggest that the local gingival inflammatory response is not reflected by obvious changes in major blood immune cell subset frequencies.

Introduction

Periodontitis is a chronic inflammatory disease caused by the colonisation of teeth by the bacterial plaque biofilm and the resultant host immune responses that occur in the surrounding periodontal tissues. The bacterial plaque biofilm induces an inflammatory infiltrate in periodontal tissue, in which the dominant immune cell populations may differ from person to person. There are wide variations in disease severity between patients with periodontal disease. Many subjects will have superficial gingival inflammation without any deeper tissue destruction, (gingivitis), whilst other subjects will experience progressive destruction of the supporting tissues of the tooth, including connective tissue damage and loss of alveolar bone (periodontitis). In the majority of cases progression of tissue damage occurs

slowly over many years and is affected by risk factors, such as oral hygiene and plaque levels, local predisposing factors, smoking, genetic factors, systemic risk factors such as diabetes and stress (Chronic Periodontitis). In a smaller number of cases tissue damage may occur rapidly, typically with an early age of onset (<35 years), and may result in rapid tooth loss (Aggressive Periodontitis) [1].

Studies have shown increased levels of pro-inflammatory cytokines such as IL-17 in serum from patients with periodontitis when compared to healthy controls [2-4].

Furthermore, studies have reported a decreased percentage of IL-17+ CD4+ T cells in peripheral blood mononuclear cells (PBMC) as well as decreased levels of IL-17 protein in serum from patients with periodontitis following non-surgical periodontal treatment [5-7].

These findings suggest that changes in cytokine levels in locally inflamed gingival tissue are mirrored by cytokine levels in the systemic circulation. Other studies have determined the frequencies of immune cell populations and cytokine producing T helper cells in peripheral blood from patients with different forms of periodontitis, i.e. chronic and aggressive periodontitis [8-10]. However, the limited existing data regarding IFN γ , IL-4 or IL-17 producing cells in peripheral blood are not definitive as both decreased and increased percentages of these cytokine-expressing cells in PBMC from patients with periodontitis have been demonstrated relative to healthy controls [8-10]. The conflicting conclusions from these

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studies may be because different classifications of periodontitis were utilised (e.g. differences in the number of teeth affected, or different age criteria for chronic or acute periodontitis), or because not all studies included patients with different forms of periodontitis.

Studies have suggested that monocytes during or after periodontal pathogen challenge could contribute to inflammation in periodontitis-associated systemic disease such as aortic inflammation [11, 12]. One study has shown an increased percentage of CD14+CD16+ monocytes in the peripheral blood of patients with chronic periodontitis compared to healthy individuals [13], but overall data on peripheral blood monocyte populations in periodontitis are scarce.

Epidemiological data suggest an association between periodontal diseases and systemic conditions such as atherosclerotic cardiovascular disease, an increased risk of preterm low birth-weight babies, and chronic inflammatory diseases such as rheumatoid arthritis, diabetes and respiratory diseases, although the mechanisms for the various relationships remain largely unknown (reviewed in [14, 15]). A possible mechanism has been established by which locally produced pro-inflammatory cytokines including IL-6, IL-1 β and TNF α in severe periodontitis can enter the systemic circulation and cause increases in the serum levels of these cytokines as well as elevated levels of C-reactive protein (reviewed in [14]).

To assess the effect of localised inflamed tissue on the systemic circulation in periodontitis, we performed a systematic analysis of immune cell subsets in peripheral blood from patients with chronic or aggressive periodontitis and periodontally healthy control subjects. Specifically, we investigated the frequencies of the main immune cell subsets (T cells, Tregs, B cells, monocytes, NK cells), and frequencies of IL-17, IFN γ , TNF α and IL-10 producing cells within CD4 $^+$ T cells, CD8 $^+$ T cells, $\gamma\delta$ T cells and CD19 $^+$ B cells. We focused on these four cytokines as they are important in regulation of inflammation-induced tissue destruction in periodontitis. Furthermore, we investigated the phenotype of peripheral blood CD14 $^+$ monocytes, and also determined the levels of a wide range of T cell-derived or monocyte-derived cytokines in the serum from patients with periodontitis and periodontally healthy controls. Overall our data do not indicate significant changes in any of these parameters.

Materials and Methods

Patient and healthy control samples

Healthy control samples were obtained from periodontally healthy volunteers. Newly diagnosed patients with periodontitis were recruited from the periodontal clinic at Guy's Hospital (see **Table I** for clinical and demographic parameters). Fifty ml of peripheral venous blood was drawn from each participant from the antecubital fossa and collected in Sodium

Heparin Plasma tubes (BD, Franklin Lakes, NJ, USA). An additional 6 ml of blood was collected in anticoagulant-free serum tubes (BD) and stored at 4°C (fridge) for 1 hour before centrifugation at 1200 rpm for 10 minutes and harvesting of the serum. The harvested serum samples were aliquoted into 500 µl amounts and stored at -80°C until further analysis. Oral and periodontal examination records, age, sex and smoking status were documented on the day of sample collection. Periodontal disease case definitions for chronic periodontitis were used according to those described by Page and Eke, and definitions for aggressive periodontitis according to those proposed by Demmer and Papapanou [16, 17], with the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥ 6 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing. Age and sex matched periodontally healthy control subjects were recruited and assessed with definitions as no probing pocket depth (PPD) >3 mm at any site, no proximal sites with attachment loss at any site, and BOP of $< 20\%$ of possible sites. All clinical investigations were conducted according to the Declaration of Helsinki principles. Ethics approval for this study was given by the NRES Committee London City Road & Hampstead Research Ethics Committee (12/LO/0376). Written informed consent was received from participants before their inclusion in the study.

Cell isolation from peripheral blood

PBMC were isolated by density gradient centrifugation using lymphocyte separation medium (LSM 1077; PAA Laboratories, Pasching, Austria or Lymphoprep; AXIS-SHIELD, Oslo, Norway). PBMC were cryopreserved within 1 hour of isolation and stored in liquid nitrogen in medium containing 90% fetal bovine serum (lot 030M3399, Sigma-Aldrich, St. Louis, MO, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich).

Ex vivo immune cell subset staining

The immune cell subsets and phenotypes that were determined are shown in **Supplementary**

Figure 1. The identification of CD4⁺ T cells, CD8⁺ T cells, CD4⁺CD45RO⁺CD25⁺CD127^{low} regulatory T cells (Tregs), $\gamma\delta$ T cells, B cells, monocytes and NK cells was performed using an eight-colour extracellular staining panel (**Supplementary Table I**). For the identification of cytokine-expressing cells, PBMC were stimulated for 3 hours with PMA (50 ng/ml) and ionomycin (750 ng/ml) (both from Sigma-Aldrich) in the presence of GolgiStop according to the manufacturer's instructions (BD, Oxford, UK). The identification of IL-17, IFN γ , TNF α or IL-10 expressing cells within CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, or CD19⁺ B cells was facilitated by staining with a ten-colour intracellular cytokine staining panel (**Supplementary Table II**). Extracellular surface staining was performed using the following monoclonal antibodies:

PE-Cy7-conjugated anti-CD3 (clone UCHT1), PerCP-Cy5.5-conjugated anti-CD4 (clone SK3), APC-Cy7-conjugated anti-CD14 (clone HCD14), Brilliant Violet 605-conjugated anti-CD19 (clone HIB19), APC-conjugated anti-CD56 (clone HCD56), PE-conjugated anti-CD25 (clone M-A251), FITC-conjugated anti-CD127 (clone A019D5), APC-Cy7-conjugated anti-CD45RA (clone HI100), Pacific Blue-conjugated anti-CD45RO (clone UCHL1), APC-conjugated anti-CD54 (clone HCD54), Pacific Blue-conjugated anti-CD86 (clone IT2.2) (all from BioLegend, London, UK), PE-CF594-conjugated anti-CD8 (clone RPA-T8), FITC-conjugated anti- $\gamma\delta$ T-cell receptor (TCR) (clone 11F2), PerCP-Cy5.5-conjugated anti-HLA-DR (clone G46-6) (all from BD Biosciences, Oxford, UK), and PE-conjugated anti-CD40 (clone LOB7/6, AbD Serotec, Kidlington, UK).

For intracellular staining, following appropriate cell surface staining, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin (Sigma) and stained intracellularly with Pacific Blue-conjugated anti-CD4 (clone SK3), Alexa Fluor 488-conjugated anti-IL-10 (clone JES3-9D7), PE-conjugated anti-IL-17A (clone BL168), APC-conjugated anti-TNF α (clone MAb11), and PerCP-Cy5.5-conjugated anti-IFN γ (clone 4S.B3) (all from BioLegend). For intranuclear staining, cells were extracellularly stained and fixed as described above followed by permeabilization with 1x FoxP3 Perm Buffer (BioLegend). Cells were then stained with Alexa Fluor 647-conjugated FoxP3 (clone 259D,

BioLegend). Cells were acquired by flow cytometry using a LSRFortessa (BD Biosciences, Franklin Lakes NJ, USA) and analysed using the FlowJo software (Tree Star, Ashland, OR). Cytometer settings were tracked over time using BD Biosciences Cytometer Setup and Tracking software to monitor cytometer performance of the LSRFortessa at the NIHR Biomedical Research Centre Flow Cytometry Core Facility in Guy's Hospital. Viable single cells were identified using a LIVE/DEAD® Fixable Blue Dead Cell stain kit (Life technologies, Carlsbad, CA).

Cytokine detection

Serum samples were collected and stored at -80°C until further use. Serum cytokine levels were determined by Luminex assay (Bio-Plex Pro™ Human Th17 Cytokine Assays, Bio-Rad Laboratories, CA, USA).

Statistical analysis

Values are expressed as mean with SEM. Data were tested for normality using D'Agostino & Pearson omnibus normality testing and where not normally distributed data were log₁₀ transformed prior to analysis (which provided normally distributed results). Comparisons between patients and healthy controls were made using one-way analysis of variance followed by Bonferroni's multiple comparison test for parametric data. Data were analysed

using Prism version 5 (GraphPad Software Inc, La Jolla, USA). For all tests, p values of less than 0.05 were considered significant. In addition, to allow for the possibility that variations in subject age might account for some of the differences seen, we carried out a bivariate linear regression analysis with diagnosis and age inputted as explanatory variables. This was analysed using SPSS v23 (IBM).

Results

Similar frequencies of major immune cell subsets in peripheral blood from patients with periodontal disease vs. periodontally healthy subjects

To determine the frequencies of the major immune cell subsets, we applied the staining panel as described in **Supplementary Table I** to cryopreserved cells from patients with chronic periodontitis, aggressive periodontitis and periodontally healthy donors. We found no statistically significant differences in the frequencies of CD4⁺, CD8⁺ and $\gamma\delta$ T cell subsets, or in CD19⁺ B cells, CD14⁺ monocytes and CD56⁺ NK cells in peripheral blood between the three groups (**Figure 1**). Frequencies of CD45RA⁺ or CD45RO⁺ cells within CD4⁺ T cell populations were also similar in the peripheral blood from the different groups (**Figure 2A** and data not shown).

Regarding Tregs, as expected [18] there was a slightly higher percentage of CD25+CD127^{low} T cells within the CD4+CD45RO+ T cell population (on average 10%) when compared to CD4+CD45RA+ or total CD4+ T cells (on average 5% and 7%, respectively), but the frequencies between the groups were not statistically significant different (**Figure 2B** and data not shown). No significant difference was observed in FOXP3 expression in CD4+CD45RO+CD25+CD127^{low} regulatory T cells between the groups (**Figure 2C**).

CD14+ monocytes from PB of patients with periodontal disease and healthy control subjects are phenotypically similar

We investigated the phenotype of CD14+ monocytes from peripheral blood of patients with periodontal disease vs. healthy control subjects. No significant differences in the expression of the markers CD14, CD16, CD40, CD54, CD86 or HLA-DR by peripheral blood CD14+ monocytes were observed among the different groups (**Figure 3**). We also investigated the frequency and phenotype (expression of the markers CD14, CD16, CD40, CD54, CD86 or HLA-DR) of CD14+CD16+ monocytes in PB (often referred to as ‘inflammatory’ monocytes [19]) but no significant differences were found between the groups (data not shown). The frequencies of CD14+CD16+ monocytes in the healthy control, chronic and aggressive

periodontitis groups were also not significantly different (median 10.3% [IQR: 3, 24]; 4.2% [IQR: 3, 16]; 4.9% [IQR: 2, 28]; and 7.5% [IQR: 3, 25], respectively).

Similar frequencies of cytokine producing cells within T cell populations from PB of patients with periodontal disease and healthy control subjects

Next, we investigated whether there were quantitative differences in cytokine producing CD4⁺ T cells in peripheral blood between the groups (**Figure 4A**). In peripheral blood from patients with chronic periodontitis, on average $1\pm 0.1\%$ of the CD4⁺ T cells expressed IL-17, $12\pm 2.3\%$ expressed IFN γ , a higher proportion expressed TNF α (on average $41\pm 3.8\%$), and a very low proportion expressed anti-inflammatory IL-10 (on average $0.3\pm 0.04\%$). However, these frequencies were not significantly different from patients with aggressive periodontitis, or periodontally healthy control subjects (**Figure 4B**).

In addition to the CD4⁺ T cell population, on average $0.4\pm 0.01\%$ of the CD8⁺ T cells expressed IL-17, $33\pm 4\%$ expressed IFN γ , $36\pm 4\%$ expressed TNF α , and a very low proportion expressed anti-inflammatory cytokine IL-10 (on average $0.1\pm 0.01\%$) in peripheral blood from patients with chronic periodontitis. In the $\gamma\delta$ T cell population in PB from patients with chronic periodontitis, on average $0.7\pm 0.1\%$ expressed IL-17, $52\pm 6\%$ expressed IFN γ , and $52\pm 4\%$ expressed TNF α (IL-10 expression was not measured in $\gamma\delta$ T cells). However, as for CD4⁺ T

cells, there were no significant differences between the groups (**Figure 5A, B** and data not shown). Very few CD19⁺ B cells expressed IL-17, IFN γ or IL-10, whilst a small proportion expressed TNF α (on average 15 \pm 3% in chronic periodontitis) but this was not significantly different between groups.

As chronic and aggressive periodontitis occur at different ages, and age itself may affect immune responses, we also carried out regression analysis to correct effects of disease diagnosis for age. The results are shown in **Supplementary Table III** and show that age had a significant effect only on % $\gamma\delta$ TCR⁺ cells and CD40 MFI. There were no significant differences with periodontal diagnoses in all tests with the exception of % $\gamma\delta$ TCR⁺IFN γ ⁺, which was significantly higher in healthy subjects compared to either aggressive or chronic periodontitis subjects.

Detection of T cell-derived and monocyte-derived mediators in serum

Finally, we determined the levels of CD4⁺ T helper cell-derived (IFN γ , IL-4, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-25, TNF α) and monocyte-derived (IL-1 β , IL-6, IL-10, IL-23, IL-33 and TNF α) mediators in the sera of the aforementioned groups by Luminex assay. Protein levels of IFN γ , IL-1 β , IL-17A, IL-21, IL-22, IL-23 and IL-25 (IL-17E) were below the lower detection limit of the Luminex assay in the majority of samples. IL-4, IL-6, IL-10, IL-17F,

IL-33 and TNF α were detectable in most serum samples, however no statistically significant differences between the groups were observed (**Figure 6 and data not shown**).

Discussion

In this study on newly diagnosed patients with chronic or aggressive periodontitis, we found no significant differences in the frequencies of major immune cell populations (CD4 $^+$ T cells, CD8 $^+$ T cells, Tregs, CD19 $^+$ B cells, CD14 $^+$ monocytes, CD56 $^+$ NK cells) and cytokine producing cells (IL-17, IFN γ , TNF α and IL-10 producing cells within CD4 $^+$ T cells, CD8 $^+$ T cells, $\gamma\delta$ T cells and CD19 $^+$ B cells), nor in the phenotype of CD14 $^+$ monocytes from the peripheral blood of chronic and aggressive periodontitis patient cohorts when compared to healthy controls.

Conflicting data have been reported regarding the frequencies of cytokine-producing CD4 $^+$ T cells in periodontitis. A previous study reported a higher percentage of IL-4 producing CD4 $^+$ T cells in PBMC from patients with chronic periodontitis vs. healthy controls, a higher percentage of TNF α producing CD4 $^+$ T cells in PBMC from patients with aggressive periodontitis vs. healthy controls, and no differences in the percentages of IFN γ or IL-10 producing CD4 $^+$ T cells between groups [8]. The authors suggested that CD4 $^+$ T cells from patients with chronic periodontitis are more committed to IL-4 production, whilst CD4 $^+$

T cells from acute periodontitis are more committed to TNF α production; they did not measure the IL-17 producing CD4⁺ T cells in PBMC [8]. Schmidt and colleagues reported slightly higher percentages of IFN γ and IL-4 producing CD4⁺ T cells in PBMC from chronic vs. aggressive periodontitis and healthy controls, and no differences in the percentages of IL-17 producing CD4⁺ T cells among the three groups [9]. The authors also reported a slightly reduced Th1 response in patients with acute periodontitis defined by a lower Th1/Th2 ratio when compared with chronic periodontitis and healthy controls [9]. Chen and colleagues recently reported significantly increased levels of IL-17A⁺ and IFN γ ⁺ CD4⁺ T cells in PBMC from chronic periodontitis vs. healthy controls [10]. Our data however show that CD4⁺ T cells from chronic or aggressive periodontitis patients did not contain significantly enhanced percentages of IL-17⁺, IFN γ ⁺, or TNF α ⁺ cells compared to age-and sex matched healthy controls.

In terms of serum cytokine levels, previous studies have shown higher levels of IL-17 and IL-1 β in serum from patients with periodontitis when compared to healthy controls [2-4, 20]. In these studies, Gümüs et al. reported the serum IL-1 β levels in chronic and aggressive periodontitis groups were higher than those in healthy control group, whilst no significant differences in serum IL-1 β levels between the periodontitis groups were noted [20]. A different study however, reported no differences in the serum level of IL-1 β between chronic

periodontitis and healthy control groups [21]. In our dataset, the serum levels of IL-17A and IL-1 β were below the lower detection limit of the Luminex assay in the majority of samples. We did detect IL-4, IL-6, IL-10, IL-17F, IL-33 and TNF α however no statistically significant differences were found between the groups. Our data on IL-4, IL-6, IL-10 and TNF α data are in line with several previously published papers [6, 21, 22] although significant differences in serum cytokines between healthy controls, aggressive and chronic periodontitis have been reported [6, 22-24].

Explanations for the differences in results between these studies are not immediately clear. Although there are some variations in study design, most particular in differences in periodontal case definitions and in ages between groups in some of these studies, it seems unlikely that these account for the observed differences. Differences in experimental and technical approaches and standardisation may explain some of these differences. In our study, to minimize inter-experimental variation, cryopreserved samples were analysed in batches of five different samples that always consisted of a mix of HC (n=1-2), CP (n=1-2) and AP (n=1-2); we also applied CS&T settings to monitor and correct for flow cytometer performance over time. Furthermore, in addition to analysing cytokine-expressing CD4 $^+$ T cells, we also analysed cytokine-expressing CD8 $^+$ T cells, $\gamma\delta$ T cells and B cells, and Foxp3-expressing Tregs. Recently, CD8 $^+$ T cells and $\gamma\delta$ T cells have been reported to be able

to produce IL-17 [25, 26] and B cells have been reported as a major source of IL-17A and IL-17F during *Trypanosoma cruzi* infection [27]. However, we did not detect significant differences in the frequencies of any of these cell populations between groups. Furthermore, the data were not only not different between groups but were also remarkably similar between groups. In a retrospective Power calculation using the data we obtained in our results, we found that our study had 96% Power to detect a mean increase of 50% in TNF+ cells with alpha as 0.05, and 74% Power to detect a similar change in IFN γ + cells. Thus, we can reasonably rule out the likelihood of a type II error in failing to detect a difference that actually existed.

Previous studies have suggested a role for monocytes from patients with periodontitis in aortic inflammation [11, 12]. These data suggested that monocytes during or after periodontal pathogen challenge could contribute to inflammation in periodontitis-associated systemic disease. In addition, we recently showed that in the presence of *Porphyromonas gingivalis* significantly higher IL-17 production was observed in anti-CD3 mAb-stimulated monocyte/CD4+ T cell co-cultures from patients with periodontitis compared to periodontally healthy controls [28]. However, our current analysis did not reveal a significant difference in the frequencies of monocytes or expression of activation markers on monocytes among patients with different periodontal diseases and periodontally healthy controls.

Our data do not exclude the possibility that differences in immune cell subsets are present at the site of inflammation in chronic and/or acute periodontitis vs. healthy gingival tissue. Indeed, studies have reported an increased percentage of IL-17-producing cells in gingival tissue from chronic periodontitis [28-30]. Furthermore, in our study we only determined relative proportions of immune cell subsets in PBMC and therefore we cannot rule out differences in absolute numbers of these immune cell types.

In conclusion, our data provide evidence that no significant alterations are present in the frequencies of major immune cell populations and cytokine producing cells in peripheral blood from patients with well-defined periodontal disease.

Acknowledgments

WCC was supported by a grant from Tri-Service General Hospital, National Defense Medical Center. We are very grateful to the patients and volunteers who consented to participate in this study. We would like to thank Dr Emily Lu (Dental Institute, King's College London) for help in patient recruitment and data collection, Dr Gina Walter and Dr Hayley Evans for flow panel advice, and Dr Susanne Heck, PJ Chana and Helen Graves for their help in flow cytometry at the Biomedical Research Centre Flow Cytometry Core Facility funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's

and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Conflict of Interest

The authors declare that they have no conflict of interests in relation to this study.

References

1. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999; **4**:1-6.
2. Schenkein HA, Koertge TE, Brooks CN, Sabatini R, Purkall DE, Tew JG. IL-17 in sera from patients with aggressive periodontitis. *J Dent Res* 2010; **89**:943-7.
3. Wang H, Luo Z, Lei L, Sun Z, Zhou M, Dan H, Zeng X, Chen Q. Interaction Between Oral Lichen Planus and Chronic Periodontitis with Th17-Associated Cytokines in Serum. *Inflammation* 2013.
4. Qi Y, Feng W, Song A, Song H, Yan S, Sun Q, Yang P. GAP > LAP > HC IL-23/IL-17 Axis in the Relationship Between Periodontitis and Coronary Heart Disease. *Int J Periodontics Restorative Dent* 2013; **33**:185-92.
5. Zhao L, Zhou Y, Xu Y, Sun Y, Li L, Chen W. Effect of non-surgical periodontal therapy on the levels of Th17/Th1/Th2 cytokines and their transcription factors in Chinese chronic periodontitis patients. *J Clin Periodontol* 2011; **38**:509-16.
6. Duarte PM, da Rocha M, Sampaio E, Mestnik MJ, Feres M, Figueiredo LC, Bastos MF, Faveri M. Serum levels of cytokines in subjects with generalized chronic and aggressive periodontitis before and after non-surgical periodontal therapy: a pilot study. *J Periodontol* 2010; **81**:1056-63.
7. Cifcibasi E, Koyuncuoglu C, Ciblak M, Badur S, Kasali K, Firatli E, Cintan S. Evaluation of Local and Systemic Levels of Interleukin-17, Interleukin-23, and Myeloperoxidase in Response to Periodontal Therapy in Patients with Generalized Aggressive Periodontitis. *Inflammation* 2015; **38**:1959-68.

8. Lima PM, Souza PE, Costa JE, Gomez RS, Gollob KJ, Dutra WO. Aggressive and chronic periodontitis correlate with distinct cellular sources of key immunoregulatory cytokines. *J Periodontol* 2011; **82**:86-95.
9. Schmidt J, Jentsch H, Stingu CS, Sack U. General immune status and oral microbiology in patients with different forms of periodontitis and healthy control subjects. *PLoS One* 2014; **9**:e109187.
10. Chen XT, Chen LL, Tan JY, Shi DH, Ke T, Lei LH. Th17 and Th1 Lymphocytes Are Correlated with Chronic Periodontitis. *Immunol Invest* 2016; **45**:243-54.
11. Miyajima S, Naruse K, Kobayashi Y, Nakamura N, Nishikawa T, Adachi K, Suzuki Y, Kikuchi T, Mitani A, Mizutani M, Ohno N, Noguchi T, Matsubara T. Periodontitis-activated monocytes/macrophages cause aortic inflammation. *Sci Rep* 2014; **4**:5171.
12. Buhlin K, Gustafsson A, Pockley AG, Frostegard J, Klinge B. Risk factors for cardiovascular disease in patients with periodontitis. *Eur Heart J* 2003; **24**:2099-107.
13. Jagannathan R, Lavu V, Rao SR. Comparison of the proportion of non-classic (CD14+CD16+) monocytes/macrophages in peripheral blood and gingiva of healthy individuals and patients with chronic periodontitis. *J Periodontol* 2014; **85**:852-8.
14. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* 2015; **15**:30-44.
15. Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K. Relationship between periodontal infections and systemic disease. *Clin Microbiol Infect* 2007; **13 Suppl 4**:3-10.
16. Demmer RT, Papapanou PN. Epidemiologic patterns of chronic and aggressive periodontitis. *Periodontol* 2000 2010; **53**:28-44.
17. Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* 2007; **78**:1387-99.
18. Walter GJ, Fleskens V, Frederiksen KS, Rajasekhar M, Menon B, Gerwien JG, Evans HG, Taams LS. Phenotypic, Functional, and Gene Expression Profiling of Peripheral CD45RA+ and CD45RO+ CD4+CD25+CD127(low) Treg Cells in Patients With Chronic Rheumatoid Arthritis. *Arthritis Rheumatol* 2016; **68**:103-16.
19. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheum* 2012; **64**:671-7.
20. Gumus P, Nizam N, Nalbantsoy A, Ozcaka O, Buduneli N. Saliva and serum levels of pentraxin-3 and interleukin-1beta in generalized aggressive or chronic periodontitis. *J Periodontol* 2014; **85**:e40-6.

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21. Cetinkaya B, Guzeldemir E, Ogus E, Bulut S. Proinflammatory and anti-inflammatory cytokines in gingival crevicular fluid and serum of patients with rheumatoid arthritis and patients with chronic periodontitis. *J Periodontol* 2013; **84**:84-93.
 22. Miranda TS, Heluy SL, Cruz DF, da Silva HDP, Feres M, Figueiredo LC, Duarte PM. The ratios of pro-inflammatory to anti-inflammatory cytokines in the serum of chronic periodontitis patients with and without type 2 diabetes and/or smoking habit. *Clin Oral Investig* 2018.
 23. Keles ZP, Keles GC, Avci B, Cetinkaya BO, Emingil G. Analysis of YKL-40 acute-phase protein and interleukin-6 levels in periodontal disease. *J Periodontol* 2014; **85**:1240-6.
 24. Gumus P, Nizam N, Lappin DF, Buduneli N. Saliva and serum levels of B-cell activating factors and tumor necrosis factor-alpha in patients with periodontitis. *J Periodontol* 2014; **85**:270-80.
 25. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 2010; **10**:479-89.
 26. Patel DD, Lee DM, Kolbinger F, Antoni C. Effect of IL-17A blockade with secukinumab in autoimmune diseases. *Ann Rheum Dis* 2013; **72 Suppl 2**:ii116-23.
 27. Bermejo DA, Jackson SW, Gorosito-Serran M, Acosta-Rodriguez EV, Amezcua-Vesely MC, Sather BD, Singh AK, Khim S, Mucci J, Liggitt D, Campetella O, Oukka M, Gruppi A, Rawlings DJ. *Trypanosoma cruzi* trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. *Nat Immunol* 2013; **14**:514-22.
 28. Cheng WC, van Asten SD, Burns LA, Evans HG, Walter GJ, Hashim A, Hughes FJ, Taams LS. Periodontitis-associated pathogens *P. gingivalis* and *A. actinomycetemcomitans* activate human CD14(+) monocytes leading to enhanced Th17/IL-17 responses. *Eur J Immunol* 2016; **46**:2211-21.
 29. Cardoso CR, Garlet GP, Crippa GE, Rosa AL, Junior WM, Rossi MA, Silva JS. Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. *Oral Microbiol Immunol* 2009; **24**:1-6.
 30. Adibrad M, Deyhimi P, Ganjalikhani Hakemi M, Behfarnia P, Shahabuei M, Rafiee L. Signs of the presence of Th17 cells in chronic periodontal disease. *J Periodontal Res* 2012; **47**:525-31.

Table I. Demographic and clinical characteristics of the study subjects

	HC (n=13)	CP (n=15)	AP (n=15)	ANOVA p-value ^a	
Age, mean years (range)	36.8 (20-63)	42.1 (36-58) [#]	30.5 (19-36) [#]	P<0.001	
Gender	Male, n (%)	6 (46)	7 (47)	8 (53)	ns
	Female, n (%)	7 (54)	8 (53)	7 (47)	ns
Number of teeth, mean	28.7	28.1	28.7	ns	
PPD, mean \pm SD mm	1.73 \pm 0.24	3.53 \pm 0.69 ^{***}	3.19 \pm 0.78 ^{***}	P<0.0001	
BOP, mean \pm SD %	10.9 \pm 7.0	32.1 \pm 14.9 [*]	34.2 \pm 30.9 ^{**}	P<0.01	
% sites of PPD > 5 mm	0 \pm 0	29.5 \pm 12.8 ^{***}	23.4 \pm 16.6 ^{***}	P<0.0001	
PISA, median (IQR) mm ²	91 (42-136)	643 (337-906) ^{***}	255 (137-1336) ^{***}	P<0.0001	

AP, aggressive periodontitis patients; BOP, bleeding on probing; CP, chronic periodontitis patients; GI, gingivitis patients; HC, periodontally healthy controls; PISA, periodontal inflamed surface area; PPD, probing pocket depth. Data were tested for normality using D'Agostino & Pearson omnibus normality testing and where not normally distributed data were log₁₀ transformed prior to analysis. Data were analysed by one-way ANOVA and the overall p-value shown as ^a. Individual groups were compared to HC values by Bonferroni's multiple comparison test for parametric data, and significant differences indicated as *, P<0.05; **, P<0.01; ***, P<0.001. Significant differences between CP and AP groups are indicated as [#]P<0.05.

Figure legends

Figure 1. Frequencies of major immune cell populations in PBMC from healthy controls and patients with periodontal disease. PBMC were isolated from peripheral blood from patients with chronic periodontitis (CP, n=15) or aggressive periodontitis (AP, n=15) as well as periodontally healthy controls (HC, n=13). (A) Representative gating strategy and (B) cumulative data showing the frequencies of cell populations within live-gated single cells. Each symbol represents an individual donor. Lines show the mean with SEM. Log-transformed data were analysed by one-way analysis of variance followed by Bonferroni's multiple comparison test.

Figure 2. Frequencies of CD4⁺ CD45RO⁺ CD25⁺ CD127^{low} regulatory T cells in PBMC from healthy controls and patients with periodontal disease. PBMC were isolated from peripheral blood from patients with chronic periodontitis (CP, n=15) or aggressive periodontitis (AP, n=15) as well as periodontally healthy controls (HC, n=13). Cells were surface stained and then permeabilised and intranuclearly stained for FOXP3. Representative dot plots and cumulative data showing the frequencies of CD4⁺CD45RO⁺ T cells (A) and CD25⁺CD127^{low} cells within CD4⁺CD45RO⁺ T cells (B). (C) Representative histogram plots showing the expression levels of FOXP3 in CD4⁺CD25⁺CD127^{low} T cells. Each symbol

represents an individual donor. Lines show the mean with SEM. Log-transformed data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test.

Figure 3. Expression of activation markers on CD14+ monocytes from healthy controls and patients with periodontal disease. PBMC were isolated from peripheral blood from patients with chronic periodontitis (CP, n=12) or aggressive periodontitis (AP, n=11) as well as periodontally healthy controls (HC, n=8). (A) Representative gating strategy to identify CD14+ monocytes. (B) Representative histogram plots and (C) cumulative data showing the geometric mean fluorescence intensity (MFI) for the surface expression of indicated markers determined by flow cytometry on CD14+ monocytes. Each symbol represents an individual donor. Lines show the mean with SEM. Log-transformed data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test.

Figure 4. Frequencies of cytokine producing CD4+ T cells in PBMC from healthy controls and patients with periodontal disease. PBMC were isolated from peripheral blood from patients with chronic periodontitis (CP, n=15) or aggressive periodontitis (AP, n=15) as well as periodontally healthy controls (HC, n=13). Cells were stimulated *ex vivo* with PMA and ionomycin for 3 hours prior to intracellular cytokine staining. (A) Representative gating strategy and (B) cumulative data showing the frequencies for the IL-17, IFN γ , TNF α and

IL-10-expressing cells within CD4⁺ T cells as determined based on fluorescence-minus-one (FMO) control. Each symbol represents an individual donor. Lines show the mean with SEM. Log-transformed data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test.

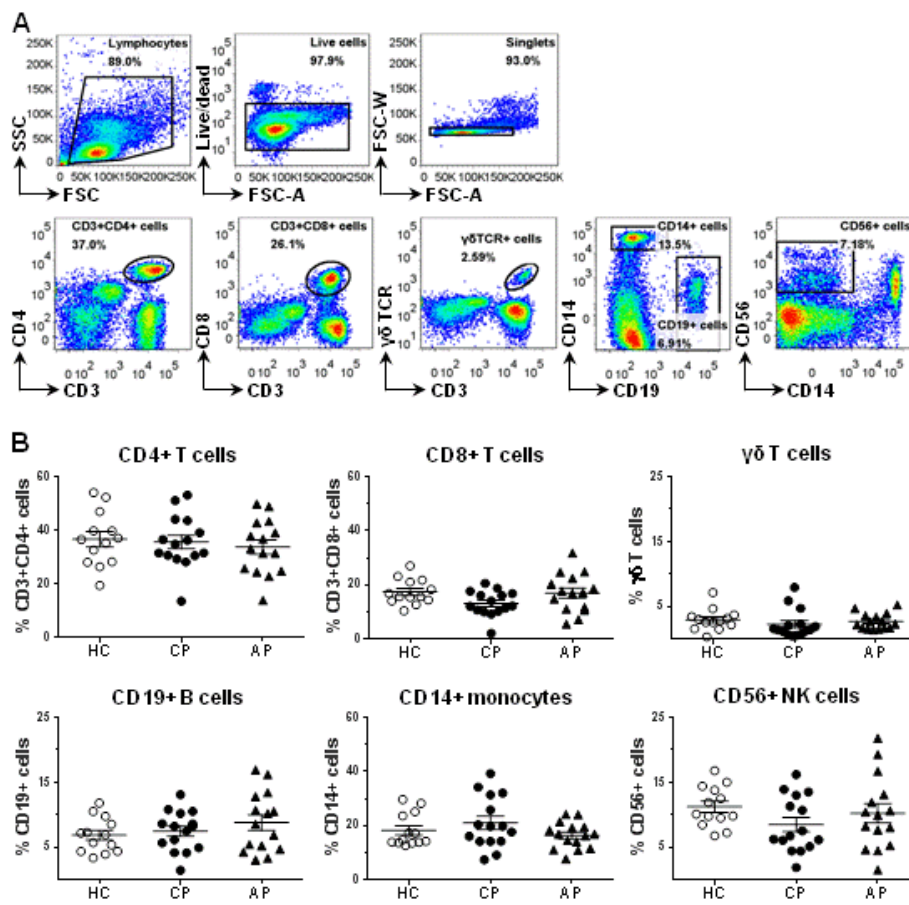
Figure 5. Frequencies of cytokine producing cells CD8⁺ and $\gamma\delta$ T cells in PBMC from healthy controls and patients with periodontal disease. PBMC were isolated from peripheral blood from patients with chronic periodontitis (CP, n=15) or aggressive periodontitis (AP, n=15) as well as periodontally healthy controls (HC, n=13). (A, B) Representative dot plots and cumulative data for the frequencies of IL-17⁺, IFN γ ⁺ and TNF α ⁺ cells within CD8⁺ T cells (A) or $\gamma\delta$ T cells (B). Each symbol represents an individual donor. Lines show the mean with SEM. Log-transformed data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test.

Figure 6. Cytokine levels in serum from patients with periodontitis or gingivitis, or from healthy controls. Serum from patients with chronic periodontitis (n=15) or aggressive periodontitis (n=15) as well as periodontally healthy controls (n=13) was analysed. Cytokine levels in 25 μ l of serum were determined by Luminex. Each symbol represents an individual subject; the median and interquartile range is shown, dotted lines indicate the minimum

detectable concentration of that particular mediator. Log-transformed data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test. *P<0.05.

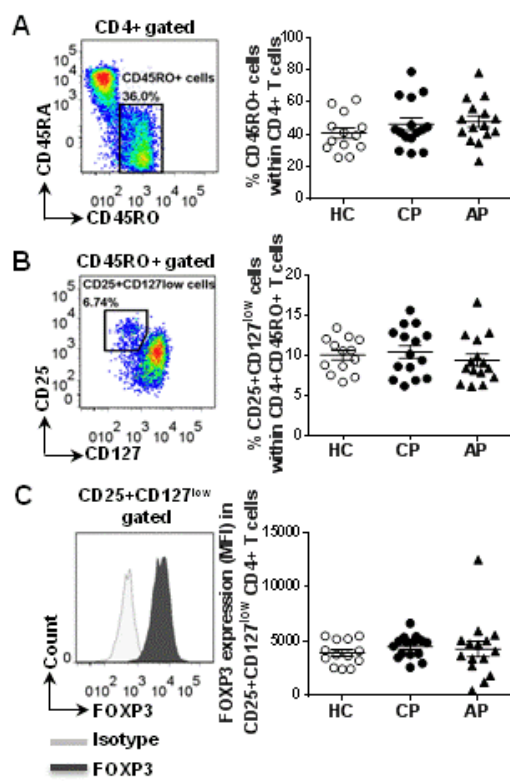
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Figure 1



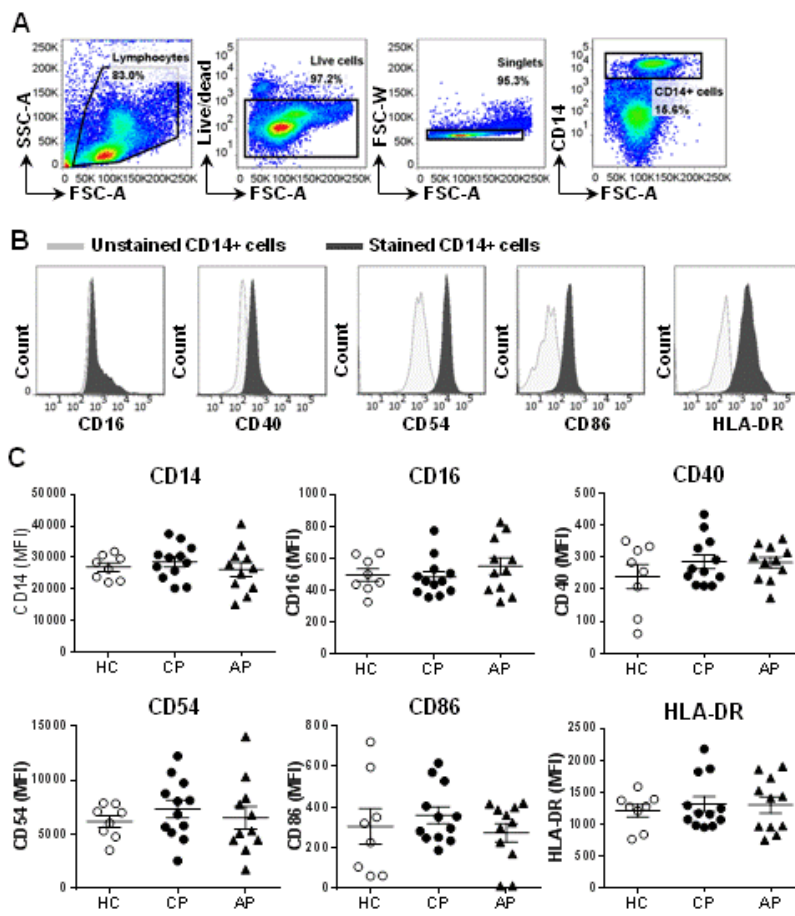
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Figure 2



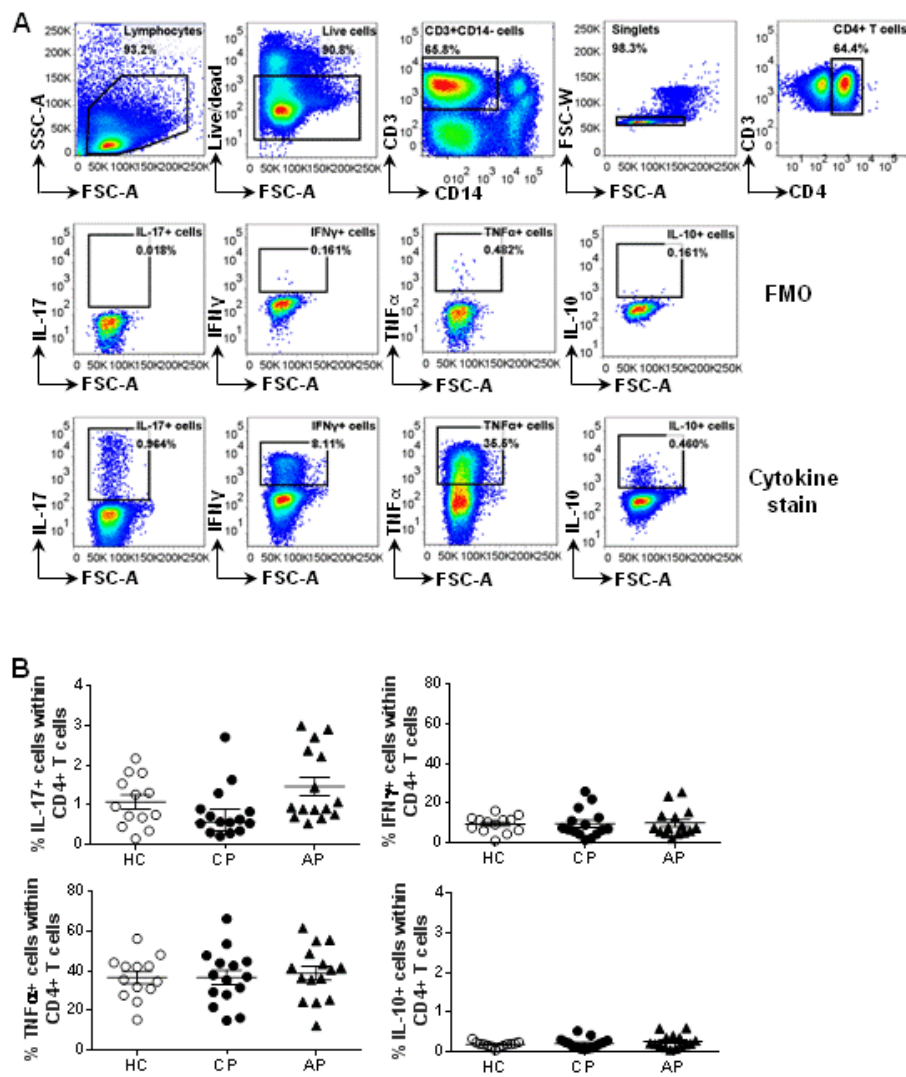
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Figure 3



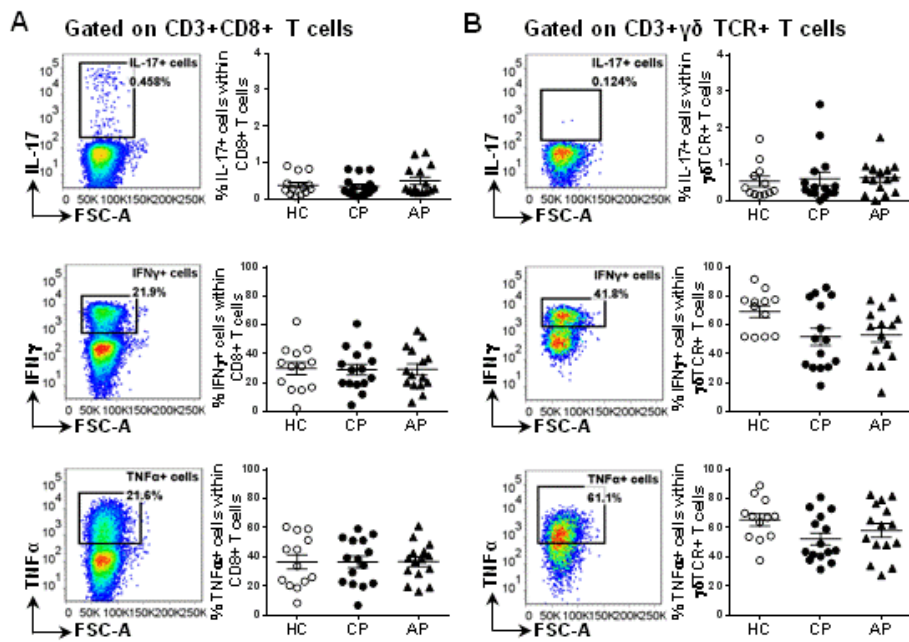
Cheng et al. Figure 3

Figure 4



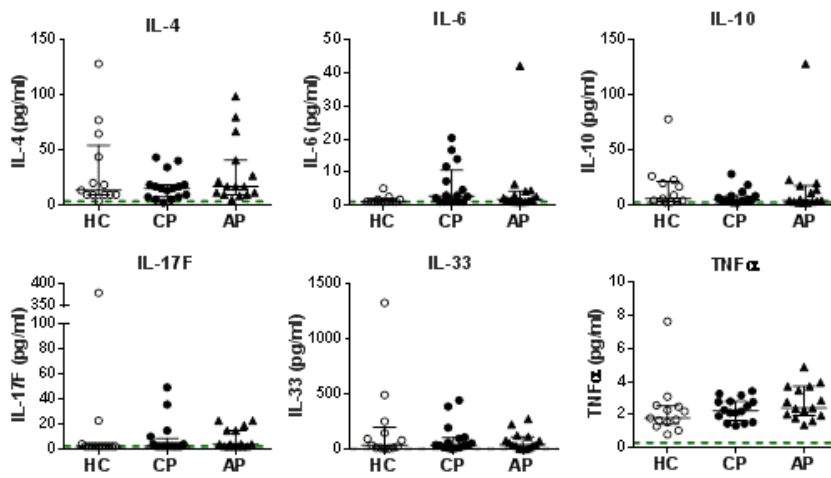
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Figure 5



Cheng et al. Figure 5

Figure 6



Cheng et al. Figure 6