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Detection and consistency of mucosal fluid T lymphocyte phenotypes and their relationship with blood, age and gender

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ABSTRACT

Innate and adaptive immune responses at mucosal surfaces play a role in protection against most infectious diseases. However, the relative importance either of mucosal versus systemic, or of cellular versus humoral immunity in protection against such infections remains unclear. We aimed to determine the relative percentages and reproducibility of detection of five major T lymphocyte phenotypes in stimulated whole mouth fluid (SWMF); to compare matched mucosal and blood phenotypes; to evaluate the consistency of phenotypes in SWMF over time; and to determine any associations with age or gender. Peripheral blood and SWMF samples were collected from 194 participants and sequential concomitant samples were collected from 27 of those and from 12 subjects living with HIV. CD3, CD4, CD8, Th1 and Th2 T lymphocyte phenotypes were determined by FACS. All the five T lymphocyte phenotypes were detected consistently by FACS in PBMC and SWMF with experimental replicates ($N = 10$; PBMC CV: 3–30%; SWMF CV: 12–36%). In longitudinal samples detection rates were reproducible in both fluids but variations were higher in SWMF (CV: 23–79.6%) than PBMC (CV: 9.7–75%). Statistically significant correlations of the percentages of all the T lymphocyte phenotypes except CD8 was seen between the two fluids. In PBMCs a negative correlation with age was found with CD3, CD4 and CD8 phenotypes, whilst a positive correlation was found in both SWMF and PBMC with the Th2 phenotype. CD3, CD4 and CD8 phenotypes in SWMF and PBMCs from an HIV-positive cohort were not significantly correlated in contrast with the HIV-negative controls. Our study provides a robust FACS protocol for the detection of the five major T lymphocyte phenotypes in SWMF which should prove useful for research with other mucosal fluids.

1. Introduction

There is increasing evidence for the importance of both innate and adaptive immune responses at mucosal surfaces in protection against infectious diseases including COVID-19. However, the relative importance either of mucosal versus systemic immunity, or of cellular versus

Abbreviations: SWMF, stimulated whole mouth fluid; PBMC, peripheral blood mononuclear cells; COVID-19, coronavirus disease 2019; SARS-CoV2, severe acute respiratory syndrome coronavirus 2; HIV, human immunodeficiency virus; FACS, fluorescence-activated cell sorting; PBS, phosphate buffered saline; CD, cluster of differentiation; SE, standard error; SD, standard deviation.

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humoral immunity in protection against viral infections remains unclear (Russell and Mestecky, 2022). It is known that in viral diseases T lymphocytes and cytokines are altered in both systemic and mucosal immunity but these factors have not been much explored in the mucosal response of the upper aero-digestive tract. Several viral diseases target sites in mucosa including salivary glands which can be infected by mumps and SARS-CoV2 (Liu et al., 2011; Xu et al., 2020).

The role of oral mucosal immunity has been well elucidated in a number of oral and systemic diseases (Feller et al., 2013; van Splunter et al., 2018; Naiff et al., 2014). The host immune response elicited by SARS-CoV2 upon dysregulation leads to high morbidity and mortality. Thus, the role of mucosal immunity in the pathogenesis of COVID-19 may be of paramount importance. Although a number of host immune factors including cytokines (notably IL-6, IL-1β, IFN-γ) and T lymphocyte phenotypes (Th1, Th2, Th-17, Tregs, Tfh cells) have been implicated in a few reports to play important roles in systemic immunity to SARS (Chen and Wherry, 2020; McElvaney et al., 2020), their role in mucosal immunity remains largely unexplored.

There has been a realisation in recent years that the distribution of T lymphocytes in mucosae differs from that found systemically, shown particularly in several mucosal diseases including candidiasis (Russell and Mestecky, 2022) and suggested in SARS-CoV2 infection (Clancy, 2023). The distribution of major leucocyte types in crevicular fluid was reported by Skapski and Lehner in 1976, and work on polymorphonuclear cells in both crevicular fluid and saliva showed that they had functional activity (Wilton et al., 1976; Scully, 2016). The percentage of T and B lymphocytes and monocytes as leucocyte subsets in saliva assayed by flow cytometry was first described by Vidovic and colleagues (Vidović et al., 2012). T-cell subsets were not determined. Whole mouth fluid is a complex hypotonic fluid representing the secretions of both major and minor salivary glands, crevicular fluid and serum transudates across epithelium. Major Tlymphocyte subtypes (CD3, CD4 and CD8) have been examined by FACS in cervical mononuclear cells obtained by cytobrush, and cytokines, but not cells, examined in a corresponding vaginal lavage (Juno et al., 2014). Identification of leucocyte types by FACS in vaginal lavage including CD4 and CD8 cells have been reported (Giraldo et al., 2012), and CD8 subtypes in bronchial lavage by Alexandrova et al. (2024).

The aims of this study were to establish whether FACS was a suitable method for enumerating immune cells in hypotonic mucosal fluids by a) determine the relative percentages and reproducibility of detection of five major T lymphocyte phenotypes in stimulated whole mouth fluid (SWMF); b) to compare mucosal T lymphocyte phenotypes with those in concomitant blood samples; c) to evaluate the consistency of phenotypes in SWMF over time; and d) to determine whether values changed in relation to age or differed according to sex. We also wished to examine T lymphocyte phenotypes in subjects known to be living with HIV and to determine if mucosal fluid T lymphocyte distribution might be impaired.

2. Methods

The study was approved by VHS-Institutional Ethics Committee (proposal#: VHS-IEC/75–2021) and the UK Oxford Research Ethics Committee (proposal#:22/SC/0105). This was a multi-center study and after obtaining written informed consent, samples were taken from 194 participants from The Voluntary Health Services, Chennai, India and Guy's & Thomas' Hospital, London, UK. Of the 194 participants, the mean age was 46.5 years (range: 18–85 years), with 76 males and 118 females.

2.1. Samples

Concomitant peripheral blood and stimulated whole mouth fluid (SWMF) samples were collected. SWMF is a mix of stimulated saliva from the major and minor salivary glands and crevicular fluid. For SWMF sample collection, patients were asked to chew a sterile paraffin wax without swallowing for 5 min and drool the collected saliva into a wide mouthed Genefix calibrated container containing preservative solution (Isohelix, UK). The volume collected was recorded. Up to 10 ml of blood were collected by venipuncture. Peripheral blood was stored at room temperature, while SWMF samples were stored at 4 °C until further processing. All samples were processed within 5–6 h of collection.

2.2. Reproducibility

Ten SWMF and concomitant blood samples were divided into two, and each sample assayed independently. The individual variation was calculated and the mean variation for each T lymphocyte phenotype determined.

2.3. Biological consistency

Second blood and SWMF samples were collected in a subset of 27 individuals after four weeks and T lymphocyte phenotypes assayed.

2.4. HIV group

Samples were collected from 12 patients living with HIV and on long term anti retroviral therapy. CD4 counts were within laboratory normal range (27–45%) in 11/12 patients.

2.5. FACS analysis of T lymphocyte phenotypes

Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficol gradient (HiSep gradient, HiMedia, India and Histopaque, Sigma-Aldrich, USA). Fifty microlitres of the isolated PBMC were used in each tube for T cell phenotyping. The volumes of the SWMF samples were noted, mixed well and centrifuged at 1500 rpm for 10 min. The pellet was then resuspended in 1.5 ml volume of the sample and the remaining supernatant was stored as 1 ml aliquots at −80 °C. Fifty microlitres from the resuspended pellet were used in each tube for T lymphocyte phenotyping. In a subset of five SWMF samples, 10 μl of the resuspended deposit were loaded onto haemocytometers to determine the total cell count.

2.6. T lymphocyte phenotyping

Cells were stained with mouse anti-human antibodies (BD Biosciences, USA) against CD3, CD4 and CD8 (APC mouse anti-human CD8: Clone: HIT8a; PerCP mouse anti-human CD4: Clone: L200; PE mouse anti-human CD4: Clone SK3; V450 mouse anti-human CD3:Clone: UCHT1 (BD Biosciences, USA) for 15 min at room temperature in the dark according to the manufacturer's instructions. The cells were then washed in cold 1× Dulbecco's phosphate buffered saline (PBS) after pelleting at 1500 rpm for 10 min at 4 °C. The cells that were to be stained for the transcription factors T-bet (Th1) and GATA-3 (Th2) were permeabilised with 1× FixPerm solution and washed in 1× transcription buffer (BD Biosciences, USA) as per manufacturer's instructions. The cells were then stained at 4 °C for 40 min with anti-human T-bet and anti-human GATA-3 antibodies (Alexa Fluor 488/Alexa Fluor 647 mouse anti-GATA3: Clone: L50–823; Alexa Fluor 647 mouse anti-T-Bet: Clone: 4B10, BD Biosciences, USA). Concentrations of the antisera were optimized using the manufacturer's instructions to 1.5-2μg/ml and the same concentration used for both SWMF and blood samples. Cells were washed in transcription buffer and finally resuspended in 1× Dulbecco's PBS. Identification of the cells was finally acquired in BD FACS Lyric or BD FACS CantoII machines (BD Biosciences, USA) and analysed using the BD FACS Suite or FLOWJO software.

2.7. SWMF cell population gate and cell viability

To identify and optimize the leucocyte populations in SWMF samples, PerCP mouse anti-CD45: Clone: 2D1 and FITC mouse anti-human CD3: Clone HIT3a (BD Biosciences, USA) were used in the initial samples as per above surface markers protocol. To check for cell viability rates, random SWMF samples were stained with 2 μl of 7-AAD (BD Biosciences, USA) just before acquisition.

2.8. Statistics and data analysis

Statistical analyses were performed using python3. Details on the specific tests applied and *p* values for each dataset are described in figure legends. *Multivariate analysis:* the effect of age and sex on T-lymphocyte phenotypes was assessed using the *statsmodels* package implemented in python3. For each cell type an ordinary least squares (OLS) model was constructed to assess whether cell count was dependent on age or sex.

3. Results

3.1. Optimization of T cell phenotyping using SWMF samples by FACS

The various cell types in SWMF include large numbers of epithelial cells, polymorphonuclear leucocytes and immune cells. An average cell count of the rounded cells in the resuspended SWMF deposit was equivalent to 8.6 × 10^5 cells/ml of SWMF, as determined in five random samples from healthy controls (range: 3.5 × 10^5–18.9 × 10^5 cells/ml).

A viability check of the leucocytes in SWMF samples was performed using 7-AAD stain during the initial optimization experiments rather than in all samples. In order to stain for the transcription factors T-bet and GATA-3 permeabilisation of cells is required, and so usual viability stains cannot be used since they leach from the dead cells into the permeabilised cells (Tario Jr and Wallace, 2014). In order to account for the quality of the SWMF samples tested because of a practical time delay of 5–6 h between sample collection (the recruitment and sample collection process takes about 4–5 h for 3–4 participants) and processing, we checked the viability of the leucocytes in the SWMF samples at two

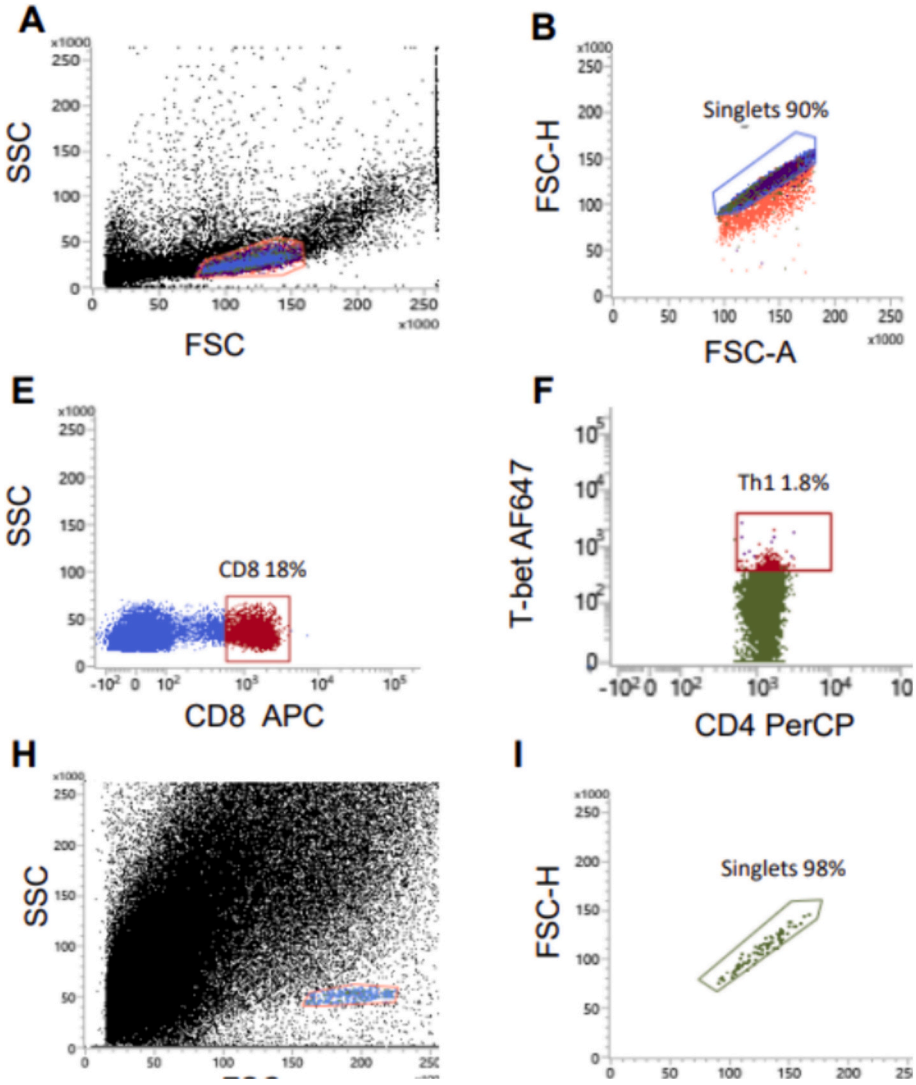

Fig. 1. Representative dot plots showing the gating strategy for flow cytometry using peripheral blood mononuclear cells (PBMCs) and stimulated whole mouth fluid (SWMF) samples. The cellular marker and the fluorochrome used are shown along the corresponding axes. A-G: PBMC sample. H-N: SWMF sample. The percentages of the CD3, CD4, CD8, Th1 and Th2 lymphocytes are shown in the corresponding dot plots adjacent to the cell label. Linear scale was used for forward scatter (FSC) and side scatter (SSC) axes. All the fluorescent channels are depicted in logarithmic scale. All percentages were calculated over the total number of cells in the initial gate of the FSC/SSC dot blots of the corresponding samples.

hours (90%) and five hours (80%) from sample collection (Supplemental fig. 1). Although there was a small decrease of 10% in the viability, the relative percentages of CD3 and CD4 T lymphocytes was similar at both time points, suggesting a proportionate impact of viability on various T lymphocyte phenotypes (Supplemental fig. 1). Hence all sample collections were done in the morning and processed by the same afternoon.

3.1.1. Gating strategy

The same gating strategy was used for both the PBMC and SWMF samples. The forward scatter (FSC) threshold was kept at 10,000 in order to gate out the dead cells from the FSC/SSC (side scatter) plot. The same FSC/SSC/fluorochrome voltage settings and compensation settings were used for both the PBMCs and SWMF samples. The leucocyte gate for SWMF samples were set in the same FSC/SSC position as in the PBMC (lymphocyte gate). In order to check the accuracy of this gate, a few initial samples were stained with CD45 and CD3 markers. The CD3+ T lymphocyte percentage (23%) gated over SSC/CD45+ cells was similar to the CD3+ T lymphocytes (21%) gated over FSC/SSC gate (Supplemental fig. 2). In the case of SWMF samples, since the gated population does not separate in the same way as gradient separated PBMCs, this population was considered as the leucocyte population, as shown in Fig. 1. For PBMCs, first the lymphocyte population was gated, then singlets were gated. For SWMF samples, the singlets were gated directly from the leucocyte population (Fig. 1). From the singlets gate, the CD3, CD4 and CD8 T lymphocytes were analysed. The Th1 and Th2 cells were gated upon the $CD4^+$ T lymphocytes. Since CD3 is a pan T lymphocyte marker, the CD4, CD8, Th1 and Th2 T lymphocyte percentages were also calculated as a percentage of the total CD3 T lymphocytes in the corresponding samples in a subset of 27 samples over two time points.

3.2. Mean and range of T lymphocyte phenotypes in SWMF and blood

In SWMF samples from 194 participants, the mean percentages of CD3–15%, CD4–10% and CD8–4% were compared with those in blood: CD3–65%, CD4–40% CD8–20% (Fig. 2A). The mean percentage of total lymphocytes (± SE) was lower in SWMF than in PBMC samples, but was expressed as a percentage of leucocytes, whereas in PBMC values were expressed as a percentage of lymphocytes. The combined CD4 plus CD8 values equaled the CD3 T lymphocyte value in both fluids. Th1 (mean percentage: 1.5%) and Th2 (mean percentage: 0.5%) phenotypes were both detectable in SWMF and there was a wide range of values, in spite of the low mean counts. Blood values for Th1 and Th2 were also very low with mean percentages of 1.35% and 0.61% respectively.

When CD4, CD8, Th1 and Th2 percentages were expressed as a percentage of the CD3 counts (Table 3), CD4 percentages were very similar between the two fluids, whereas CD8 values were lower in SWMF and both Th1 and Th2 values were higher in SWMF than in PBMCs.

3.3. Reproducibility of techniques

The reproducibility of testing was analysed in duplicates from 10 samples of both SWMF and blood and this showed a variation of <7% in blood for the major cell phenotypes (CD3, CD4, CD8), but was greater for Th1 and Th2 phenotypes (17% and 30% respectively; Fig. 2B). In general the mean of individual variances was greater in SWMF for all the major T lymphocyte phenotypes with 12 and 13% for CD3 and CD4, 24% for CD but over 35% for both Th1 and Th2 (Table 1). Statistically significant correlations between duplicates of all five phenotypes were found in both fluids ($p < 0.001$; Fig. 2B 2B).

3.4. Consistency within fluids over time

The individual consistency of T lymphocyte values in SWMF was determined by repeating the analysis with samples from 27 participants after a period of four weeks. Significant correlations were seen with all five T cell phenotypes in both SWMF and blood (from $p < 0.05$ for Th2 to $p < 0.001$ for Th1, CD3, CD4 and CD8, Fig. 3). For CD4 and CD8, mean variances in SWMF were 25% and 35% respectively (compared with 17% and 20% in blood) and for Th1 and Th2 were 61% and 79% respectively (63% and 75% in blood; Table 2).

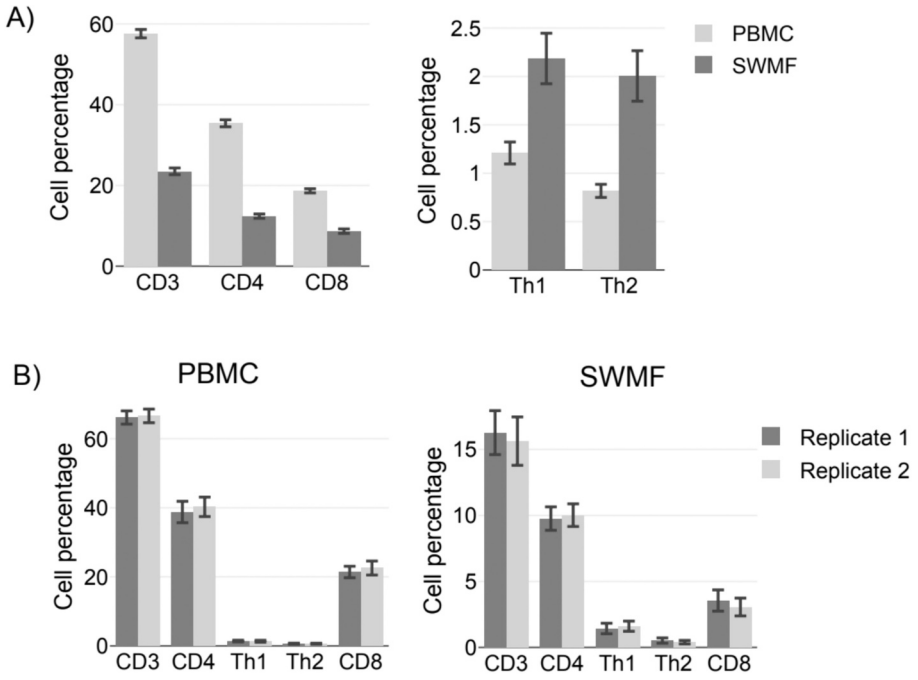

Fig. 2. T lymphocyte phenotypes as a percentage of lymphocytes in peripheral blood mononuclear cells (PBMC) and leucocytes in stimulated whole mouth fluid (SWMF). A) Overall percentages of the major T lymphocyte phenotypes. Mean ± SE of CD3, CD4, CD8, Th1, Th2 T lymphocytes in PBMC and SWMF ($N = 194$). B) Reproducibility of T lymphocyte phenotyping in PBMC and SWMF ($N = 10$ replicates). Ten samples of each fluid were divided into two and each assayed separately. Significance set at <0.05 using Wilcoxon rank-sum test.

Table 1
Reproducibility in the identification of T lymphocyte phenotypes in SWMF (stimulated whole mouth fluid) and in PBMC (peripheral blood mononuclear cells). T lymphocyte phenotypes expressed as a percentage of lymphocytes in PBMC and leucocytes in SWMF. Samples ($N = 10$ for each fluid) were divided into two and each assayed separately.

T lymphocyte phenotypes	Mean	SD	SE	Mean % individual replicate variance
PBMC				
CD3	66.39	6.31	1.35	3.14
CD4	39.52	9.63	2.05	7.69
CD8	21.96	6.02	1.28	7.18
Th1	1.35	0.83	0.18	17.23
Th2	0.62	0.5	0.11	30.4
SWMF				
CD3	15.95	5.68	1.21	12.48
CD4	9.89	2.83	0.6	13.34
CD8	3.3	2.42	0.52	24.29
Th1	1.52	1.27	0.27	38.77
Th2	0.45	0.56	0.12	35.68

SD = standard deviation; SE = standard error. The individual coefficients of variation of replicates were calculated and the mean shown.

3.5. Comparison of individual values in SWMF and blood

A comparison was made on the relationship between individual level of T lymphocyte phenotypes in SWMF and blood in the cohort of 194 participants. Using linear regression, these showed significant positive correlations with CD3 ($p < 0.05$), CD4, ($p < 0.03$) but interestingly no significant relationship with CD8 (Fig. 4). Spearman rank correlations were used for Th1 and Th2 since they did not show normal distributions and significant positive relations between blood and SWMF were evident with Th1 (rho 2.9, $p < 0.001$) and with Th2 (rho 0.23, $p < 0.01$; Fig. 4).

3.6. Relationship of T lymphocyte phenotypes with gender and age

An analysis of the association of T lymphocyte phenotypes in blood in relation to gender showed no significant mean differences between males and females for four of the five cell phenotypes examined but a higher mean percentage in Th1 values in males ($p < 0.05$) in blood but not SWMF (Fig. 5).

In peripheral blood, there was a gradual lessening of values with age to give significant negative correlations with CD3 ($p < 0.005$) and CD8 ($p < 0.001$). However, there appeared to be a significant positive correlation with blood Th2 percentages ($p < 0.05$; Fig. 6), but no obvious changes with age for either CD4 or Th1 phenotypes. In SWMF no

Table 2
Consistency of individual T lymphocyte phenotype concentrations in SWMF and PBMC over a period of 4 weeks. T lymphocyte phenotypes as a percentage of lymphocytes in PBMC and leucocytes in SWMF. (N = 27 for each fluid). SD = standard deviation; SE = standard error. Variance was calculated as a within-subject coefficient of variation that allows comparison of variance between visit 1 and visit 2 for each cell type. The variance for the cell type of each individual was calculated and the mean variance determined.

T lymphocyte phenotypes	Mean	SD	SE	Mean % individual variance
PBMC				
CD3	55.44	14.6	1.99	9.65
CD4	30.12	11.54	1.57	17.25
CD8	20.78	7.48	1.02	20.16
Th1	1.05	0.68	0.09	63.01
Th2	0.57	0.49	0.07	75.19
SWMF				
CD3	19.43	6.35	0.86	23.01
CD4	11.14	4.53	0.62	25.55
CD8	5.11	3.17	0.43	34.96
Th1	1.48	1.59	0.22	61.25
Th2	0.66	0.65	0.09	79.6

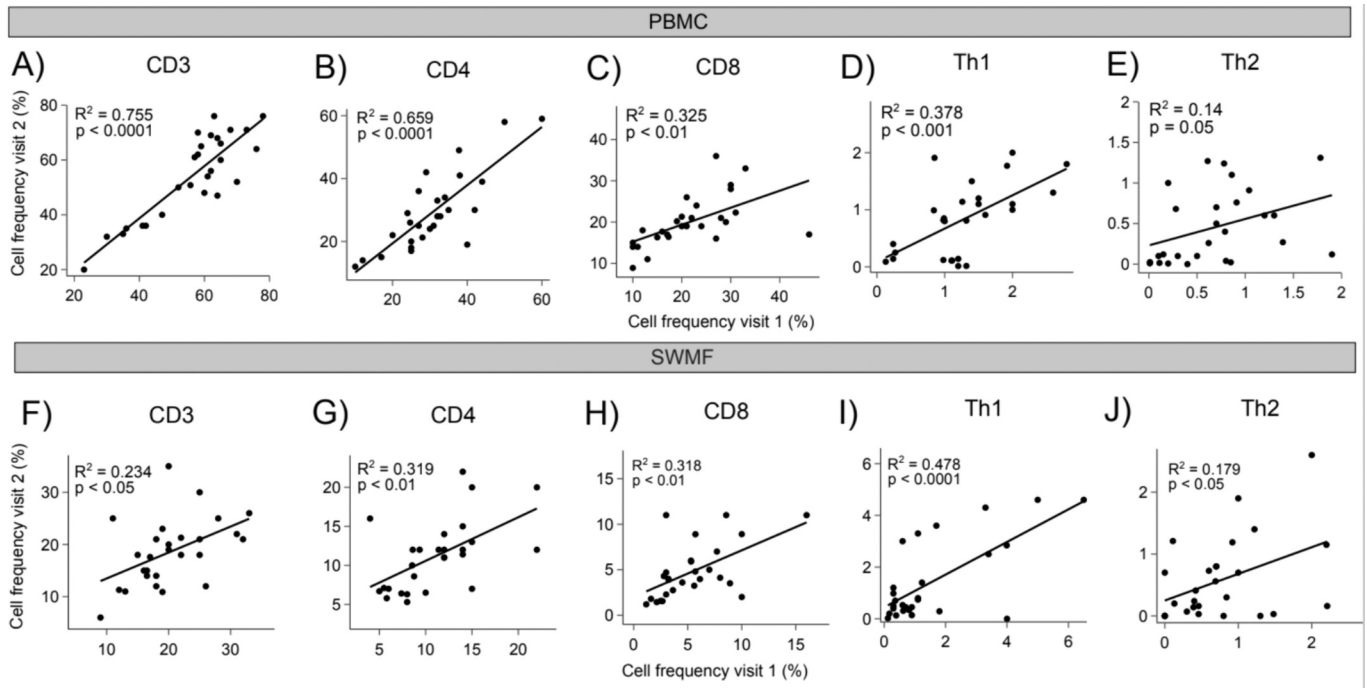

Fig. 3. Longitudinal assessment of T lymphocyte phenotypes in PBMC and SWMF. Consistency of individual phenotype percentages in PBMC and SWMF samples taken four weeks apart ($N = 27$). Panels show correlations between samples at visit 1 and at visit 2 four weeks later. Values are percentages of lymphocytes (PBMC) or leucocytes (SWMF). Linear regression correlation coefficients and statistical significances are shown. A) CD3 T lymphocytes in PBMC. B) CD4 T lymphocytes in PBMC. C) CD8 T lymphocytes in PBMC. D) Th1 T lymphocytes in PBMC. E) Th2 T lymphocytes in PBMC. F) CD3 T lymphocytes in SWMF. G) CD4 T lymphocytes in SWMF. H) CD8 T lymphocytes in SWMF. I) Th1 T lymphocytes in SWMF. J) Th2 T lymphocytes in SWMF.

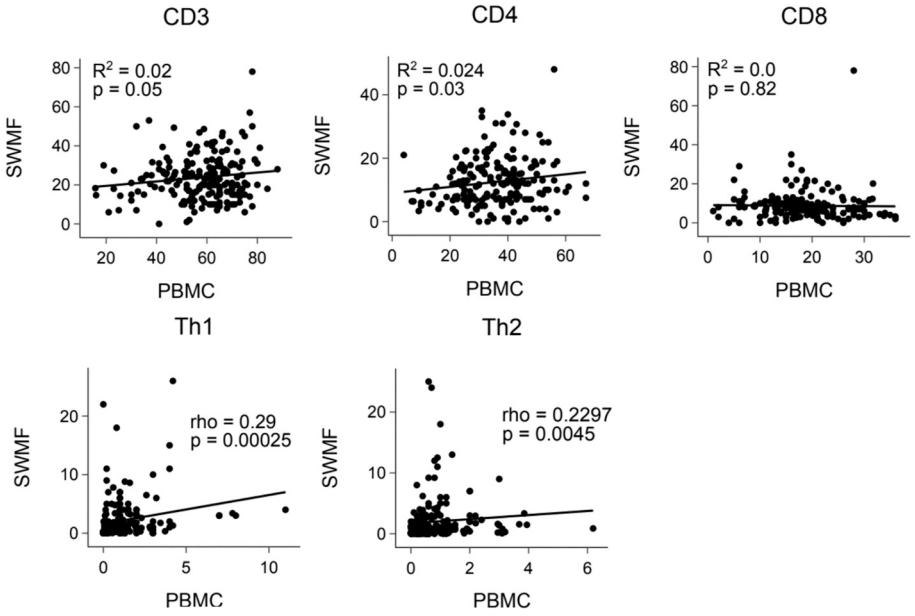

Fig. 4. Relationship of individual T lymphocyte phenotypes in SWMF and PBMC in 194 participants. Y axis: SWMF T lymphocyte percentages of each phenotype as a proportion of leucocytes and X-axis: PBMC T lymphocytes as a percentage of total lymphocytes. Linear regression R^2 value and significances are shown for CD3, CD4 and CD8 T lymphocytes. For Th1 and Th2 T lymphocytes, Spearman rank correlation rho values and their corresponding significances are shown.

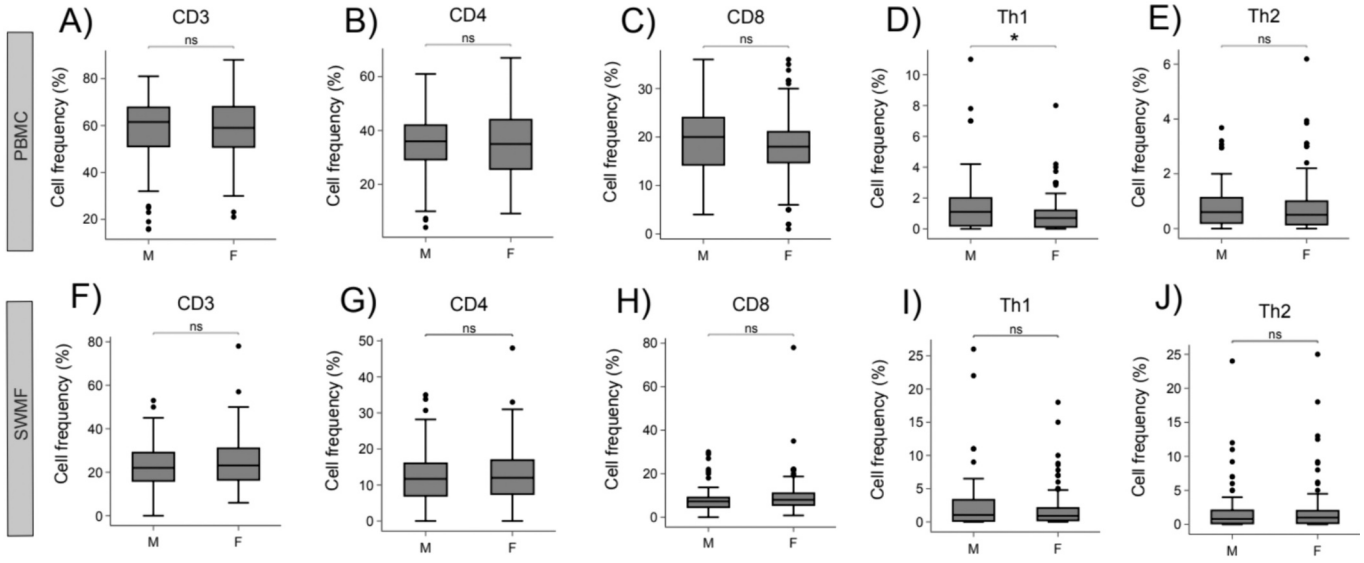

Fig. 5. Percentage of T lymphocyte phenotypes in PBMC and SWMF with relation to gender. $N = 194$ participants. M = male ($n = 76$), F = female ($n = 118$). * $p < 0.02$ for Th1 T lymphocytes in PBMC; n.s. = not significant for all other panels using Mann-Whitney U test. A) CD3 T lymphocytes in PBMC. B) CD4 T lymphocytes in PBMC. C) CD8 T lymphocytes in PBMC. D) Th1 T lymphocytes in PBMC. E) Th2 T lymphocytes in PBMC. F) CD3 T lymphocytes in SWMF. G) CD4 T lymphocytes in SWMF. H) CD8 T lymphocytes in SWMF. I) Th1 T lymphocytes in SWMF. J) Th2 T lymphocytes in SWMF.

obvious relationship between the percentages of four of the five T lymphocyte phenotypes and age was apparent except for a positive correlation was seen with Th2 ($p < 0.05$; Fig. 6).

Multivariate analysis of demographic information as confounding variables of cell type abundance showed that most cell types are not dependent on age and sex. In PBMC, CD3, CD8 and Th2 abundance were significantly associated with age (p-value <0.05) while only Th2 was associated with age in SWMF. The only significant association with sex was Th1 in PBMC. (Table 4).

3.7. Major T lymphocyte phenotypes in HIV-positive patients taking long-term ART

In SWMF, mean CD4 T lymphocyte percentages in the HIV group were lower than in the control series (6% vs 10%; $p < 0.01$) whereas CD8 percentages in SWMF in the HIV group and controls were not significantly different (11% vs 9%; Fig. 7A). The mean CD3 T lymphocyte percentages in SWMF (22% vs 20%) or blood (61% vs 58%) were similar in the HIV group and the overall series. Blood CD4 T lymphocyte percentages were similar. The CD8 mean percentage in blood was higher in the HIV group compared with the control group (24% vs 18%; $p < 0.02$; Fig. 7A). Whilst comparisons of individual CD3, CD4 or CD8 values in

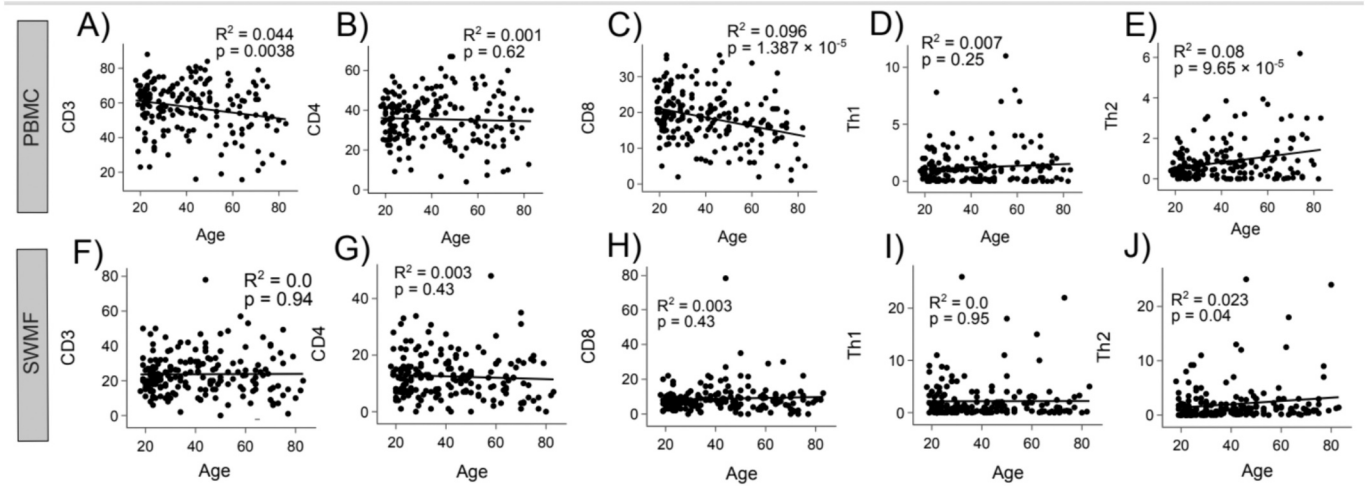

Fig. 6. Relationship between percentage values of T lymphocyte phenotypes in PBMC and SWMF with relation to age in 194 subjects. Linear regression correlations: Regression lines, R^2 values and significances are shown. A) CD3 T lymphocytes in PBMC. B) CD4 T lymphocytes in PBMC. C) CD8 T lymphocytes in PBMC. D) Th1 T lymphocytes in PBMC. E) Th2 T lymphocytes in PBMC. F) CD3 T lymphocytes in SWMF. G) CD4 T lymphocytes in SWMF. H) CD8 T lymphocytes in SWMF. I) Th1 T lymphocytes in SWMF. J) Th2 T lymphocytes in SWMF.

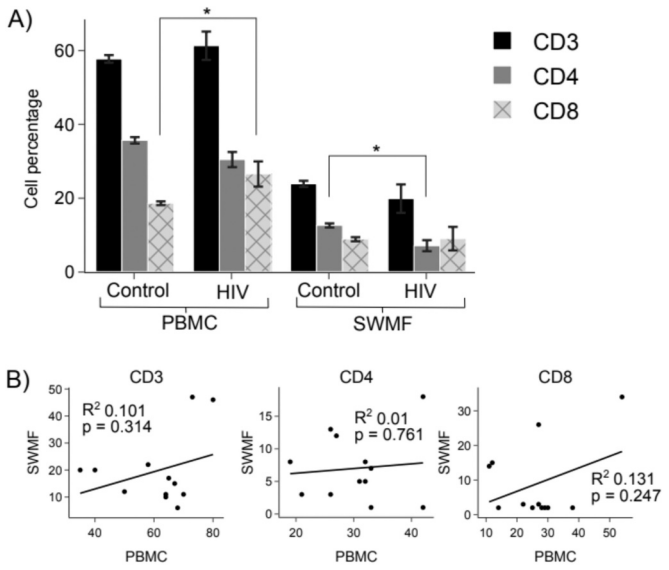

Fig. 7. Major T lymphocyte phenotypes in PBMC and SWMF of persons living with HIV infection ($N = 12$), compared with series control group ($N = 194$). A) Percentages of T lymphocyte counts in PBMC and SWMF. Mean ± SE of CD3, CD4 and CD8 T lymphocytes are shown. $^*p < 0.02$ using Wilcoxon rank-sum test. B) Linear regression correlations between PBMC and SWMF in HIV positive patients. Linear regression R^2 value and corresponding significances are shown for CD3, CD4 and CD8 T lymphocytes.

blood and SWMF suggested positive correlations, none were statistically significant (Fig. 7B).

4. Discussion

This study shows that five major T lymphocyte phenotypes can be detected in a mucosal fluid on a reproducible basis. Stimulated whole mouth fluid (SWMF) is a hypotonic fluid and there is increasing recognition that saliva can be useful as a diagnostic fluid in many infectious and autoimmune diseases, especially as its collection is non-invasive, simple and without the requirement for extensive training for collection associated with e.g. blood (Ji et al., 2014; Russell and Mestecky, 2022). However, standardized and validated methods for assessment of mucosal immunity in clinical settings remain limited (Wang et al., 2020), though some nasal, vaginal and bronchial fluid sampling techniques provide a less invasive technique for those mucosal fluids (Alexandrova et al., 2024, Giraldo et al., 2012, Jochems et al., 2017, Thwaites et al., 2018). Measurement of mucosal responses to infectious agents such as SARS-CoV2 may indicate both disease and its severity.

Analysis of autoimmune antibodies in saliva can be diagnostic in conditions such as mucous membrane pemphigoid and pemphigus (Ali et al., 2016a; Ali et al., 2016b) and the detection of specific secretory IgA antibodies can indicate involvement of mucosae in the generation of immune responses (Russell and Mestecky, 2022; Clancy, 2023; Tsai, 2023). More recently saliva has been a valuable mucosal fluid to analyze cytokine responses indicating both systemic and mucosal immunity (Liukkonen et al., 2016; Gupta et al., 2024). However, very few studies have examined the role of lymphocyte phenotypes in mucosal fluids such as saliva in relation to disease. This probably reflects the lack, until recently, (Vidović et al., 2012; Gillum et al., 2017) of the methodology to analyze the fluid cellular compartment, along with the observation that in a hypotonic fluid, there may be loss and disintegration of leucocytes, necessitating speed of assessment or fixation.

Determination of leucocyte subsets in human saliva by flow cytometry was nicely demonstrated by Vidović et al. (2012) who concluded that flow cytometry could be used as a reproducible method for the analysis of leucocyte types in human saliva including granulocytes, monocytes and lymphocytes. They showed that the predominant leucocyte cell type was polymorphonuclear. Percentages of mononuclear cells ranged from 0.3% to 7.2%, with monocytes comprising the highest percentage, followed by T lymphocytes and B lymphocytes. With the described gating strategy, CD3 T lymphocyte percentages obtained in this study were similar to those obtained in saliva samples by Vidović et al. (2012) and Gillum et al. (2017). This suggests that the FACS optimization and defined gating strategy in our study are reliable and reproducible. The current study extends these observations and demonstrates that it is possible to quantify T lymphocyte subtypes in a mucosal fluid, analyze their consistency over time and show that the determinations can be reproducible. Studies using FACS for the identification of leucocyte populations in vaginal lavage and cervical mononuclear cells (Giraldo et al., 2012) and CD8 lymphocyte subtypes in bronchial lavage (Alexandrova et al., 2024) have also shown the potential value of mucosal fluids in the analysis of host immune responses.

The origin of salivary leucocytes is likely to be mainly from the crevicular fluid (Skapski and Lehner, 1976; Wilton et al., 1976) though

reports have suggested that lymphocytes can also be found in parotid saliva, especially associated with diseased salivary glands (Selifanova et al., 2020). The numbers of mononuclear and polynuclear cells in saliva have been shown to be related to the extent of gingival inflammation (Aps et al., 2002; Coopman et al., 2014) irrespective of age or gender (Sreenivasan and Prasad, 2022). In spite of being contained in a hypotonic fluid, PMNs in saliva have been shown to be capable of active phagocytosis, although at a lower level than in blood (Scully, 2016). It is generally assumed that the lymphocytes are also increased in oral inflammation in a similar way, suggesting an upregulation reflected by increased concentrations of salivary T lymphocyte cytokines in relation to periodontitis (Medara et al., 2021).

In general, the percentages in blood for T lymphocyte phenotypes in this study were consistent with those in the literature (Bisset et al., 2004; Uppal et al., 2003). We found that mucosal T lymphocytes ($CD3^+$) comprised on average 17% of the total leucocyte population which compares well with other studies using similar techniques (Gillum et al., 2017), but values for Th1 and Th2 cells using flow cytometry were not readily available. For the three major T lymphocyte phenotypes (CD3, CD4 and CD8) in blood the mean variance was 7% or less. Mean Th1 and Th2 proportions in blood were 1.4% and 0.6% respectively, with greater variances of 17% and 30% respectively, reflecting these very low mean values. The reproducibility of our methodology was determined by duplication of samples and examining each independently (Table 1).

Variances in SWMF samples were, as expected, generally greater than in blood and were approximately 13% for CD3 and CD4, 24% for CD8 but over 35% for both Th1 and Th2 (Table 1). With Th1 and Th2 there was a wider range of values in spite of the low mean counts, and it will be of interest to determine if the higher values might reflect responses to concurrent mucosal or systemic disease. When expressed as a percentage of CD3 positive cells in SWMF, Th1 at 7% and Th2 at 3% appeared to be greater than in PBMCs, suggesting that values of these phenotypes on mucosal fluids may not always be directly related to those in blood (Table 3).

The consistency of individual mucosal T lymphocyte phenotype values in SWMF has not been previously reported to our knowledge. We repeated sampling after 4 weeks in 27 participants (Fig. 3, Table 2). Significant correlations between duplicates of all five phenotypes were found in both fluids ($p < 0.001$) with mean of individual variances between 23 and 34% for the three main phenotypes compared with 9–20% in PBMCs. This suggests that T lymphocyte phenotypes in SWMF can be usefully sampled on a sequential basis and that SWMF may be a relevant fluid to determine mucosal immunity in relation to infectious disease.

One of the key objectives of this study, in addition to verifying a method to reproducibly measure T lymphocyte phenotypes in SWMF, was to determine the relationship between such phenotypes in blood and those in mucosal fluids, in this case SWMF. Overall, there were significant positive correlations between the percentages of CD3, CD4 ($p < 0.01$) and with Th1 and Th2 phenotypes ($p < 0.005$) in blood and SWMF (Fig. 4). However, no obvious correlation with CD8 lymphocyte values was apparent (Fig. 4), suggesting that mucosal phenotype distribution is not necessarily a direct reflection of those in blood. This was supported by the finding that proportionate distribution of Th2 and Th1 lymphocytes as a percentage of CD3 positive cells appeared to be significantly increased in SWMF compared with PBMC (Table 3). Comparison of blood and mucosal lymphocyte cytokines may be of interest in confirming and expanding these findings.

Analyses of blood T lymphocyte phenotypes in relation to gender have been reported in the literature. In a study of Indian blood donors, females had significantly higher CD4 counts ($p < 0.05$), percentage of CD4 lymphocytes ($p < 0.01$), and CD4:CD8 ratio ($p < 0.01$; Uppal et al., 2003). They also found that males had a significantly higher percentage of CD8 lymphocytes ($p < 0.01$). However, we were not able to confirm these results and with the methodology used found no significant mean differences for any of the five T lymphocyte phenotypes examined except a possible increase in Th1 values in males in blood (Fig. 5). With SWMF, analysis of samples from 76 males and 118 females revealed no differences in relation to gender with any of the five T lymphocyte phenotypes.

A general diminution in systemic immune responses have been reported in relation to age, and have been suggested as a major factor in the increased susceptibility of elderly persons to infectious diseases (Zheng et al., 2022; Costagliola et al., 2021). For example, T lymphocyte lymphocytopenia is regarded as an omnipresent predictor of morbidity and mortality in consequence of SARS-CoV disease (Putter and Seghatchian, 2023) and with the marked increase in risk of death with age in SARS-CoV2 infection it could be an important predictor.

In the current study, significant negative correlations with age were found in peripheral blood CD3 ($p < 0.005$), and CD8 ($p < 0.001$) T lymphocyte percentages and interestingly a weak but significant positive correlation was found with Th2 values ($p < 0.05$; Fig. 6). The effect of aging on Th2/Th1 ratios in blood has been investigated in other studies and our findings in PBMC are in agreement with previous research that demonstrated a higher Th2/Th1 ratio in older individuals (Mansfield et al., 2012). These studies highlighted a shift in immune and inflammatory responses, which supports the observation of higher susceptibility to certain infections in older individuals.

Although a number of studies have investigated mucosal tissue lymphocytes in relation to age, we are unaware of studies examining the relationship of mucosal fluid T lymphocyte phenotypes with age although reduced expression of some nasal mucosal cytokines in adults compared with children has been reported (Koch et al., 2022) and Thome et al. (2014) demonstrated by T cell mapping increased compartmentalisation over time. In this study in SWMF, a weak positive correlation was seen with Th2 and age ($p < 0.05$) as found in blood by Mansfield et al. (2012) but values of the other phenotypes did not appear to be changed with age and did not seem to follow the trends found in blood (Fig. 6). These findings were supported by a multivariate analysis of demographic information as confounding variables of cell type abundance which showed that most cell types are not dependent on age and sex. (Table 4) ().

Differences in the T lymphocyte subsets have been studied in relation to ethnicity but Uppal et al. (2003) also did not find any statistically significant differences in those of Dravidian heritage versus those of Aryan heritage. In the current study, the pooled population was drawn from different ethnicities and further studies may reveal differences between them.

Differences in CD8 distribution were also found in the HIV positive participants. Blood levels of CD4, as expected, were significantly lower than in controls whereas mean CD8 values were slightly higher. In SWMF, mean CD4 T lymphocyte percentages in the HIV group were significantly lower than in the overall series whereas CD8 values were similar (Fig. 7). In contrast to the main series, we did not find a significant relationship between the levels in SWMF and blood of CD3 and CD4, but trends were similar. This may reflect more variability or the smaller numbers of patients seen. Assuming that the origin of lymphocytes in SWMF is mainly from blood, these findings might indicate that

Table 3
T lymphocyte phenotypes in SWMF and in PBMC expressed as percentage of CD3 cells (values are mean ± standard deviation). $N = 27$. Visit 1 at day 0, visit 2 at four weeks.

	PBMC		SWMF	
	Visit 1	Visit 2	Visit 1	Visit 2
CD4	54.78 ± 14.17	53.63 ± 12.50	54.64 ± 13.38	61.21 ± 15.23
CD8	39.75 ± 20.07*	37.99 ± 9.38	27.85 ± 15.06*	26.76 ± 14.56*
Th1	2.33 ± 1.27	1.54 ± 1.31	7.16 ± 6.34**	7.19 ± 7.50**
Th2	1.34 ± 1.54	0.87 ± 0.96	3.77 ± 3.00**	3.54 ± 4.62*

* CD8 in SWMF lower than in PBMC ($p < 0.02$).
** Th1 ($p < 0.001$) and Th2 ($p < 0.01$) significantly greater in SWMF than in PBMC.

Table 4
Multivariate analysis of the effect of age and sex on T- lymphocyte phenotypes in SWMF and PBMC. Significance values shown.

Multivariate analysis				
	PBMC		SWMF	
	Age	Sex	Age	Sex
CD3	0.0028	0.7016	0.9701	0.2628
CD4	0.6245	0.6121	0.4104	0.5764
CD8	1.11E-05	0.1737	0.4406	0.1810
Th1	0.2429	0.0048	0.9661	0.2856
Th2	0.0001	0.9961	0.0407	0.8965

transfer to mucosal tissues and into SWMF may be impaired in treated persons living with HIV.

Together, these findings suggest that five major T lymphocyte phenotypes can be detected reproducibly in a mucosal fluid, are relatively consistent in value over time, may differ in distribution from those in blood, are similar in males and females and in contrast to blood, were not found to decrease with age.

5. Conclusions

Several methods have been developed in research laboratories for measuring aspects of mucosal immune responses and there has been a need for standardized techniques. This study shows that five major T lymphocyte phenotypes can be detected in a mucosal fluid on a reproducible basis and can therefore be potentially analysed in assessment of mucosal T lymphocyte related immunity along with cytokines. Stimulated whole mouth fluid is representative of the whole oral cavity and possibly of other mucosal fluids. CD3, CD4 and CD8 T lymphocytes were abundant and could be assessed with reproducible accuracy in SWMF. Th1 and Th2 cells were of low concentrations but could also be assessed reproducibly. Longitudinal studies revealed that concentrations of all five T lymphocyte phenotypes in SWMF showed consistency over time. Significant correlations between values in blood and SWMF were found with CD3, CD4, Th1 and Th2 T lymphocytes ($p < 0.01$ to 0.05) but interestingly no significant relationship with CD8 T lymphocytes. CD3 and CD T lymphocyte values showed a negative correlation with age in blood, but Th2 showed a positive relationship with age in both fluids. No firm relation was apparent in SWMF with other cell types with age or was there any gender specific differences detected. Transfer to mucosal tissues and into SWMF may be impaired in treated persons living with HIV. Studies of T lymphocyte phenotypes in mucosal fluids can be performed reproducibly and may reveal important relationships with mucosal diseases.

CRediT authorship contribution statement

Shervin Dokht Sadeghi Nasab: Writing – review & editing, Resources, Investigation, Data curation. **Muruganantham Lillimary Eniya:** Writing – review & editing, Resources. **Albert Judith:** Writing – review & editing, Resources. **Frederick Clasen:** Formal analysis, Data curation. **Beulah Faith:** Writing – review & editing, Resources. **Selvamuthu Poongulali:** Resources. **Jayaraman Bhagavad Gita:** Writing – review & editing, Resources. **Chakrapani Ashok:** Resources, Investigation. **Velmurugan Raghavi:** Resources. **Subramanian Vedavalli:** Resources. **Chandra Lavanya:** Writing – review & editing, Resources. **Kannan Ranganathan:** Writing – review & editing, Funding acquisition, Conceptualization. **Gunaseelan Rajan:** Writing – review & editing, Funding acquisition, Conceptualization. **Nagalingeswaran Kumarasamy:** Writing – review & editing, Funding acquisition, Conceptualization. **David Moyes:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Mark Ide:** Writing – review & editing, Resources, Conceptualization. **Saeed Shoaie:** Formal analysis, Data curation. **Yuko Kurushima:** Resources. **Daljit Jagdev:** Resources. **Mina Pun:** Resources. **Newell Johnson:** Funding acquisition, Conceptualization. **Priya Kannian:** Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Stephen Challacombe:** Writing – original draft, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2024.113731.

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