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Peripheral neutrophil phenotypes during management of periodontitis

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ABSTRACT

Background and Objectives: Neutrophils are emerging as a key player in periodontal pathogenesis. The surface expression of cellular markers enables functional phenotyping of neutrophils which have distinct roles in disease states. This study aimed to evaluate the effect of periodontal management on neutrophil phenotypes in peripheral blood in periodontitis patients over one year.

Materials and methods: Peripheral blood and the periodontal parameters, mean probing depth and percentage of sites with bleeding on probing (%BOP), were collected from 40 healthy controls and 54 periodontitis patients at baseline and 3-, 6- and 12-months post-treatment. Flow cytometry was used to identify $CD11b^{+}$, $CD16b^{+}$, $CD62L^{-}$ and $CD66b^{+}$ expression on neutrophils, neutrophil maturation stages as promyelocytes ($CD11b^{-}CD16b^{-}$), metamyelocytes ($CD11b^{+}CD16b^{-}$) and mature neutrophils ($CD11b^{+}CD16b^{+}$), and suppressive neutrophil phenotype as bands ($CD16^{dim}CD62L^{bright}$), normal neutrophils ($CD16^{bright}CD62L^{bright}$) and suppressive neutrophils ($CD16^{bright}CD62L^{dim}$) .

Results: $CD62L^{-}$ expression decreased with treatment. No differences were observed in neutrophil maturation stages in health or disease upon treatment. Suppressive and normal neutrophils showed a reciprocal relationship, where suppressive neutrophils decreased with treatment and normal neutrophils increased with treatment. In addition, %BOP was associated with suppressive neutrophils.

Conclusion: This study demonstrates that management of periodontitis significantly modifies distinct neutrophil phenotypes in peripheral blood. Suppressive neutrophils may play a role in the pathogenesis of periodontitis. However, their exact role is unclear and requires further investigation.

KEYWORDS

Periodontitis; Periodontal debridement; Granulocyte Precursor Cells; Suppressive Neutrophils

INTRODUCTION

Neutrophils are a key player in the chronic inflammation seen in periodontitis. They are persistently recruited to the gingival crevice during inflammation, where they form a wall to block bacterial invasion into the underlying connective tissue[1]. Neutrophil extravasation through the post-capillary venules of the gingival plexus is tightly regulated through sequential expression of adhesion molecules. The initial stage of neutrophil adhesion from a freely circulating to a rolling state at reduced speeds is mediated by selectins, whereas integrins facilitate secondary 'firm' adhesion[2]. Once neutrophils reach the site of inflammation and are activated, they facilitate microbial clearance through a number of different processes including phagocytosis, generation of reactive oxygen species, release of granular contents, cytokine production and formation of neutrophil extracellular traps (NETs)[3].

Previous studies have investigated CD11b, CD16b, CD62L and CD66b membrane markers in periodontitis to evaluate neutrophil adhesion and activation. CD11b non-covalently binds to CD18 to form the functional integrin heterodimer CD11b/CD18, and is involved in neutrophil adhesion and migration through the endothelium. Activation of neutrophils leads to a rapid increase in surface expression[4,5], where CD11b/CD18 can also act as a complement receptor and transmembrane signalling adaptor[6]. CD62L (L-selectin) is part of the selectin family of cell adhesion molecules. It is expressed at high levels on resting circulating neutrophils[7], but is shed rapidly during activation[8]. The same stimuli that cause up-regulation of CD11b expression can induce an equally rapid loss of CD62L during inflammation through proteolytic cleavage of the extracellular domain[9]. CD16b (FcγRIIIb) is the low affinity receptor for IgG1 and IgG3. CD16b plays a role in recognition and phagocytosis of IgG opsonised bacteria which triggers neutrophil activation, pro-inflammatory cascade and the production and release of anti-microbial compounds[10]. CD66b is a GPI-anchored glycoprotein of the carcinoembryonic antigen family. CD66b is involved in regulating CD11/CD18 function[11-13], and CD66b cross-linking causes activation or priming in neutrophils and induces an increase in calcium and oxidative burst[14], exocytosis of specific granules[15], aggregate formation[16] and release of the pro-inflammatory cytokine IL-8[17,18].

Neutrophils develop from progenitor cells in the bone marrow in several stages before exhibiting the characteristic segmented nuclear appearance seen in mature cells. Immature granulocytes are present rarely or in low numbers in health[19], but their numbers increase in peripheral circulation during infection[20,21], inflammation[22,23] or neoplastic processes[24]. Bacteria are the primary aetiological agents in the development of periodontitis and the differential expression of CD11b and CD16 may

be used to distinguish the various stages of neutrophil maturation in response to the microbial challenge in periodontitis[25,26].

Neutrophils have previously been considered a homogenous population but mounting evidence suggests that distinct neutrophil subsets exist which have diverse roles in inflammation and cancer[27]. A subset of neutrophils with suppressive function can be identified based on the cell surface expression of CD16 and CD62L[28]. In addition to acute systemic inflammation, these $CD16^{bright}/CD62L^{dim}$ suppressive neutrophils were also detected in patients with allergic rhinitis[29], severe viral respiratory infections in infants[30], head and neck squamous cell carcinoma[31] and other neoplasms[32]. Suppressive neutrophils display an activated phenotype with a hypersegmented nucleus, mediate T cell regulation in a CD11b manner[28], form NETs, and migrate to tumour sites and perform anti-tumour functions such as inhibition of proliferation and induction of apoptosis in cancer cells[31].

Previous studies that have evaluated CD11b, CD16b, CD62L and CD66b surface expression in peripheral neutrophils have only done so in cross-sectional studies and report conflicting results. Higher CD11b[33] expression and lower CD62L[34,35] and CD16[36] expression in periodontitis compared to health have been reported, whereas other studies reported no significant differences between health and disease[37-41].

The aims of this study were three-fold. The first aim was to assess the longitudinal variation in the expression of the four adhesion and activation marker in periodontitis. Secondly, neutrophil maturation stage based on CD11b and CD16b expression was evaluated, where $CD11b^{-}CD16b^{-}$ neutrophil were promyelocytes, $CD11b^{+}CD16b^{-}$ neutrophils were metamyelocytes and $CD11b^{+}CD16b^{+}$ neutrophils were mature neutrophils. Finally, the suppressive neutrophil phenotype was evaluated, where $CD16^{bright}CD62L^{bright}$ neutrophils were phenotypically normal mature neutrophils, $CD16^{dim}CD62L^{bright}$ neutrophils exhibited a banded nuclear morphology of cells recently released from bone marrow (bands) and $CD16^{bright}CD62L^{dim}$ were suppressive neutrophils.

MATERIALS AND METHODS

Study population

The study was approved by the Human Ethics Sub-Committee at the University of Melbourne (1339812.3) and the Human Ethics Research Committee at Dental Health Services Victoria (279).

Sample size calculations were based on previously published work[42]. Informed written consent was obtained from each participant at the commencement of the study.

Fifty four subjects with periodontitis 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 the periodontitis group were at least two non-adjacent sites per quadrant exhibiting probing depths (PD) ≥5mm, excluding the third molars[43]. Subjects were >21 years, had a minimum of 16 teeth (excluding third molars) and were systemically healthy with no periodontal treatment or antibiotic use within the preceding 6 months. Exclusion criteria included pregnancy or lactation and medical conditions affecting the progression of periodontitis (e.g. diabetes) or requiring pre-medication prior to treatment.

For the control group (*n*=40), the inclusion criteria were a gender and age (±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, and peripheral blood were collected once from the healthy controls, and at baseline before treatment and 3-, 6- and 12-months during supportive periodontal therapy from the periodontitis group. Supportive periodontal therapy involved supra- and sub-gingival debridement every three months. PD and BOP were recorded at six sites around each tooth using a William's probe (Hu-Friedy Mfg. Co., Chicago, USA). PD was measured to the nearest millimeter from the base of the gingival sulcus to the free gingival margin. BOP was assessed visually following probing to the base of the sulcus and recorded as present or absent up to 30 seconds after probing. Smoking status was defined as current smoker or non-smoker at baseline.

Neutrophil phenotyping

Peripheral blood was collected in potassium-EDTA tubes (BD Biosciences, NSW, Australia) and whole blood (20µL) was incubated for 30 minutes at 4^0C protected from light with pre-diluted fluorochrome-conjugated monoclonal antibodies against CD11b-PECy7 (3µL, clone-ICRF44), CD16b-PE (7µL, clone-CLBgran11.5), CD62L-APC (10µL, clone-DREG-56) and CD66b-FITC (15µL, clone-G10F5). All antibodies were purchased from BD Bioscience and the volumes used were optimised based on manufacturer's recommendation. After incubation, red blood cell lysis was performed by adding 1mL of cold MilliQ water (MilliporeSigma, MA, USA) and vigorously pipetting up and down for 30 seconds. After 30 seconds, 1mL of 2x phosphate buffered saline (PBS) was added to stop lysis. The cells were centrifuged at 800RCF for 5 minutes at 2^0C and the supernatant aspirated. The cells

were resuspend in 200μL of PBS and acquired by flow cytometry on BD LSRFortessa™ X-20 (BD Bioscience, CA, USA). Data analysis was performed on FlowJo software (v10, Tree Star Inc., OR, USA). The gating strategy to phenotype neutrophils is displayed in figure 1. CD62L is rapidly lost on activation, thus, neutrophils without CD62L represent an activated phenotype.

Statistical analysis

Independent samples *t*-tests with unequal variances were used for comparison between health and periodontitis at each timepoint. Linear mixed models (LMMs) were used to determine changes in the mean of the outcome variable over timepoint. The base model for variation over timepoints included timepoint, age and sex as fixed effects and subject as random effect. Pairwise comparisons of estimated marginal means were used to assess significant differences. To assess the effect of periodontal management, mean PD and %BOP were added as fixed effects to the base model. Visual inspection of residual plots did not show any obvious deviations from normality. To assess difference in smoking status, independent samples *t*-tests with unequal variances were used for comparison between current smokers and non-smokers at each timepoint in health and periodontitis and smoking status was added as a fixed effect to the base LMMs. Spearman's correlations were used for associations with the periodontal parameters at each timepoint.

The level of significance was set at $p \leq 0.05$ for all variable. Statistical analyses were performed in SPSS (v23, IBM SPSS Statistics for Windows, IBM Corp., NY, USA) and graphs were prepared using GraphPad Prism (v8, GraphPad Software, CA, USA).

RESULTS

Cohort demographics

The cohort demographics for healthy controls and periodontitis subjects are displayed in table 1. No significant differences were observed between healthy and periodontitis subjects at baseline with regards to age, sex or smoking status. The periodontal parameters, mean PD and %BOP, were significantly higher at all timepoints in periodontitis compared to health. With treatment, both parameters significantly decreased at all subsequent timepoints compared to baseline.

CD11b, CD16b, CD62L and CD66b expression on neutrophils

Neutrophils and their expression of $CD11b^+$, $CD16b^+$, $CD62L^-$ and $CD66b^+$ are displayed in figure 2. Neutrophils were significantly higher at 3- and 6-months in periodontitis compared to health. $CD62L^-$ expression was significantly higher at 12-months compared to health, and with treatment, $CD62L^-$ expression significantly decreased at all subsequent timepoints compared to baseline. CD11b, CD16b and CD66b were all highly and constitutively expressed on neutrophils. There was a statistically significant increase in $CD66b^+$ expression at 6-months compared to baseline, but no differences were observed in $CD11b^+$ or $CD16b^+$ expression.

The CD16b antibody clone used in this study only bound to neutrophils expressing one of the two neutrophil antigen (NA) molecules. No statistical differences were observed between groups at all timepoints in the frequency of $CD16b^+$ expression (table 1) with regards to age, gender, mean PD or %BOP.

When using a combination of markers (figure 3), $CD16b^+CD66b^+CD11b^+$ identified 95% of neutrophils (mean±SD in health = 95.47±2.51 and baseline = 95.71±3.51). $CD16b^+CD66b^+CD11b^+CD62L^-$ cells, which represent an activated neutrophil phenotype, were significantly decreased at 3- and 6-months in comparison to health and baseline.

Smokers had significantly higher neutrophil proportions at baseline (mean difference (*d*)=9.56%) and 6-months (*d*=6.69%) in comparison to non-smokers in periodontitis (supplementary table 1).

Neutrophil maturation

Mature neutrophils were the most abundant cell type in peripheral blood, followed by metamyelocytes and promyelocytes (figure 4). These proportions of immature granulocytes and mature neutrophils remained constant in health and disease upon treatment.

Suppressive neutrophils

When phenotyping neutrophils based on CD16b and CD62L expression, normal neutrophils were the largest subset followed by suppressive neutrophils then bands (figure 5A). The small proportion of bands remained relatively constant in health and disease (figure 5B). Age was a significant predictor for bands in the base model for timepoint (estimate of fixed effect (*b*) = 0.03, standard error (SE) = 0.01), and a 1 year increase in age was associated with 0.03% increase in bands. Smoking status also

had an effect on bands where smokers had significantly lower proportions compared to non-smokers at 6-months (*d*=0.31%).

Normal and suppressive neutrophils behaved in reciprocal ways (figure 5C, D), where normal neutrophils were significantly increased and suppressive neutrophils were significantly decreased at 3- and 6-months post-treatment compared to health and baseline.

Association with periodontal parameters

Mean PD and %BOP were correlated to various neutrophil subsets (table 2). In health, %BOP was significantly positively correlated with $CD16b^{+}CD66b^{+}CD11b^{+}CD62L^{-}$ neutrophils (ρ=0.401). At 3-months, %BOP was significantly positively correlated with $CD16b^{+}CD66b^{+}CD11b^{+}$ neutrophils (ρ=0.505) and $CD11b^{+}CD16b^{+}$ mature neutrophils (ρ=0.468), and negatively correlated with promyelocytes (ρ=-0.547). At 6-months, mean PD was significantly negatively correlated with total neutrophils (ρ=-0.379), and at 12-months post-treatment, mean PD was significantly positively correlated with $CD66b^{+}$ expressing neutrophils (ρ=0.378) and $CD16^{bright}CD62L^{bright}$ normal neutrophils (ρ=0.514).

When mean PD and %BOP were added as fixed effects to the base statistical model, %BOP (*b*=0.22, SE=0.10) was a significant predictor for suppressive neutrophils and a 1% increase in %BOP was associated with a 0.22% increase in suppressive neutrophils.

DISCUSSION

Neutrophils are actively recruited to the gingival sulcus during periodontal inflammation. In normal conditions, 50% of peripheral neutrophils are marginating through post-capillary venules throughout the body[44]. Furthermore, neutrophil release from the bone marrow in response to proinflammatory stimuli is a rate-limiting step in the host response to pathogens[45]. Thus, a study of markers that facilitate neutrophil adhesion and activation will improve our understanding of the role of neutrophils in periodontitis.

CD62L or L-selectin is highly expressed on resting circulating neutrophils[7], but is shed during activation[8,9,46]. $CD62L^{-}$ expression was not significantly different between health and baseline in this study, however, there was a significant decrease post-treatment at all timepoints compared to

baseline. The greatest decrease in $CD62L^-$ expressing cells was between baseline and 3-months. Likewise, mature activated $CD16b^+CD66b^+CD11b^+CD62L^-$ cells decreased post-treatment compared to baseline. Previous studies that evaluated CD62L expression were cross-sectional and reported significantly lower expression in rapidly progressive periodontitis[34] and adult periodontitis[35] compared to healthy controls, while other studies observed no significant differences[37,40,47]. It is unclear at this stage as to what stimulus leads to the shedding of CD62L in peripheral blood. These neutrophils may have been activated due to systemic bacterial dissemination seen in periodontitis[48,49,50] or may represent a subset that is being actively recruited to periodontal sites. The latter scenario may be more likely as kinetic studies report that CD62L shedding is rapid and only 10-15% surface expression was found after 30 minutes[51,52]. It has been proposed that this shedding of CD62L may provide a rapid means for neutrophil de-adhesion necessary for detachment from endothelial cells before transmigrations into sites of inflammation[9].

Several CD16b clones are available for use in flow cytometry including ID3, REA589, 245514 and MM0272-5L11. The clone used in this study was CLBgran11.5 which binds with neutrophils expressing the NA1 molecule. CD16b monoclonal antibodies recognise the biallelic NA1/NA2 antigens, which differ in five nucleotides (nucleotide 141, 147, 227, 277 and 349) which leads to changes in four amino acids at positions 36, 65, 82 and 106, one of which is a silent mutation[53,54]. In this study, 64% of subjects in health and 58% in periodontitis at baseline were detected by the CD16b antibody representing the NA1NA1 homozygous forms. Although there were no differences in NA1NA1 expression in health and periodontitis groups, this effectively reduced the sample size for the study when CD16b was used in the analysis. CD16b is the low-affinity Fc receptor for immunoglobulin isotypes and is involved in adhesion and phagocytosis of opsonised bacteria. *In vitro* studies have reported that neutrophils homozygous for NA2 bind IgG3 less efficiently than NA1 homozygous neutrophils, and IgG1 or IgG3 opsonised particles are more efficiently ingested upon interaction with the NA1 allotype[55,56]. In periodontitis, a meta-analysis of fourteen studies reported that the NA2 phenotype was associated with an odd ratio of 2.01 for aggressive periodontitis compared to NA1, and the homozygous NA2NA2 phenotype was associated with an odd ratio of 1.40 for chronic periodontitis compared to the NA1NA1 and NA1NA2 phenotype[57]. Copy number variations of *FCGR3B* gene have also been reported to be associated with several other chronic inflammatory diseases, such as systemic lupus erythematosus[58], rheumatoid arthritis[59] and immune-mediated glomerulonephritis[60].

Neutrophils have traditionally been considered a homogenous population because of their short lifespan, restricted transcriptional activity and inability to reverse transmigrate into peripheral blood.

However, recent studies demonstrate that they exhibit plasticity and heterogeneity that enables them to respond in a flexible manner in different disease states[61-64]. One such subset is the $CD16^{bright}CD62L^{dim}$ suppressive neutrophil which displays a hypersegmented nuclear morphology and is involved in suppressing T cell proliferation via hydrogen peroxide release into the immunological synapse in a CD11b-dependent manner[28] and cell-cell contact through programmed death-ligand 1[65]. A recent study that used functional enrichment analysis showed that suppressive neutrophils were enriched in proteins involved in immune regulation[66] and interferon signalling[65]. In the present study, while there were no differences between health and baseline in periodontitis in both normal and suppressive neutrophils, these two subsets showed a reciprocal relationship upon treatment. Suppressive neutrophils significantly decreased with treatment at 3- and 6-months and were significantly associated with %BOP. This decrease with treatment may be associated with the decrease in inflammation in response to treatment. There is increased prevalence of systemic bacterial dissemination in periodontitis[50], and the magnitude of bacteraemia is associated with the number of sites with BOP[67]. The regulatory role of suppressive neutrophils may be required to restrain excessive and potentially harmful T cell activation in peripheral blood as a result of this bacteraemia and to maintain compartmentalisation between peripheral circulation and gingival tissues. At the resolution of inflammation at 12-months post-treatment, $CD16^{bright}CD62L^{bright}$ normal neutrophils were positively correlated with mean PD. Indeed, suppressive neutrophils showed lower bacterial containment[68] and may have immunoregulatory[61] rather than antibacterial functions. Further studies are needed to assess the functional role of suppressive neutrophils as well as their usefulness as a diagnostic or therapeutic marker in periodontitis.

Immature granulocytes were present at constant but low levels in health and during treatment in periodontitis. Promyelocytes and metamyelocytes have reduced effector functions compared to mature neutrophils[19] and their role in periodontitis is unclear. The chronic nature of periodontal disease and sampling well after establishment of plaque biofilm deposits may preclude detection of immature granulocyte release from bone marrow stores in this study. A previous study reported significantly more promyelocytes in early-onset periodontitis compared to health and suggested that there may be an abnormal haematopoietic mechanism involved in early-onset periodontitis pathogenesis[36].

Smoking had an effect on neutrophil proportions where smokers had higher total neutrophil at baseline and 6-months compared to non-smokers. Higher neutrophil proportions in peripheral blood of smokers were also observed in other studies conducted in healthy subjects[69-71]. Smoking has several effects on neutrophil functions including impairment of migration and chemotaxis[72],

phagocytosis[73] and respiratory burst[74,75], and these effects maybe more evident in diseased gingival tissues. This study found no differences between smokers and non-smokers in $CD62L^-$ or CD11b expression which facilitates neutrophil entry into gingival tissues. However, this study only characterised current smokers and non-smokers, and previous history of smoking or the duration required to return to baseline after smoking cessation, and smoking quantity and history in current smokers may also affect expression of cell surface markers. Another limitation of this study is the small number of smokers in the cohort which did not allow for more robust statistical comparison to be made. Our results show that a larger prospective study with well-defined smoking categories are needed to expand and corroborate our results.

In this study as well as others[37,39,76], no significant differences were observed in CD11b, CD16b and CD66b expressing neutrophils in health and disease in peripheral blood. However, others have reported significant differences in the expression of these markers in oral neutrophils in comparison with peripheral neutrophils[77-80]. Oral neutrophils may provide a more dynamic picture of cell subsets involved in periodontitis as these neutrophils are at the interface between periodontal tissues and the oral environment. Indeed, a previous study showed that neutrophils from sites with periodontal disease display increased number of gene changes compared to oral neutrophils in healthy periodontium[81]. Further studies that track the migration of activated and suppressive neutrophils from peripheral blood to the gingival tissues may provide a greater understanding of the role of these cells in periodontitis.

Several factors may affect extracellular surface marker expression on neutrophils. For example, CD62L expression was shown to be increased during active bone marrow release[82], but neutrophil ageing[83] and several pharmaceuticals like non-steroidal anti-inflammatory drugs[84] and steroids[85] cause a loss of surface CD62L. Antigens such as CD10, CD11b, CD13, CD16 and CD66b have large intracellular storage pools and experimental manipulations can mobilise these from their intracellular location and increase their surface expression[86,87]. Furthermore, neutrophil apoptosis could increase surface expression of some antigens, such as CD13, and decrease others such as CD15 and CD16[88].

In conclusion, this study found that CD11b, CD16b and CD66b were all highly expressed on neutrophils, whereas $CD62L^-$ was significantly decreased upon treatment. There was no variation in immature granulocyte proportions with treatment. Suppressive and normal neutrophils showed a reciprocal relationship upon treatment where suppressive neutrophils were decreased and normal neutrophils were increased at 3- and 6-months compared to baseline. To the best of our knowledge,

this is the first study that evaluated the longitudinal variation of these neutrophil phenotypes in peripheral blood in periodontitis.

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FIGURE & TABLE LEGENDS

Table 1. Cohort demographics and periodontal parameters.

† significantly different to health

‡ significantly different to baseline

§ significantly different to 3-months post-treatment

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

Table 2. Correlations between periodontal parameters and neutrophil subsets.

Spearman's correlation coefficients (ρ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and various neutrophil subsets in health and at baseline, 3-, 6- and 12- months in periodontitis. Significant correlations ($p \leq 0.05$) are represented in bold.

Fig. 1. Gating strategy for phenotyping neutrophils.

Neutrophils **(A)** were identified based on forward scatter area (FSC-A) and side scatter area (SSC-A). Doublets were excluded using FSC-A and forward scatter height (FSC-H) followed by SSC-H and SSC-A. Single expression **(B)** of $CD11b^+$, $CD16b^+$, $CD62L^-$ and $CD66b^+$, as well as a combination of markers **(C)** as $CD16b^+CD66b^+CD11b^+$ and $CD16b^+CD66b^+CD11b^+CD62L^-$ were identified on neutrophils. Neutrophil maturation stages **(D)** were phenotyped as promyelocytes ($CD11b^-CD16b^-$), metamyelocytes ($CD11b^+CD16b^-$) and mature neutrophils ($CD11b^+CD16b^+$). Suppressive neutrophil

subsets **(E)** were phenotyped as bands ($CD16^{dim}CD62L^{bright}$), normal ($CD16^{bright}CD62L^{bright}$) or suppressive ($CD16^{bright}CD62L^{dim}$).

Fig. 2. Surface expression of neutrophil markers.

Neutrophils **(A)** (as a percentage of total cells) and $CD11b^+$ **(B)**, $CD16b^+$ **(C)**, $CD62L^-$ **(D)** and $CD66b^+$ **(E)** expressing cells (as a percentage of neutrophils) in health and periodontitis at baseline, 3-, 6-, and 12-months. Box and whisker plots represent the median, interquartile range and 10^{th} – 90^{th} percentile; ⌐¬ above the plots represent $p \leq 0.05$.

Fig. 3. Combinations of neutrophil surface markers.

$CD16b^+CD66b^+CD11b^+$ **(A)** and $CD16b^+CD66b^+CD11b^+CD62L^-$ **(B)** expressing neutrophil subsets (as a percentage of neutrophils) in health and periodontitis at baseline, 3-, 6-, and 12-months. Box and whisker plots represent the median, interquartile range and 10^{th} – 90^{th} percentile; ⌐¬ above the plots represent $p \leq 0.05$.

Fig. 4. Neutrophil maturation stages.

Bar graph showing neutrophil maturation stages **(A)** and box and whisker plots showing promyelocytes **(B)**, metamyelocytes **(C)** and mature neutrophils **(D)** (as a percentage of neutrophils) in health and periodontitis at baseline, 3-, 6-, and 12-months. Bars with error bars represent mean and standard deviation (SD); † all subsets are significantly different ($p \leq 0.05$) to each other within the same timepoint. Box and whisker plots represent the median, interquartile range and 10^{th} – 90^{th} percentile; ⌐¬ above the plots represent $p \leq 0.05$.

Fig. 5. **Suppressive neutrophil subsets.**

Bar graph showing suppressive neutrophil subsets **(A)** and box and whisker plots showing bands **(B)**, normal **(C)** and suppressive neutrophils **(D)** (as a percentage of neutrophils) in health and periodontitis at baseline, 3-, 6-, and 12-months. Bars with error bars represent mean and SD; † all subsets are significantly different ($p \leq 0.05$) to each other within the same timepoint. Box and whisker plots represent the median, interquartile range and 10^{th} – 90^{th} percentile; ⌐¬ above the plots represent $p \leq 0.05$.

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Table 1. Cohort demographics and periodontal parameters.

	Health (*n* = 40)	Periodontitis			
		Baseline (*n* = 54)	3-months (*n* = 46)	6-months (*n* = 44)	12-months (*n* = 37)
Sex (*n*, % male)	14 (35%)	20 (37%)			
Age at baseline (years, mean ± SD)	49.30 ± 10.62	53.28 ± 11.44			
Smokers (*n*, %smokers)	6 (15.0%)	15 (27.8%)	12 (26.1%)	10 (22.7%)	7 (18.9%)
CD16b present (n, % present)	25 (62.5%)	31 (57.4%)	25 (54.3%)	23 (52.3%)	19 (51.4%)
Periodontal parameters					
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†‡

† significantly different to health

‡ significantly different to baseline

§ significantly different to 3-months post-treatment

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

Table 2. Correlations between periodontal parameters and neutrophil subsets.

Neutrophil phenotypes	Health		Periodontitis							
			Baseline		3-months		6-months		12-months	
	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP	Mean PD	%BOP
Total Neutrophils	0.068	0.243	-0.073	-0.226	0.039	-0.050	**-0.379**	-0.343	-0.149	0.068
$CD11b^+$ neutrophils	-0.077	-0.066	-0.033	0.131	-0.052	0.212	-0.296	-0.223	0.193	0.315
$CD16b^+$ neutrophils	0.136	-0.063	0.142	-0.034	0.091	0.445	0.208	0.226	0.179	-0.086
$CD62L^-$ neutrophils	0.037	0.200	-0.161	-0.139	-0.176	-0.321	0.197	0.171	0.002	-0.187
$CD66b^+$ neutrophils	0.228	0.104	0.213	0.264	0.174	0.063	0.157	0.241	**0.378**	0.344
$CD16b^+CD66b^+CD11b^+$ neutrophils	0.094	-0.051	0.170	-0.057	0.143	**0.505**	0.205	0.290	0.217	-0.006
$CD16b^+CD66b^+CD11b^+CD62L^-$ neutrophils	0.209	**0.401**	-0.185	-0.264	0.172	-0.172	0.461	0.168	-0.325	-0.086
Promyelocytes ($CD11b^-$ $CD16b^-$)	0.020	-0.311	-0.162	0.127	-0.229	**-0.547**	0.081	0.194	-0.266	-0.113
Metamyelocytes ($CD11b^+CD16b^-$)	-0.010	0.218	-0.292	-0.133	-0.036	-0.372	-0.272	-0.393	-0.223	0.000
Mature neutrophils ($CD11b^+CD16b^+$)	0.124	-0.045	0.175	0.042	0.081	**0.468**	0.205	0.279	0.232	0.017
Bands ($CD16^{dim}CD62L^{bright}$)	-0.032	-0.255	-0.148	-0.112	-0.325	-0.198	-0.172	-0.388	-0.260	-0.308
Normal neutrophils ($CD16^{bright}CD62L^{bright}$)	-0.116	-0.238	0.098	0.095	-0.018	0.328	-0.316	-0.112	**0.514**	0.412
Suppressive neutrophils ($CD16^{bright}CD62L^{dim}$)	0.169	0.266	-0.098	-0.120	0.022	-0.084	0.446	0.171	-0.360	-0.236

Spearman's correlation coefficients (ρ) between the periodontal parameters, mean probing depth (PD) and percentage of sites exhibiting bleeding on probing (%BOP), and various neutrophil subsets in health and at baseline, 3-, 6- and 12- months in periodontitis. Significant correlations ($p \leq 0.05$) are represented in bold.

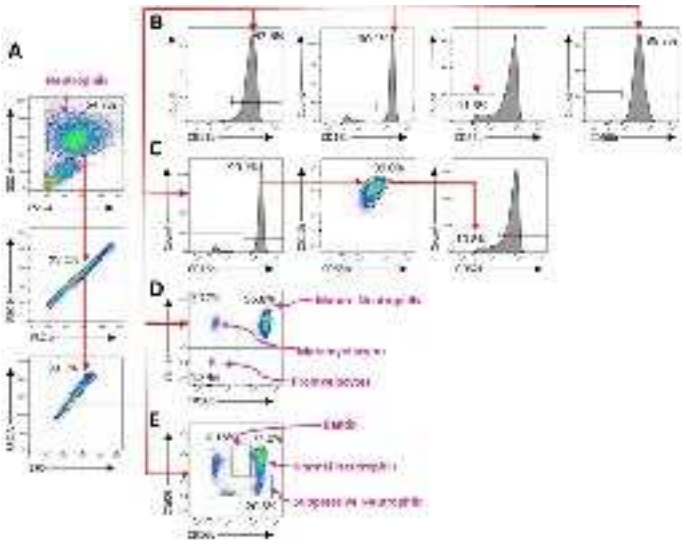

Fig. 1

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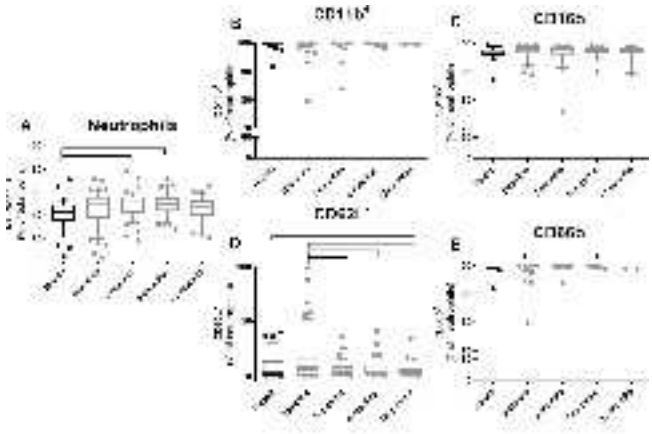

Fig. 2

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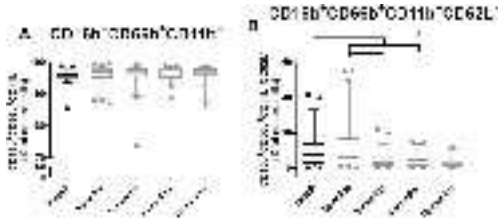

Fig. 3

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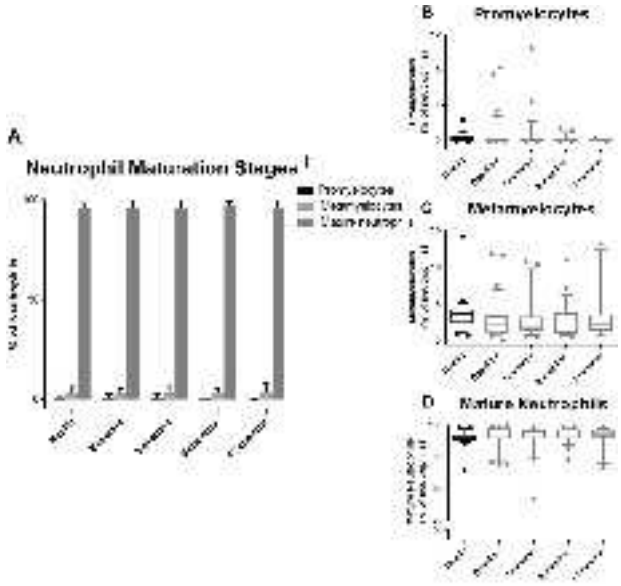

Fig. 4

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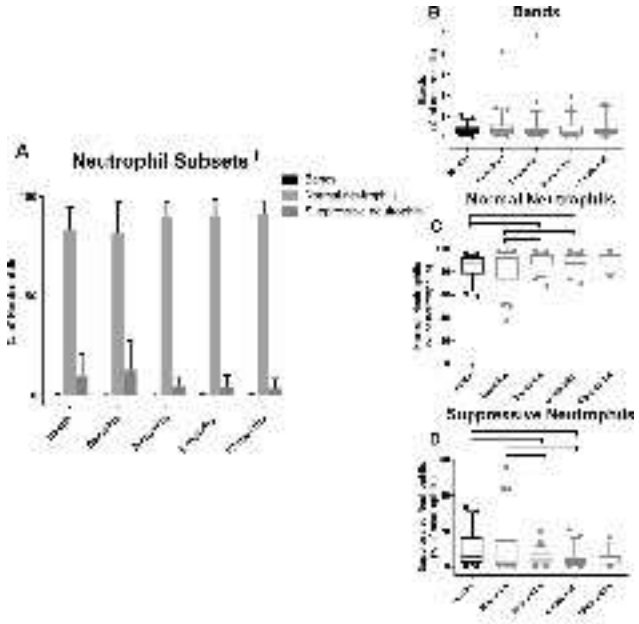

Fig. 5

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