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ARTICLE

The neutrophil subset defined by CD177 expression is preferentially recruited to gingival crevicular fluid in periodontitis

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Abstract

In recent years, the concept of distinct subpopulations of human neutrophils has attracted much attention. One bona fide subset marker, exclusively expressed by a proportion of circulating neutrophils in a given individual, and therefore dividing neutrophils in two distinct subpopulations, is the glycoprotein CD177. CD177 is expressed on the plasma and granule membranes of 0–100% of circulating neutrophils depending on the donor. Several in vitro studies have linked CD177 to neutrophil transmigration, yet very few have looked at the role of CD177 for tissue recruitment in vivo. We investigate whether the $CD177^+$ and $CD177^-$ neutrophil subsets differ in their propensity to migrate to both aseptic- and microbe-triggered inflamed human tissues. Microbe-triggered neutrophil migration was evaluated in samples of gingival crevicular fluid (GCF) from patients with periodontitis, whereas neutrophil migration to aseptic inflammation was evaluated in synovial fluid from patients with inflammatory arthritis, as well as in exudate from experimental skin chambers applied on healthy donors. We found that the proportion of $CD177^+$ neutrophils was significantly higher in GCF from patients with periodontitis, as compared to blood from the same individuals. Such accumulation of $CD177^+$ neutrophils was not seen in the two models of aseptic inflammation. Moreover, the proportion of $CD177^+$ neutrophils in circulation was significantly higher in the periodontitis patient group, as compared to healthy donors. Our data indicate that the $CD177^+$ neutrophil subset is preferentially recruited to the gingival crevice of periodontitis patients, and may imply that this subtype is of particular importance for situations of microbe-driven inflammation.

KEYWORDS
HNA-2a, inflammatory arthritis, NB1, neutrophil subpopulation, PMN, skin chamber

1 | INTRODUCTION

Whereas it is well established that adaptive immune cells (e.g., B- and T-lymphocytes) exist in multiple variants, neutrophils have traditionally been viewed as a homogenous cell population, with all cells being functionally equal and equipped with the same set of granule proteins and molecular markers. However, evidence of functional heterogeneity within the neutrophil population, as well as the concept of

Abbreviations: AASV, Anti-neutrophil cytoplasmic antibody associated systemic vasculitis; APC, Allophycocyanin; CD177, Cluster of differentiation 177; fMLF, N-Formylmethionyl-leucyl-phenylalanine; GCF, Gingival crevicular fluid; GPCR, G protein coupled receptor; GPI, Glycosylphosphatidylinositol; HNA-2a, Human neutrophil antigen 2a; KRG, Krebs-Ringer phosphate buffer; NB1, Neutrophil antigen B1; OLFM4, Olfactomedin 4; PR3, Protease 3; SLE, Systemic lupus erythematosus.

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neutrophil subsets based on the presence of distinct molecular markers, have emerged.[1,2] Some of the described subsets could represent cells with varying maturation- or activation status activation status, as the composition of neutrophil surface molecules can be altered by cellular maturation or activation.[3,4] However, there are also molecular markers that are expressed by only a proportion of neutrophils in blood samples from a given individual, independently of maturation- or activation status.[5-7] Neutrophils from an individual can thereby be divided into bona fide subsets, characterized by the expression or absence of such specific molecular markers.

Two bona fide subset markers are olfactomedin 4 (OLFM4)[5,7] and CD177.[6,8,9] CD177, also known as neutrophil antigen B1 (NB1) or human neutrophil antigen 2a (HNA-2a), is a glycosylphosphatidylinositol (GPI)-linked glycoprotein expressed on the plasma membrane and in granule membranes of neutrophil granulocytes.[10] The relative proportion of CD177-expressing neutrophils in circulation varies between individuals with a bimodal, or in some cases even trimodal,[9] expression pattern that is relatively stable over time.[6] In humans, the proportion of CD177-expressing neutrophils varies between 0% and 100% of circulating neutrophils depending on the donor. About 1–10% of healthy donors lack CD177-expressing neutrophils completely.[6,8,9,11] Whereas percentages of $CD177^+$ neutrophils in circulation are relatively constant in the same individual, increased blood levels of $CD177^+$ neutrophils have been reported in a diverse range of conditions, such as pregnancy, severe bacterial infections, polycythaemia vera, anti-neutrophil cytoplasmic antibody associated systemic vasculitis (AASV), and systemic lupus erythematosus (SLE).[12-14] Proportions of $CD177^+$ neutrophils in blood can also be transiently increased in individuals treated with G-CSF.[15,16]

The biologic purpose of neutrophil subsets is not entirely clear and whether subsets actually function differently is a matter under intense investigation, as is the biologic function of the subset markers per se. As CD177 is GPI linked,[17] and therefore lacks the capacity of intracellular signaling, it is often categorized as an inert adhesion molecule. Studies have shown that CD177 has the ability to interact with PECAM-1 (expressed on endothelial cells, platelets, monocytes, and granulocytes), which is a key player in neutrophil migration from bloodstream to tissue.[18] Such interaction has been reported to promote transmigration of $CD177^+$ cells over endothelial layers in vitro.[19,20] Furthermore, it has been shown that CD177 has the ability to bind and present protease 3 (PR3) on the neutrophil plasma membrane.[21,22] PR3 is a member of the neutrophil serine protease family and surface presentation of PR3 (on CD177) has been suggested to contribute to the possible migration advantage of $CD177^+$ cells by its ability to degrade extracellular matrix or junctional proteins.[23]

Periodontitis is an inflammatory disease that is characterized by progressive destruction of tooth-supporting structures and tooth loss, accompanied by an altered composition of the oral microflora.[24] The healthy oral cavity is heavily colonized by commensal bacteria, which stimulate a continuous recruitment of neutrophils to gingival tissues and into the gingival crevices. Accumulation of bacteria in the gingival margin and crevice lead to visible inflammation of the gingiva, a condition termed gingivitis. Gingivitis is present in a large part of the human population and is not associated with tissue destruction.[25] However, an imbalance in the interplay between oral microorganisms and host response can lead to development of a destructive inflammatory reaction, periodontitis, with increased inflow of gingival crevicular fluid (GCF), deepening periodontal pockets, breakdown of alveolar bone, and eventually tooth loss. This condition is also associated with an outgrowth of gram-negative, anaerobic, and proteolytic bacteria, which under healthy circumstances are traceable but outcompeted by species that are not dependent on an anoxic environment and inflow of proteinaceous components.[26] Whether the host response or the changed oral microflora is the instigating trigger in the development of periodontitis is still a matter of debate. Whereas there are theories suggesting a superior role of single bacterial species,[27] others consider the inflammatory response as an environmental change that drives the alterations of the periodontal microbiome.[28,29] Although bacterial invasion of gingival tissues has been reported in certain periodontal conditions, the inflammatory reaction in periodontitis is primarily triggered by colonization of the gingival crevice rather than invasion. Consequently, periodontitis can be referred to as a microbe-driven inflammation, rather than an infection in its traditional meaning.[29]

Whereas the gingival tissue in periodontitis is dominated by other types of immune cells,[30] neutrophils constitute 80–95% of the leukocytes present in GCF.[31-33] GCF neutrophils are continuously washed into the oral cavity where they are mixed with saliva and neutrophils originating from other mucosal tissues.[34] As this mix of cells, termed oral neutrophils, have undergone transmigration and chemotaxis toward the oral biofilm, they exhibit typical traits that are associated with a primed neutrophil phenotype.[35,36] Studies have shown that oral neutrophils are recruited in larger numbers and are in a higher state of activation in periodontitis patients as compared to healthy controls.[37]

After their maturation in the bone marrow, neutrophils enter the blood stream as terminally differentiated cells waiting for signals of infection, inflammation, or tissue damage.[38] Bacterium- and/or host-derived inflammatory signals originating from the inflammatory site stimulate local endothelial cells to expose adhesion molecules, and provide a chemotactic gradient that enable neutrophils to transmigrate from the bloodstream to the tissue. In the present study we investigate whether the $CD177^+$ and $CD177^-$ neutrophil subsets differ in their propensity to migrate from blood to inflamed human tissues by using three different in vivo models, representing both microbe-driven and aseptic inflammation. Microbe-driven inflammation was represented by periodontitis, whereas inflammatory arthritis and experimental skin chambers served as models of aseptic inflammation. Inflammatory arthritis is a group of diseases characterized by a continuous state of chronic inflammation of joints, interrupted by flares of acute inflammation, which leads to accumulation of leukocytes in the (normally acellular) synovial fluid. Neutrophil recruitment to the inflamed joints in these patients is triggered by nonmicrobial stimuli.[39,40] The skin chamber technique is an experimental method of acute inflammation where skin blisters are formed on the forearm of healthy volunteers, in which neutrophil recruitment is induced by endogenous chemokines such as IL-8 and C5a.[41-43]

We found that the proportions of CD177$^+$ neutrophils were higher in GCF from periodontitis patients, as compared to blood from the same donor. The accumulation of CD177$^+$ neutrophils in inflammatory exudate was not seen in two models of aseptic inflammation. Results from in vitro chemotaxis experiments showed a slight migratory advantage of CD177$^+$ neutrophils, as compared to the CD177$^-$ subtype. Further, an in vitro apoptosis assay revealed a higher rate of apoptosis in the CD177$^+$ subtype, as compared to the CD177$^-$ subtype. In line with this, the accumulation of CD177$^+$ neutrophils in GCF of periodontitis patients cannot be the result of a general difference in cell death between the neutrophil subtypes. Our data show that CD177 is dispensable for the ability of neutrophils to transmigrate from blood to tissues in general. On the other hand, the accumulation of CD177$^+$ cells in GCF of periodontitis patients may indicate that CD177$^+$ neutrophils possess a migratory advantage to sites of microbe-driven inflammation. We also recognized that periodontitis patients had significantly higher levels of CD177$^+$ neutrophils in circulation as compared to healthy donors. The increased proportion of CD177$^+$ neutrophils in this patient group could be due to a genetic predisposition to develop disease, or be a result of yet unknown external factors.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was approved by the regional Ethical board of Gothenburg, Sweden (no. 118-16 and S010-03 with amendment T536-07). Written informed consent was obtained from all patients and healthy controls who donated peripheral blood and tissue exudates. Buffy coats were obtained from the blood bank at Sahlgrenska University Hospital (Gothenburg, Sweden). Ethics approval for buffy coats was not needed as they were provided anonymously and could not be traced back to a specific individual.

2.2 | Isolation of human neutrophils from buffy coat and peripheral blood

Neutrophils were separated from peripheral blood of patients and healthy donors or from buffy coats with dextran sedimentation and Ficoll-Paque gradient centrifugation, as described by Boyum.[44] The neutrophils (95–98% pure) were washed in Krebs-Ringer phosphate buffer (KRG), resuspended in KRG supplemented with Ca^{2+} (1 mM), and kept on ice until use.

2.3 | Isolation of human transmigrated neutrophils

GCF neutrophils: Fourteen patients, diagnosed with periodontitis stage III–IV,[45] were recruited from the Specialist Clinic of Periodontics in Gothenburg, Public Dental Health Services, Region Västra Götaland, Sweden. The included periodontal pockets had a probing pocket depth (PPD) ≥6 mm, bleeding on probing and radiographic bone loss ≥50% of root length.[45] Gingival pockets were flushed with 50 μl saline and the fluid was collected with a micropipette fitted with a gel-tip, 5 times/site, 2–4 sites per patient (site and patient characteristics are given in Supporting Information Table S1). Samples containing visible signs of bleeding were excluded and the remaining samples were put on ice in low-binding tubes (Protein LoBind tubes, Eppendorf, Hamburg, Germany). The cells were diluted in KRG supplemented with Ca^{2+}, stained and analyzed by flow cytometry (Accuri C6; BD, Franklin Lakes, NJ, USA). Only GCF samples containing at least 10,000 neutrophils were included for subsequent analyses.

Synovial fluid neutrophils: Nine patients, diagnosed with inflammatory arthritis, were recruited from the Rheumatology Unit at Sahlgrenska University Hospital. Synovial fluid was collected during therapeutic joint aspiration and filtrated through 40 μm nylon cell strainers (BD) as previously described.[46] The cells were centrifuged at 330 ×*g*, for 10 min, at 4°C, resuspended in KRG supplemented with Ca^{2+}, followed by staining and flow cytometric analysis (Accuri C6). Inflammatory arthritis patient characteristics are presented in Supporting Information Table S1.

Skin chamber neutrophils: The skin chamber technique was applied on eight healthy volunteers as previously described.[41,42] Cells from skin chamber exudate were collected after 24 h and were pelleted at 9300 ×*g*, for 7 s. The exudate cells were resuspended in KRG supplemented with Ca^{2+}, followed by staining and analysis by flow cytometry.

Peripheral blood samples from all study subjects were collected in heparinized tubes before collection of exudate cells. Blood and exudate neutrophils were analyzed in parallel in all models of inflammation, and samples from the same individual were used for comparisons.

2.4 | Staining of GCF neutrophils for visualization by light microscopy

A total of 200 μl of GCF neutrophils (1×10^6 cells/ml) was cytocentrifuged at 130 ×*g* for 5 min, and the slides were allowed to dry at room temperature. The slides were stained with May-Grünwald and Giemsa (Sigma, St Louis, MO, USA) according to manufacturer's instructions. Micrographs were taken using a light microscope (Olympus [model: BX41TF], Hamburg, Germany), at 40× magnification.

2.5 | Analysis of CD177 expression in blood and exudate neutrophils

Cells (20,000–200,000/sample) from blood and exudate were stained with a phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD177 antibody (clone MEM-166; Abcam, Cambridge, United Kingdom; diluted 1:10) for 30 min on ice. To identify GCF neutrophils, cells were co-stained with an allophycocyanin (APC)-conjugated monoclonal mouse anti-human CD15 antibody (clone HI98; BD; diluted 1:20) or an APC-conjugated monoclonal mouse anti-human CD45 antibody (clone MEM-28; Abcam; diluted 1:50). GCF neutrophils were gated on the basis of side scatter and CD45/CD15 properties. Isotype controls and unstained samples were analyzed, with the

TABLE 1 Flow cytometer setup

Instrument: BD Accuri C6 Flow Cytometer				
Laser lines	488 nm	488 nm	640 nm	640 nm
Emission filters	585/40	533/30	>670	675/25
Flourochrome	PE	Fluo-3	Fura-Red	APC

exception of two samples containing low cell numbers. When isotype control was not applicable, the gate was set at the point distinctly dividing neutrophils in a $CD177^-$ and a $CD177^+$ population. Gating procedures for GCF samples are described in Supporting Information Fig. S1. Synovial fluid neutrophils were identified by co-staining with an APC-conjugated monoclonal mouse anti-human CD45 antibody, as mentioned earlier, and were gated on the basis of side scatter and CD45 properties. One inflammatory arthritis patient and one healthy donor displayed a trimodal CD177 expression pattern in blood. In these cases, the gate was set after the isotype control and the intermediate as well as the high peak were regarded as being $CD177^+$ (Supporting Information Fig. S2C). Neutrophils from skin chamber exudate were gated on the basis of forward and side scatter. All samples were analyzed by flow cytometry (Accuri C6). Information on Flow cytometer setup is given in Table 1.

2.6 | Evaluation of viability of GCF neutrophils

Five samples of GCF neutrophils were stained with APC-conjugated annexin V (Invitrogen, Carlsbad, CA, USA; diluted 1:20) and analyzed by flow cytometry. The viability of skin chamber and synovial fluid neutrophils has been previously reported.[47,48]

2.7 | Analysis of CD177 expression after in vitro chemotaxis

Isolated neutrophils from peripheral blood samples of healthy donors were pelleted and resuspended in KRG supplemented with Ca^{2+} and 0.3% BSA at 2×10^6 cells/ml. The cells were loaded on a 3 μm membrane (ChemoTX Disposable Chemotaxis System; Neuroprobe, Inc., Gaithersburg, MD, USA.) and were allowed to migrate toward chemotactic stimuli or buffer for 90 min, 37°C, 5% CO_2. Chemotaxis was evaluated using light microscopy. Migrated cells were then collected (remaining cells were detached using Versene solution [0.2 g EDTA/L]) and analyzed for CD177 expression as mentioned earlier. Samples (pre- and post-chemotaxis) from the same donor were compared. Chemotaxis was stimulated with N-Formylmethionyl-leucyl-phenylalanine (fMLF; 10 nM; Sigma), IL-8 (4.6 nM; R&D, Minneapolis, MN, USA), or a sterile filtered supernatant of a cultured periodontitis bacterial sample.

The paper point bacterial sample for preparation of the supernatant was collected from a gingival pocket (PPD ≥ 6 mm) of a periodontitis patient. The sample was cultured on blood agar plates under aerobic and anaerobic conditions in parallel, at 37°C for 72 h. Colonies were dissolved in KRG supplemented with Ca^{2+}, filtered through 0.2 μm filters (Corning, Corning, NY, USA) and stored at −80°C.

2.8 | Measurement of neutrophil cytosolic Ca^{2+} concentration by flow cytometry

Intracellular [Ca^{2+}] was measured as described by Buck et al.[49] Briefly before analysis, buffy-coat neutrophils labelled with Fluo-3 AM (ThermoFisher, Waltham, Massachusetts, USA; 3.6 μg/ml) and Fura-Red (ThermoFisher, Waltham, Massachusetts, USA; 10 μg/ml) were stained with PE-conjugated monoclonal mouse anti-human CD177 antibody as mentioned earlier. The cells were preincubated for 5 min at 37°C, baseline fluorescence was monitored for 30 s, followed by stimulation with bacterial supernatant (identical to that used for chemotaxis experiments). Fluorescence emission of the two calcium dyes were analyzed simultaneously and a ratio was calculated.[50] Neutrophils were gated on basis of CD177, and intracellular [Ca^{2+}] responses were compared between the two subsets.

2.9 | Analysis of cell death and CD177 expression after 20 h in culture

Buffy-coat neutrophils were resuspended in RPMI 1640) with phenol red (Gibco, Waltham, MA, USA) with 10% FCS and 1% penicillin-streptomycin (ThermoFisher, Waltham, MA, USA) at 5×10^6 cells/ml. The cell suspension was incubated for 20 h at 37°C under either aerobic or anaerobic conditions. Before and after incubation, neutrophils were co-stained with anti-human CD177 antibody (as mentioned earlier) and APC-conjugated annexin V and analyzed by flow cytometry. At least 10,000 events were acquired, and proportions of viable (annexin-V negative) $CD177^+$ neutrophils before and after incubation were compared in samples from the same donor.

2.10 | Statistical analyses

Differences in proportions of $CD177^+$ neutrophils between blood and exudate, differences in proportions of viable $CD177^+$ cells before and after 20 h incubation, and differences in proportions of $CD177^+$ neutrophils before and after in vitro chemotaxis were analyzed using the Wilcoxon's matched-pairs test. Differences in proportions of $CD177^+$ neutrophils between blood and exudate (in percentage points) were compared between the patient groups (periodontitis patients, inflammatory arthritis patients, and skin chamber subjects) using a Kruskal-Wallis with Dunn´s multiple comparison test. Differences in percentage of $CD177^+$ neutrophils in blood between periodontitis patients and healthy donors were analyzed using the Mann-Whitney test. All statistical analyses were performed in GraphPad Prism software (version 8.2.1; San Diego, CA, USA). A *P*-value less than 0.05 was considered statistically significant and level of significance is indicated in the figures: ns > 0.05, * <0.05, ** <0.01, and *** <0.001.

2.11 | Online supplemental material

(i) Supporting Information Fig. S1 presents our gating strategy for identification of neutrophils in GCF with anti-CD15 antibody and anti-CD45 antibody. (ii) Supporting Information Fig. S2 shows variants of CD177 expression-pattern in GCF from periodontitis patients, and

how CD177$^-$ and CD177$^+$ neutrophils were distinguished in an arthritis patient displaying a trimodal CD177 expression-pattern in blood. (iii) Supporting Information Fig. S3 shows that CD177 can be up-regulated by degranulation to the surface of CD177$^+$ neutrophils, whereas the relative sizes of the CD177$^+$ and CD177$^-$ populations remain unchanged. (iv) Supporting Information Fig. S4 shows that CD177$^+$ and CD177$^-$ neutrophils are equally recruited to GCF in periodontally healthy donors. (v) Supporting Information Fig. S5 shows that CD177$^+$ and CD177$^-$ neutrophils have a similar rate of apoptosis under anaerobic conditions. (vi) Supporting Information Table S1 contains characteristics and clinical parameters of patients and healthy donors.

3 | RESULTS

3.1 | Basic characterization of CD177 expression on peripheral blood leukocytes

We initially performed a basic characterization of the CD177 expression on human leukocytes. To investigate whether CD177 is expressed by blood cells other than neutrophils, leukocytes from heparinized whole blood from healthy controls were double-stained with anti-CD45 antibody and anti-CD177 antibody, and analyzed by flow cytometry. In samples from the CD177 positive donors ($n = 23$), neutrophils showed a clear bimodal expression pattern, that is, CD177 was present only on a proportion of neutrophils from a given blood sample. In contrast, CD177 was completely absent from the surface of monocytes and lymphocytes in all blood samples (Fig. 1A). We found that 23 out of 27 sampled individuals had a bimodal CD177 expression pattern on neutrophils (Fig. 1B) and that 4 donors (including donor 3 in Fig. 1B) were CD177-null and lacked CD177-expressing neutrophils altogether (median proportion of CD177$^+$ cells in all healthy donors was 51.2%). To study whether CD177 can be up-regulated to the surface of CD177$^-$ neutrophils, and thereby alter the relative size of the CD177$^+$ and CD177$^-$ subpopulations, neutrophils were analyzed for CD177 expression before and after degranulation triggered by short (20 min) treatment with TNF-α ($n = 3$). TNF-α stimulation induced an increase in mean fluorescence intensity of the CD177$^+$ neutrophils, but did not alter the signal from CD177$^-$ cells, that is, the relative proportions of the subpopulations remained unchanged (Supporting Information Fig. S3A).

3.2 | CD177$^+$ neutrophils are preferentially recruited to GCF in periodontitis

As an in vivo model of inflammation driven by bacteria we collected neutrophils from gingival pockets in patients diagnosed with periodontitis ($n = 13$). The GCF samples contained abundant neutrophils (median 200,000 neutrophils/patient, range: 42,000–1,700,000 neutrophils/patient) along with epithelial cells and clusters of bacteria (Fig. 2A, B). A majority of GCF neutrophils (≈95%) were viable as determined by annexin V staining and flow cytometry (Fig. 2C). May-Grünwald/Giemsa stained GCF samples studied with light microscopy showed neutrophils with clear multi-lobulated nuclei (Fig. 2A), as opposed to the condensed nuclei displayed by apoptotic cells.[51] To investigate whether CD177 expression implicated a recruitment advantage in microbe-driven inflammation, we compared proportions of CD177$^+$ neutrophils in blood and GCF samples from the same individual. The proportions of CD177$^+$ neutrophils in blood and GCF differed significantly ($P = 0.0002$), showing an accumulation of CD177$^+$ cells in GCF (Fig. 2D, E).

GCF sampling of gingival crevices in periodontally healthy donors resulted in a lower neutrophil yield (median 120,000 neutrophils/patient, range: 38,000–720,000 neutrophils/patient), as compared to samples from periodontitis patients. Similar proportions of CD177$^+$ cells were found in blood and GCF from periodontally healthy donors (Supporting Information Fig. S4). The clear bimodal CD177 expression pattern of blood neutrophils was not as distinct in GCF samples from periodontitis patients (Fig. 2E and Supporting Information Fig. S2A, B display variations) or healthy donors (Supporting Information Fig. S4).

3.3 | CD177$^+$ and CD177$^-$ neutrophils are recruited equally to synovial fluid in inflammatory arthritis and skin chamber exudate in healthy donors

To study the role of CD177 in in vivo transmigration to an aseptic inflammatory tissue, we next compared proportions of CD177$^+$ neutrophils in blood and synovial fluid of patients diagnosed with inflammatory arthritis. Similar proportions of CD177$^+$ cells were found in blood and synovial fluid from the same patient (Fig. 3A), indicating no recruitment advantage for CD177$^+$ neutrophils when migrating from blood to synovial fluid in inflammatory arthritis. We next applied an experimental skin chamber technique where in vivo transmigrated neutrophils can be sampled in a controlled manner.[41,42] As in the inflammatory arthritis model, no difference in proportions of CD177$^+$ cells could be seen in blood and skin chamber exudate (Fig. 3B). The results from both models of aseptic inflammation (Fig. 3A, B) are thus distinctly different from our findings in the periodontitis model (Fig. 2D, E). The mean difference in proportions (in percentage points) of CD177$^+$ neutrophils between blood and exudate is significantly higher in the periodontitis group as compared to the two groups that represent aseptic inflammation (Fig. 4).

3.4 | Comparison of in vitro migration of the CD177$^+$ and CD177$^-$ neutrophil subtypes in response to chemoattractants derived from a periodontitis bacterial sample

We next aimed to reiterate the in vivo results of accumulation of CD177$^+$ neutrophils to the site of microbe-driven inflammation by applying an in vitro chemotaxis assay ($n = 7$). A supernatant from a cultured bacterial sample from the gingival pocket of a periodontitis patient was used as a chemotactic stimulus, mimicking the microbial stimuli of a periodontal pocket. The chemotactic peptide, fMLF, and the endogenous chemokine IL-8 served as controls for bacterial and

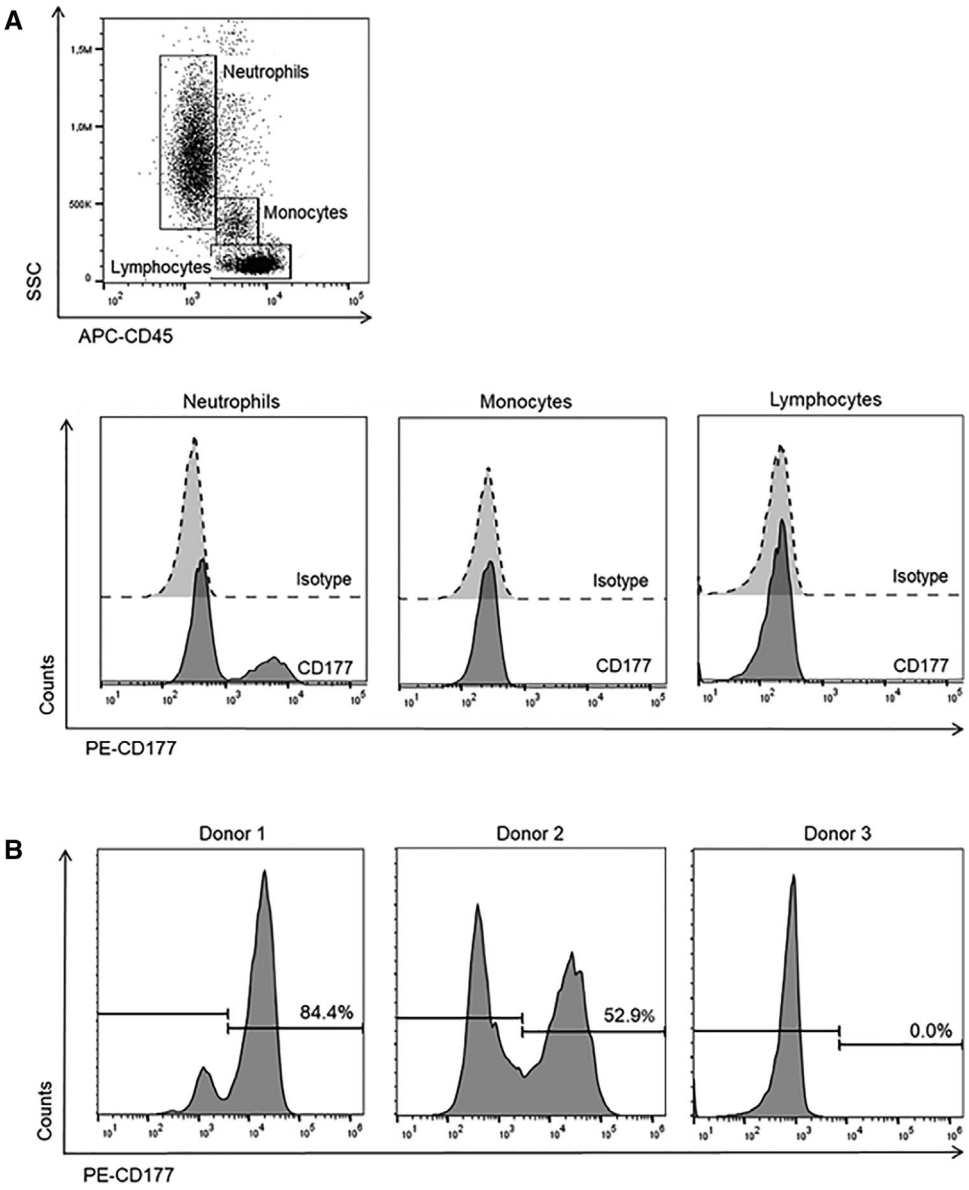

FIGURE 1 Basic characterization of CD177 expression. (A) Leukocytes from peripheral blood of healthy donors were co-stained with APC-conjugated anti-CD45 and PE-conjugated anti-CD177 antibodies, and analyzed by flow cytometry. Surface expression of CD177 was analyzed in the three indicated leukocyte populations, neutrophils, monocytes and lymphocytes. The histograms are representative of 13 independent experiments and show CD177 expression of the indicated cell types together with an isotype control antibody. (B) Histograms from three healthy individuals representing the varying proportions of $CD177^+$ neutrophils in blood are shown, including a CD177-null individual (donor 3)

endogenous chemotactic stimuli, respectively (Fig. 5A). We noted a slight but statistically significant increase in the proportion of $CD177^+$ neutrophils after chemotaxis toward the patient sample supernatant ($P = 0.03$) and IL-8 ($P = 0.02$), as compared to the baseline level of the same donor (Fig. 5B, D). There was no statistically significant difference in proportion of $CD177^+$ neutrophils before and after migration toward fMLF (Fig. 5C). Neutrophil chemotaxis is modulated by agonist binding to G-protein-coupled receptors (GPCRs) and one critical process that is triggered by GPCR ligation is a transient increase in intracellular Ca^{2+} concentration. To further investigate differences in neutrophil response to chemotactic stimulation between the two subtypes ($CD177^+/CD177^-$), the transient increase in intracellular Ca^{2+} concentration was compared between $CD177^+$ and $CD177^-$ neutrophils ($n = 4$). Both neutrophil subsets displayed a similar increase in intracellular Ca^{2+} concentration after stimulation with the bacterial culture supernatant (Fig. 5E), indicating similar activation of chemotactic GPCRs in $CD177^+$ and $CD177^-$ neutrophils.

3.5 | Differential cell death between the $CD177^+$ and $CD177^-$ neutrophil subtypes

The reason for the $CD177^+$ neutrophil accumulation in GCF does not have to be a migratory difference (i.e., that the $CD177^+$ neutrophils migrate faster and/or more efficiently into the gingival pocket),

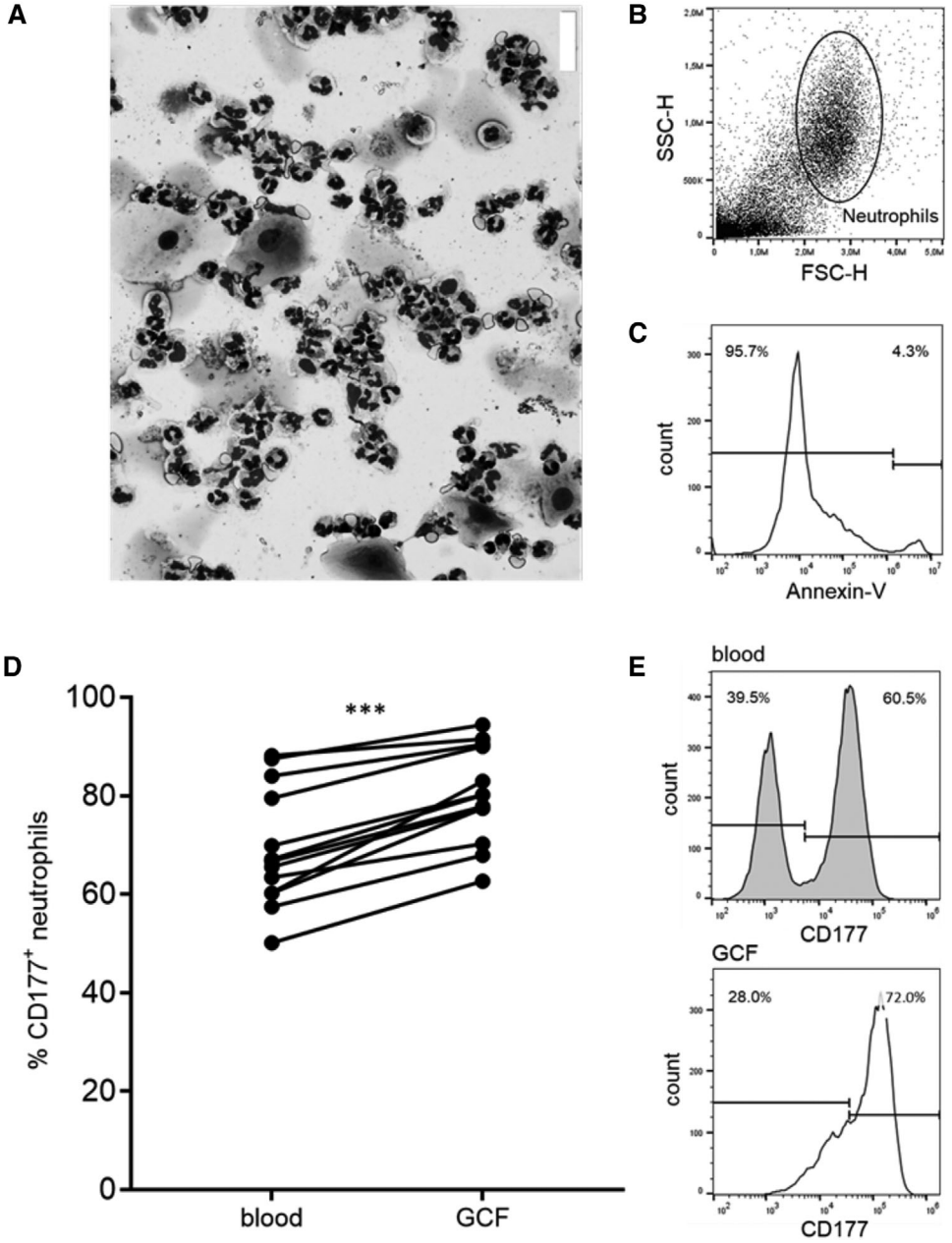

FIGURE 2 **Neutrophil expression of CD177 before and after in vivo recruitment to gingival crevicular fluid (GCF) in periodontitis patients.** (A) GCF samples ($n = 4$) were cytospun and stained with Giemsa and May-Grünwald for microscopic evaluation. The microscopic image shows neutrophils with a clear multi-lobulated nucleus, endothelial cells, and bacteria. The scale bar represents 20 μm. (B) The GCF content was evaluated by flow cytometry before further analysis. The dot plot shows a distinct neutrophil population, gated on the basis of forward and side scatter, together with a population of smaller and less granular particles that are probably representing bacteria. (C) Neutrophils from GCF samples ($n = 5$) were stained with annexin-V and analyzed by flow cytometry to determine viability (annexin-V negative cells). The histogram is representative for the neutrophil GCF samples, which all displayed >90% viable cells. (D) Neutrophils from peripheral blood and GCF from periodontitis patients ($n = 13$) were evaluated for CD177 expression by flow cytometry. The graph depicts the proportion of $CD177^+$ neutrophils in blood and GCF from periodontitis patients. Wilcoxon's matched-pairs test ($P = 0.0002$) (E) Histograms of neutrophil CD177 expression in blood and GCF (from one patient out of 13)

but could potentially also be due to a difference in longevity. Our in vivo results could be explained if $CD177^-$ neutrophils die faster in the gingival pocket than $CD177^+$ cells do, leaving a larger proportion of $CD177^+$ cells in the GCF. To study differences in the lifespan of $CD177^+$ and $CD177^-$ neutrophils, buffy-coat neutrophils ($n = 10$) were incubated under aerobic conditions for 20 h and the proportion of $CD177^+$ neutrophils among the viable cells was measured before (Fig. 6A) and after 20 h incubation (Fig. 6B). We found a significant ($P = 0.004$) decrease in the proportion of $CD177^+$ neutrophils among the viable cells after 20 h incubation (Fig. 6C). Consequently,

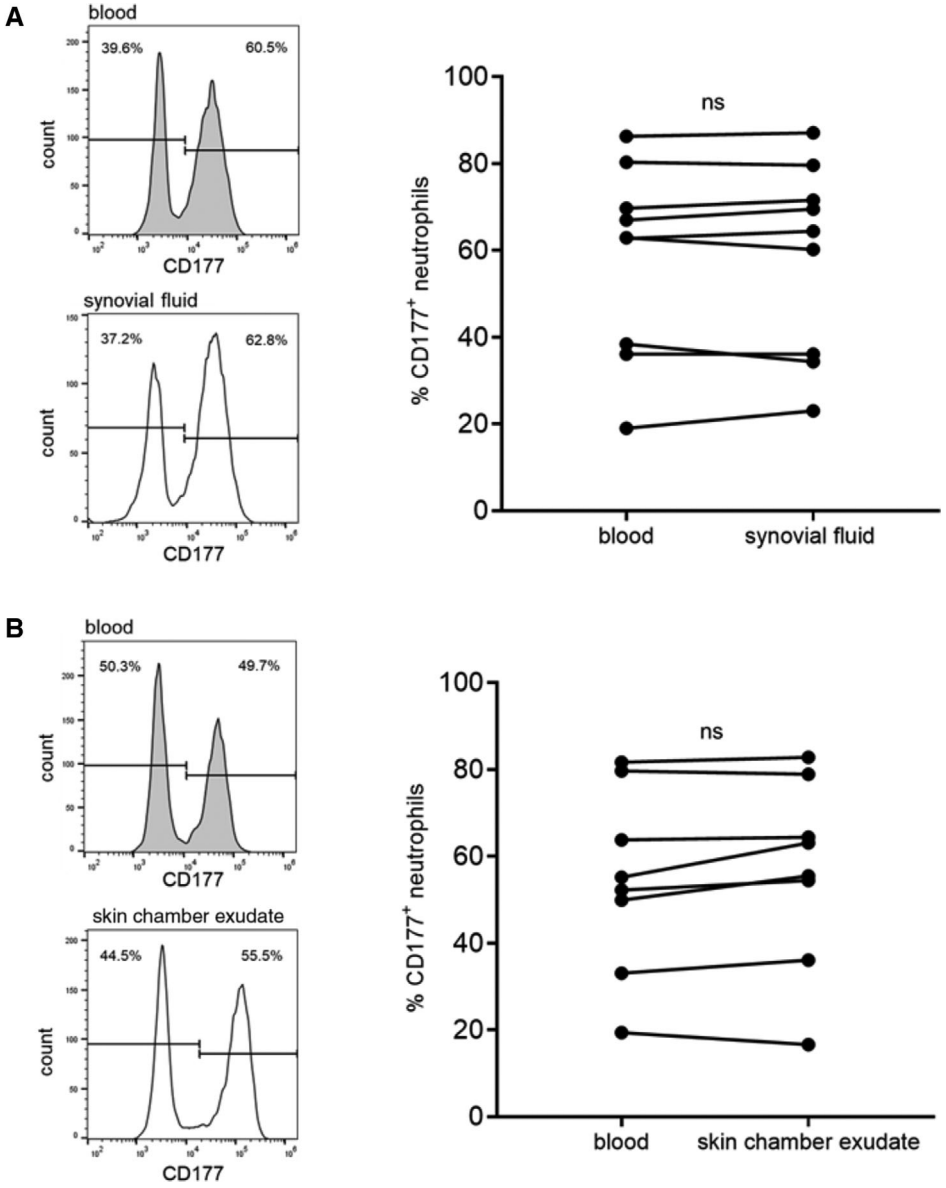

FIGURE 3 **Neutrophil expression of CD177 before and after in vivo transmigration to synovial fluid in arthritis patients and skin chamber exudate in healthy donors.** (A) Neutrophils from peripheral blood and synovial fluid from inflammatory arthritis patients ($n = 9$) were evaluated for their CD177 expression by flow cytometry. Representative histograms (from one patient) is shown (left) as is a graph with proportions of CD177$^+$ neutrophils in blood and synovial fluid from 9 patients (right). Wilcoxon's matched-pairs test ($P = 0.742$). (B) Neutrophils from peripheral blood and skin chamber exudate from healthy subjects ($n = 8$) were evaluated for their CD177 expression by flow cytometry. Representative histograms (from one subject) are shown (left) as is a graph with proportions of CD177$^+$ neutrophils from blood and skin chamber exudate from 8 healthy donors (right). Wilcoxon's matched-pairs test ($P = 0.148$)

a larger proportion of the CD177$^+$ neutrophils have undergone apoptosis, as compared to the CD177$^-$ neutrophils, after 20 h incubation. These results implicate that the CD177$^+$ neutrophil subtype have a higher rate of apoptosis as compared to the CD177$^-$ subtype, and cannot explain the accumulation of CD177$^+$ cells in GCF. The environment in deep gingival pockets is largely anaerobic, which might have an impact on the rate of apoptosis in the two neutrophil subtypes (CD177$^+$/CD177$^-$). To investigate this issue, buffy-coat neutrophils were also incubated anaerobically for 20 h and analysis of CD177$^+$ neutrophil proportions were performed, at 0 and 20 h ($n = 4$). There was no difference in the proportion of CD177$^+$ neutrophils between the two measurements under anaerobic conditions (Supporting Information Fig. S5). In summary, a difference in longevity cannot explain why the CD177$^+$ neutrophil subtype is accumulated in GCF in periodontitis patients.

3.6 | Proportions of CD177$^+$ neutrophils in circulation of periodontitis patients

As mentioned earlier, increased blood levels of CD177$^+$ neutrophils have been reported in patients with inflammatory conditions, for example, AASV and SLE.[12-14] Considering these observations, we

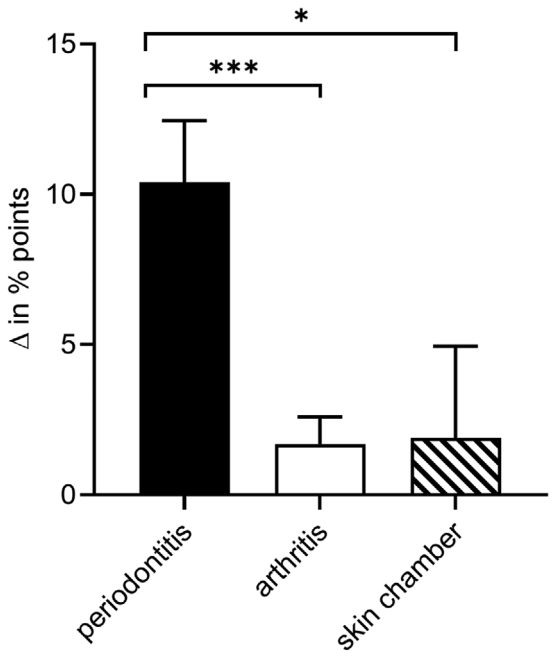

FIGURE 4 Comparison of the difference in proportions (in percentage points) of CD177$^+$ neutrophils between blood and exudate in the three in vivo models. The difference (Δ in percentage points) in proportion of CD177$^+$ neutrophils in blood and exudate from each donor in the three in vivo models of inflammation (periodontitis, inflammatory arthritis, and skin chambers) were calculated. Differences in proportions of CD177$^+$ neutrophils (in percentage points) are presented as mean + SD. Kruskal-Wallis test and Dunn´s multiple comparison test. Periodontitis—arthritis ($P = 0.0007$), periodontitis—skin chamber ($P = 0.0159$)

compared proportions of CD177$^+$ neutrophils in blood from healthy donors ($n = 27$) and patients diagnosed with severe periodontitis ($n = 14$). Periodontitis patients had significantly ($P = 0.019$) larger proportions of circulating CD177$^+$ neutrophils than healthy donors (Fig. 7). Out of 27 healthy donors, 4 lacked CD177 expressing neutrophils completely (CD177-null) and the remaining donors had CD177$^+$ cell proportions between 19.8% and 84.9% (median of all healthy donors = 51.2% CD177$^+$ cells). No donors in the periodontitis group were CD177-null or had a CD177$^+$ proportion <50.1%. CD177 was expressed on 50.1–88.1% (median 66.9%) of circulating neutrophils in periodontitis patients.

4 | DISCUSSION

The existence of a "granulocyte specific antigen" was first described in 1960 by Lalezari et al. when studying newborns suffering from agranulocytosis[52] and this antigen was later identified as CD177 (or NB1-antigen, HNA-2a).[8] In this study we confirm that CD177 is expressed by neutrophils, although being absent from lymphocytes and monocytes, and that a majority of healthy individuals have a bimodal expression pattern of this surface marker. In our group of healthy donors, 23 out of 27 donors showed a bimodal expression of CD177 in blood neutrophils (range 19.8–84.9% CD177$^+$ neutrophils). The median proportion of CD177$^+$ neutrophils in all healthy donors was 51.2%, which is in line with what others have reported regarding CD177 membrane expression in healthy individuals.[6,9,12,53] Four (14.8%) of the healthy donors in our study lacked CD177$^+$ neutrophils completely. Earlier studies show that 1–10% of healthy individuals are CD177-null, completely lacking CD177$^+$ neutrophils.[6,9,11] The group of individuals who are CD177-null does not seem to be immunocompromised, but are at risk of developing anti-CD177 alloantibodies if exposed to the antigen. This has been reported to occur after blood transfusion, leading to transfusion related acute lung injury, or during bone marrow transplantation or pregnancy with a CD177-expressing foetus.[54,55] Whereas the CD177-null phenotype is caused by a single nucleotide polymorphism that creates a stop codon in the coding region of CD177,[56,57] the mechanism underlying the bimodal (sometimes even trimodal) CD177 expression is not completely understood. However, recent studies suggest a combination of complex genetic and epigenetic factors.[57-59] As CD177 is located in granule membranes, as well as in the plasma membrane of neutrophils, it can be further upregulated to the surface of the CD177$^+$ cells upon degranulation. The mean fluorescence intensity of the CD177$^+$ population was increased after in vitro stimulation with TNF-α, whereas the CD177– population remained negative (with unaltered mean fluorescence intensity).

When proportions of CD177$^+$ neutrophils in blood and exudate were compared, we saw an enrichment of CD177$^+$ neutrophils in the GCF of patients diagnosed with periodontitis. Cells lacking CD177 were clearly capable of transmigrating to the gingival pocket, as GCF neutrophils were not exclusively CD177$^+$. This was also confirmed by the presence of neutrophils in GCF samples from one healthy control subject who was CD177-null (Supporting Information Figs. S3B and S4). Nevertheless, the proportion of CD177$^+$ neutrophils was increased in GCF of periodontitis patients, as compared to blood. We did not find an accumulation of the CD177$^+$ neutrophil subtype in GCF samples from gingival crevices of periodontally healthy subjects. This could be due to a different arsenal of chemotactic factors being released by the altered microbial community in the periodontal pocket, as compared to chemotactic factors derived from the healthy oral microbiome. However, it should be kept in mind that the shallow gingival crevices of periodontally healthy subjects are more difficult to sample than deep periodontal pockets, with a greater risk of contamination of non-GCF neutrophils (e.g., from saliva). Thus, a direct comparison between GCF samples from periodontitis patients and healthy subjects should be done with caution.

The enrichment of the CD177$^+$ neutrophil subset was not evident in synovial fluid of patients with inflammatory arthritis or in skin chamber fluid of healthy controls. These data could indicate a recruitment advantage for CD177$^+$ neutrophils in periodontitis, but not a tissue recruitment advantage in general. We and others have previously shown that the recruitment process varies between different tissues and that neutrophils isolated from different human exudates may have profoundly different phenotypes.[46,60-63] One major difference between GCF and the other tissue exudates presented, is that the inflammation in periodontitis is triggered by bacteria, whereas

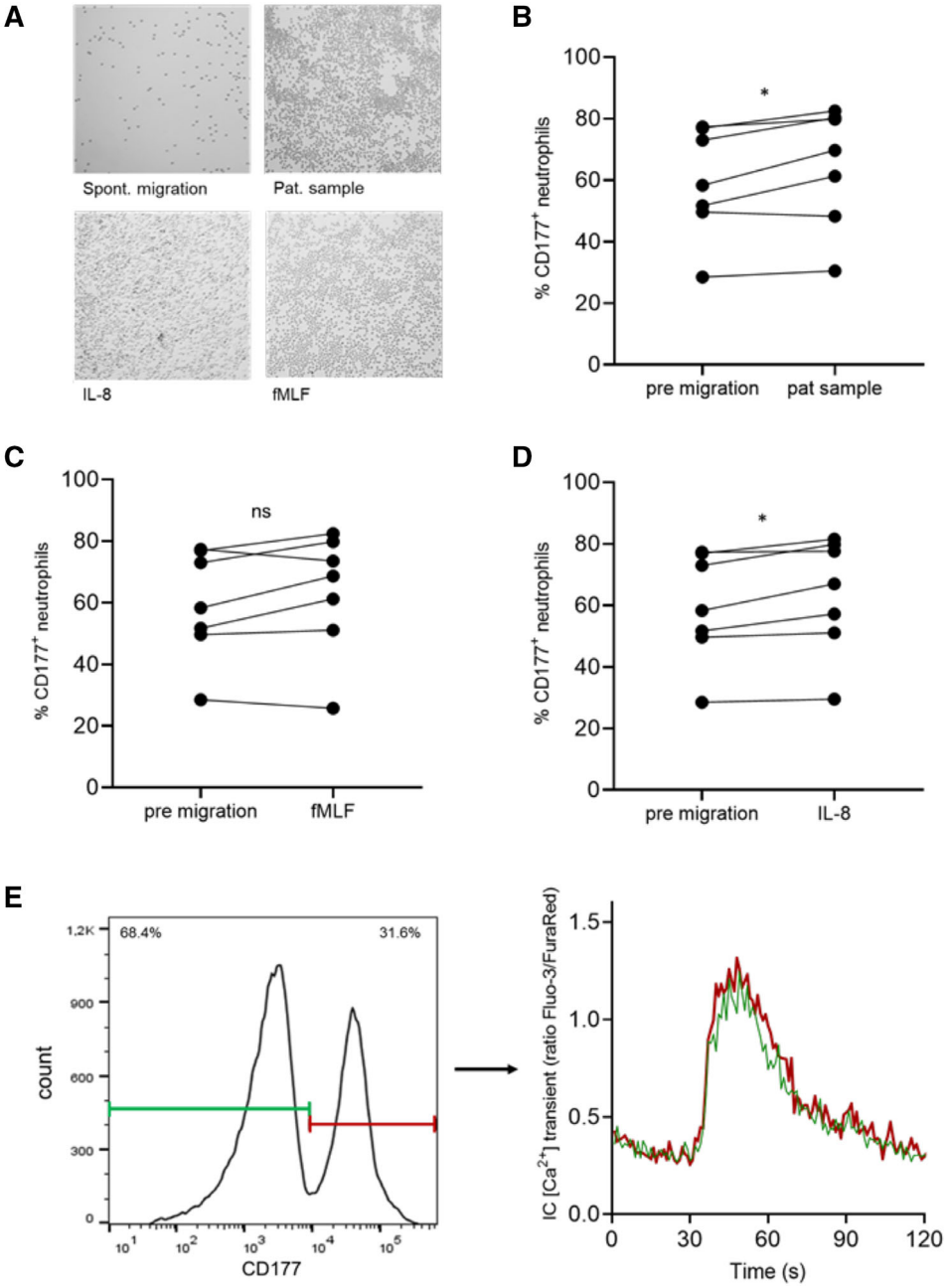

FIGURE 5 **In vitro migration of CD177$^+$ and CD177$^-$ neutrophil subtypes.** Neutrophils isolated from peripheral blood samples were allowed to migrate over a membrane (pore size 3 μm) for 90 min, toward IL-8 (4.6 nM), fMLF (10 nM), sterile filtered supernatant of a culture of a periodontitis bacterial sample, or buffer. Proportions of CD177$^+$ neutrophils in the sample were measured by flow cytometry before (pre migration) and after migration. (A) Micrographs of migrated cells from one representative experiment, ×10 magnification. Spontaneous migration (Spont. migration), supernatant of periodontitis patient bacterial sample (Pat. sample), IL-8, and fMLF. (B) Percentages of CD177$^+$ neutrophils before and after chemotaxis toward periodontitis patient bacterial sample ($n = 7$). Wilcoxon's matched-pair test ($P = 0.031$). (C) Percentages of CD177$^+$ neutrophils before and after chemotaxis toward fMLF (10 nM) ($n = 7$). Wilcoxon's matched-pair test ($P = 0.156$). (D) Percentages of CD177$^+$ neutrophils before and after chemotaxis toward IL-8 (4.6 nM) ($n = 7$). Wilcoxon's matched-pair test ($P = 0.0156$). Buffy-coat neutrophils were co-stained with the calcium dyes Fluo-3 and Fura-red, followed by staining with an anti-CD177 antibody. The cells were stimulated with a culture supernatant of a bacterial sample from the gingival pocket of a periodontitis patient, and intracellular calcium signals were analyzed by flow cytometry ($n = 4$). Neutrophils were gated for CD177$^+$ and CD177$^-$ neutrophils and the intracellular calcium signals were compared between the two subtypes. (E) The histogram shows gating of CD177$^+$ (red) and CD177$^-$ (green) neutrophils in one representative experiment. The graph shows the increase in intracellular Ca^{2+} concentration induced in the CD177$^-$ (green) and CD177$^+$ (red) subset, respectively, after stimulation with the periodontitis bacterial culture supernatant

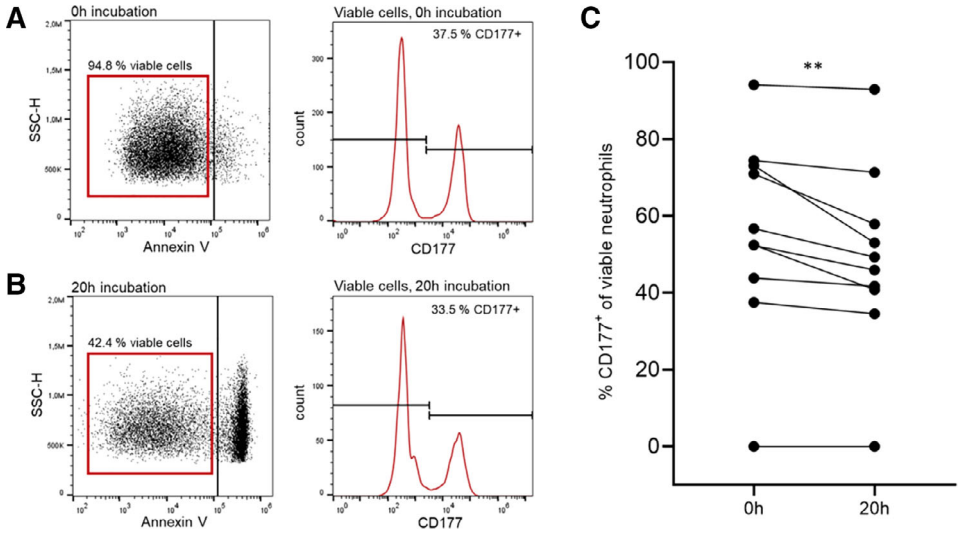

FIGURE 6 **Differential cell death between the CD177$^+$ and CD177$^-$ neutrophil subtypes.** Buffy-coat neutrophils were incubated for 20 h under aerobic conditions and the proportions of CD177$^+$ neutrophils of all viable cells were measured before and after incubation. The mean proportion of viable cells after 20 h incubation was 43 ± 10%. (A) The dot plot shows viable (red gate, annexin-V negative) cells in a scatterplot before incubation (0 h) from one representative experiment. The histogram shows proportions of CD177$^+$ and CD177$^-$ neutrophils in the population of viable cells. (B) The dot plot shows viable (red gate, annexin-V negative) cells in scatterplots after 20 h incubation from the same representative experiment, and the histogram displays percentages of CD177$^+$ and CD177$^-$ neutrophils among the viable cells. (C) Graph shows proportions of CD177$^+$ neutrophils of all viable cells before (0 h) and after 20 h incubation from 10 independent experiments. Wilcoxon's matched-pair test ($P = 0.0039$)

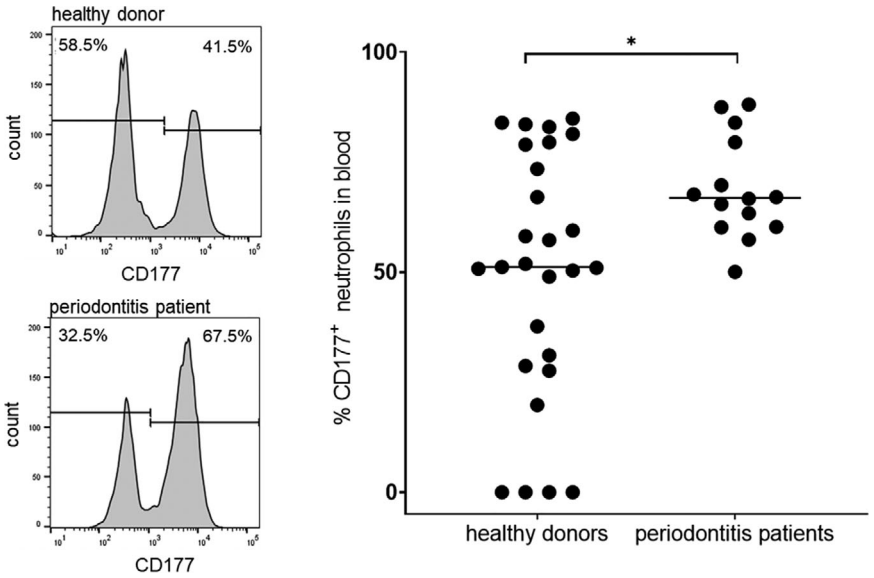

FIGURE 7 **Proportion of CD177$^+$ neutrophils in blood from healthy donors vs. blood from patients diagnosed with periodontitis.** Neutrophils from peripheral blood of periodontitis patients and healthy donors were evaluated for their CD177 expression by flow cytometry. The histograms show the distribution of CD177$^+$ and CD177$^-$ neutrophil populations in blood from one healthy donor and one periodontitis patient. Individual proportions of circulating CD177$^+$ neutrophils in all healthy donors ($n = 27$) and periodontitis patients ($n = 14$) are shown and median values are indicated in the graph. Blood samples from periodontitis patients are the same as those presented in Figure 2. Mann-Whitney test ($P = 0.019$)

the inflammation in both inflammatory arthritis and the skin chamber model are aseptic and triggered by endogenous signals. Along these lines a decrease in neutrophil migration toward *Staphylococcus aureus* infected skin was observed in CD177-deficient mice, compared to a similar skin infection in wild-type mice.[64] As mentioned earlier, several studies have reported that CD177 contributes to neutrophil transmigration in vitro, due to its association with PECAM-1 and PR3.[19,20,23] To our knowledge, only one previous report has looked at proportions of human CD177$^+$ neutrophils from blood and after transmigration in vivo. This report found similar proportions of CD177$^+$ neutrophils in blood and peritoneal fluid of dialysis patients, regardless whether the patients presented with acute peritonitis or not.[65] This appears to contradict our results as most instances of acute peritonitis during peritoneal dialyses are triggered by (bacterial or fungal) infections.[66] However, the nature of the inflammatory triggers of the acute peritonitis patients studied by Wang et al. is not stated, which makes it difficult to draw conclusions.

Other studies have demonstrated a migration advantage of CD177$^+$ neutrophils over endothelial cell layers in vitro, after stimulation with the bacteria derived formylated peptide fMLF as well as endogenous cytokines such as IL-8.[19,23] However, the inflammatory response in periodontitis is maintained by a highly variable and

complex poly-microbial community, including both aerobic and anaerobic bacterial species.[29] Although fMLF and IL-8 can contribute to the periodontal inflammation in vivo, the combination of bacterial species that is present in the gingival pocket would likely result in a different inflammatory response than the response stimulated by an isolated chemoattractant. We therefore wanted to address the issue of whether the complex chemotactic stimuli originating from the periodontal pocket have an impact on the fact that CD177$^+$ and CD177$^-$ neutrophils behave differently during transmigration to this in vivo site. We prepared a supernatant from a cultured clinical bacterial sample from the gingival pocket of a periodontitis patient that (along with fMLF and IL-8) was used as chemotactic stimuli in an in vitro chemotaxis assay. A slight accumulation of CD177$^+$ neutrophils was noted after migration over the membrane when cells were stimulated with the periodontitis bacterial sample supernatant or the endogenous chemokine IL-8, whereas CD177$^+$ and CD177$^-$ neutrophils appeared to migrate equally when stimulated with fMLF. It seems unlikely that the minimal difference in migration between the CD177$^+$ and CD177$^-$ cells that was noted in our in vitro chemotaxis assay would have a decisive effect on neutrophil migration in the in vivo situation. Furthermore, stimulation with the supernatant from the periodontitis bacterial sample induced a similar increase in intracellular Ca^{2+} concentration in the CD177$^+$ and CD177$^-$ subpopulations, indicating a similar chemotactic receptor activity. Thus, the possible migratory advantage of CD177$^+$ neutrophils over the CD177$^-$ subtype is likely dependent on superior adhesion properties, which may vary depending on tissue type, rather than on a difference in chemotactic receptor signaling. One should be aware that CD177 per se may provide a functional difference to the CD177$^+$ neutrophils, but it is also possible that other molecules, yet unknown and uniquely expressed/absent by this subset, give the cell a distinct function. One such factor could be certain chemotactic receptors such as those described to display bimodal expression patterns on human neutrophils.[67]

A difference in recruitment efficacy between the CD177$^+$ and CD177$^-$ subtypes is not the only possible explanation to the in vivo enrichment of the CD177$^+$ neutrophils in GCF from periodontitis patients. Our in vivo results could be explained if CD177$^-$ neutrophils die faster in the gingival pocket than CD177$^+$ cells do, leaving a larger proportion of CD177$^+$ cells in the gingival pocket. However, our results indicate that CD177$^-$ neutrophils, to the contrary, have a slightly slower rate of apoptosis than CD177$^+$ neutrophils. Although the in vitro milieu of this assay is not entirely representing the in vivo situation in periodontitis, these results indicate that the accumulation of CD177$^+$ neutrophils in the gingival pocket are a result of a recruitment advantage rather that a difference in cell death between the neutrophil subtypes. If the CD177$^+$ neutrophils have a higher rate of apoptosis in vivo, the difference in migration of the CD177$^+$ and CD177$^-$ neutrophils that we found in the periodontitis model could be an underestimation of the actual difference in migration between the subtypes.

There are indications that the relative abundance of bona fide neutrophil subsets can play a role in morbidity or severity of several inflammatory diseases. Alder et al. found that pediatric septic shock patients with high proportions of OLFM4$^+$ neutrophils in blood were at higher risk of massive organ failure and death, as compared to patients featuring low proportions of OLFM4$^+$ neutrophils.[68] Moreover, higher levels of CD177$^+$ neutrophils in blood have been reported earlier in patients with clinical conditions such as polycythaemia vera and severe bacterial infections, as compared to healthy controls.[13] Abdgawad et al. could not confirm higher levels of CD177$^+$ neutrophils in polycythaemia vera patients, but they and others have detected significantly higher levels of CD177$^+$ neutrophils in blood from patients diagnosed with AASV and SLE compared to healthy controls.[14,53] Interestingly, we saw significantly higher proportions of CD177$^+$ neutrophils in blood from periodontitis patients (median 66.9 %) as compared to healthy donors (median 51.2 %). Worth noting is that no information on periodontal status is available for a majority of the healthy donors in our study. As periodontitis is a widespread disease affecting, in its severe form, 10% of the population globally,[69,70] there are probably some individuals with periodontitis (manifest or subclinical) among the group of "healthy blood donors." Consequently, the data shown could be an underestimation of the actual difference between healthy controls and periodontitis patients.

In conclusion, we confirm that CD177 is expressed on neutrophils with a bimodal expression pattern. We show that the CD177$^+$ neutrophil subtype is preferentially recruited to inflammatory exudate in periodontitis whereas this recruitment advantage is not evident during migration to inflamed joints in arthritis patients or experimental skin chambers in healthy donors. Increased levels of CD177$^+$ neutrophils in blood of periodontitis patients was detected, as compared to healthy controls. Further studies are needed to reveal if the overexpression of CD177$^+$ blood neutrophils in periodontitis patients are a result of external factors, or if the proportion of CD177$^+$ neutrophils influence susceptibility to this disease.

AUTHORSHIP

Experiments were conducted by A.D.R., F.A., P.T., A.W., L.D., and A.K. Results were analyzed by A.D.R., F.A., P.T., A.W., A.K., J.B., and K.C. S.T.M. was in charge of clinical evaluation and recruitment of periodontitis patients, and L.B. and L.D. were in charge of clinical evaluation and recruitment of inflammatory arthritis patients. A.D.R. and J.B. wrote the paper, which was critically revised by all authors.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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