Characterization of neutrophil function in Papillon-Lefèvre syndrome

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ABSTRACT

Papillon-Lefèvre syndrome is a rare, inherited, autosomal-recessive disease, characterized by palmo-plantar keratosis and severe prepubertal periodontitis, leading to premature loss of all teeth. Papillon-Lefèvre syndrome is caused by a mutation in the cathepsin C gene, resulting in complete loss of activity and subsequent failure to activate immune response proteins. Periodontitis in Papillon-Lefèvre syndrome is thought to arise from failure to eliminate periodontal pathogens as a result of cathepsin C deficiency, although mechanistic pathways remain to be elucidated. The aim of this study was to characterize comprehensively neutrophil function in Papillon-Lefèvre syndrome. Peripheral blood neutrophils were isolated from 5 patients with Papillon-Lefèvre syndrome, alongside matched healthy control subjects. For directional chemotactic accuracy, neutrophils were exposed to the chemoattractants MIP-1α and fMLP and tracked by real-time videomicroscopy. Reactive oxygen species generation was measured by chemiluminescence. Neutrophil extracellular trap formation was assayed fluorometrically, and proinflammatory cytokine release was measured following overnight culture of neutrophils with relevant stimuli. Neutrophil serine protease deficiencies resulted in a reduced ability of neutrophils and is responsible for the activation of NSPs. The loss of CTSC activity causes the subsequent cessation of NSP activity.

Introduction

PLS is a rare, inherited, autosomal-recessive disease, characterized by diffuse palmo-plantar keratosis and a severe prepubertal periodontitis, leading to an edentulous state in adolescence [1]. Chronic periodontitis is a disorder initiated by dysbiosis within the oral plaque biofilm. It progresses as a result of an abnormal host inflammatory-immune response, characterized by exaggerated and nonresolving chronic inflammation, and leads to periodontal tissue damage and bone loss [2]. PLS has an estimated prevalence of 1–4 cases per million with signs and symptoms appearing between 1 and 4 yr of age, typically resulting in permanent tooth loss during adolescence. There is no predominant racial or sexual predilection; however, one third of reported cases involve parental consanguinity [3]. It is noteworthy that systemic immunodeficiency in PLS is relatively mild, with 15–20% predisposed to recurrent infections [4], yet the local periodontitis is profoundly aggressive.

PLS is caused by mutations in the gene encoding the lysosomal CTSC (CTSC gene); >50 mutations in the gene have been reported as responsible for PLS, with phenotypes ranging from specific loss of function (resulting in a deficiency) to complete absence of the enzyme. CTSC is expressed in high levels in neutrophils and is responsible for the activation of NSPs. The loss of CTSC activity causes the subsequent cessation of NSP activity

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Abbreviations: A. actino = Aggregatibacter actinomycetemcomitans, AFU = arbitrary fluorescence unit, AMP = antimicrobial peptide, CG = cathepsin G, CGD = chronic granulomatous disease, CTSC = cathepsin C, gPRES = glucose PBS, HAX1 = hematopoietic lineage-cell-specific protein, 1-associated protein X-1, IL-8 = IL-8/CXCL8, MMP9 = matrix metalloproteinase 9, MOI = multiplicity of infection, MPO = myeloperoxidase, MIP = myeloid-related

(continued on next page)
Failure of NSPs to eliminate periodontal bacteria is thought to be the underlying cause of the severe periodontal disease in patients with PLS; however, comprehensive and systematic characterization of neutrophil function in PLS has been hampered by the rarity of the disease and case studies limited to individual patients.

Neutrophils represent the first line of defense against microbial pathogens. Neutrophils resolutely survey tissues for microorganisms, and during tissue infections, they extravasate the circulation into the tissues, where they undergo directional movement toward the source of infection via chemical gradients (chemotaxis). This highly regulated recruitment process is orchestrated by the release of chemoattractants that may be host derived, such as IL-8 and MIP-1α, or pathogen derived (e.g., fMLP). Neutrophils function by eradicating pathogens via phagocytosis and subsequent intracellular and extracellular killing mechanisms in situ as a vital part of innate and acquired immunity.

Phagocytosis involves pathogen internalization and destruction via reactive ROS generation following the assembly of NADPH oxidase at the phagosome membrane, the fusion of 4 distinct types of neutrophil granules (azurophilic/primary, specific/secondary, gelatinase/tertiary, and secretory), and release of AMPs into the phagosome. The azurophilic granules contain several microbialidal agents, including MPO, defensins, and various NSPs that include NE, CG, PR3 [5], and the recently described NSP4 [7].

NSPs can be released extracellularly following limited granule exocytosis [8], binding to the external cell surface, where they target a variety of host chemokines, cytokines, growth factors, and cell-surface receptors contributing to pro- and anti-inflammatory processes. PR3 has been shown to cleave active IL-8, increasing its potency [9], and to splice the inactive membrane-bound proform of TNF-α [10], resulting in activation. Conversely, NSP processing affects the activity of chemokines, such as MIP-1α [11] and IL-8 [12], resulting in their inactivation. In addition, NSPs have been shown to inactivate IL-6 at inflammatory sites [13]; thus, NSPs appear to play a key role in orchestrating immunostimulatory signals.

One recently described neutrophil-mediated, antimicrobial strategy involves the production of NETs, which comprise decondensed nuclear chromatin associated with granule-derived antimicrobial proteins, including NSPs [14]. The proposed role of NETs is to facilitate neutrophil containment and destruction of pathogenic organisms, although the mechanisms that lead to NET formation are not completely understood. NE and ROS have been shown to be fundamental to NET release [15]. In addition, the specific granule-derived AMP cathelicidin [human cathelicidin 18 (hCAP18)/LL-37] is cleaved by PR3, forming LL-37, which is also known to facilitate NET formation [16].

For decades the consensus has been that patients with PLS suffer a severe prepubertal periodontitis as a result of NSP deficiencies failing to eliminate causative pathogens, in particular, A. actinomycetemcomitans [17]. However, such proposed mechanisms fail to explain the severe periodontal inflammation, associated alveolar bone destruction, and subsequent tooth loss that characterizes PLS periodontitis. This relates, in part, to a lack of systematic evaluation of different aspects of neutrophil function, which given the diverse range of NSP-dependent processes that in turn require CTSC activity, are likely to reveal a more complex series of linked events that culminate in the destructive, localized, chronic periodontal inflammation that characterizes PLS. Moreover, in the last decade, it has become clear that neutrophils are not merely short-lived destructor cells, armed with a prepackaged cytotoxic arsenal, but in fact, survive for 5.4 d in the circulation [18] and are capable of more subtle synthetic activities, elaborating various signaling molecules, including proinflammatory cytokines [19] and MRPs; of note are the calcium-binding proteins S100A8 and S100A9, which represent ~45% of proteins within the neutrophil cytosol [20]. S100s, also known as calgranulins, are involved in many proinflammatory immune processes, including phagocyte migration, chemotaxis, and antimicrobial defense.

Aims

In this study, we aimed to characterize comprehensively peripheral blood neutrophil function from a group of patients with PLS (aged between 9 and 14 yr) for the first time, using novel assays of directional chemotactic accuracy, NET formation, ROS, and cytokine release to explain the likely mechanisms underpinning the local, severe periodontal inflammation in the absence of significant systemic immune dysfunction in PLS.

MATERIALS AND METHODS

Blood collection and neutrophil isolation

Whole blood (up to 18 ml) was collected from 5 adolescents from 5 different families with PLS at Birmingham Children’s Hospital and 5 gender-matched control subjects. Exclusion criteria included smoking and pregnancy. Ethical approval was provided by the West Midlands/The Black Country National Research Ethics Service Committee (14/WM/1175).

Gender-matched, young, healthy control subjects (aged 19–21 yr) were recruited from students within the University of Birmingham Dental School. Neutrophils were isolated by Percoll density centrifugation using 2 discontinuous gradients—1.079 and 1.096—followed by erythrocyte lysis (0.83% NH4Cl containing 1% KHCO3, 0.04% EDTA, and 0.25% BSA), as previously described [21]. Cells were resuspended in gPBS (1 mM glucose) and cations (1 mM MgCl2, 1.5 mM CaCl2). The cells were diluted accordingly. Cell viability was determined by Trypan blue dye exclusion (typically, >98%) and cell purity by cytospin. Plasma was also prepared by centrifugation and stored at −80°C before further use. For all subsequent assays, plasma was used neat/diluted, according to relevant assay manufacturers’ instructions. As a result of low neutrophil counts, it was not possible for all 5 patient samples to be used in all assays; the numbers included are specified accordingly.

NET assays

NET DNA was quantified as described previously by our group [22]. In brief, neutrophils were resuspended in RPMI 1640 and added to a preblocked, 96-well microplate (1 × 105 in 150 µl/well). After a 30 min baseline incubation period (37°C), selected wells were stimulated with PMA (50 nM), HOCl (0.75 nM), or periodontally relevant bacterial stimuli (opsonized Staphylococcus aureus, A. actinomyces, b, and Fusobacterium nucleatum) at an MOI of 1000:1 to
ensure NET stimulation by the bacteria. NET DNA was digested after 4 h of incubation by the addition of micrococal nuclease (15 µl at 1 unit/ml) for 20 min to digest extracellular DNA, after which the cells were pelleted at 1200 relative centrifugal force for 10 min. Supernatant (150 µl) was collected into a black microplate, and Sytox Green (Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA; 15 µl, 10 µM) was added. Fluorescence was read in AFUs using a fluorometer (Twinkle LB 970; Berthold Technologies, Oak Ridge, TN, USA). NETs were also visualized after 4 h incubation with an epifluorescence microscope (Nikon Eclipse TE300).

NET-bound components were assayed within neutrophil supernatants using the following 96-well microplates: 1) NE, equal volumes (100 µl) of supernatants and 0.5 M Nε-methylsuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were mixed; after 2 h incubation (37°C), absorbance was measured at 405 nm; 2) NET-bound MPO, equal volumes (50 µl) of supernatant and 3,3′,5,5′-tetramethylenediamine substrate solution were mixed and incubated (20 min at room temperature); subsequently, the reaction was stopped by addition of sodium phosphate (50 µl, 1 M), and absorbance was measured at 540 nm; 3) NET-bound CG, equal volumes (50 µl) of supernatant and α-naphthyl-Ala-Ala-Pro-Phe-p-nitroanilide were mixed and incubated (2 h, 37°C), after which absorbance was measured at 405 nm.

**ECL assay of ROS production**

ROS were variously analyzed using the chemiluminescent reagents luminol, isoluminol, and lucigenin to detect total, extracellular ROS, and superoxide production, respectively, as described previously [19, 22]. In brief, a white, 96-well microplate was preblocked by addition of PBS with 1% BSA (200 µl/well) and incubated overnight at 4°C; before use, the liquid was aspirated off. Neutrophils were suspended in G PBS and seeded into the preblocked, 96-well microplate (1 × 10⁷ in 100 µl/well). Chemiluminescent reagents were added to the appropriate wells: luminol (30 µl, 3 mM), isoluminol (30 µl, 3 mM), or isoluminol (60 µl, 3 mM), with HRP (15 µl, 6 U). After a 30 min baseline incubation period, selected wells were stimulated with PMA (25 nM) to activate PKC directly without cell-surface receptor ligation or bacterial stimuli (opsonized S. aureus and heat-killed A. actinomycetemcomitans). All assays were performed at 37°C using a Berthold Technologies microplate luminometer (LB96v).

**Chemotaxis assay**

The Insall chamber was used for a novel, directional chemotaxis assay [23]. Neutrophils at 1 × 10⁶/ml were suspended in RPMI 1640, and 400 µl was added to 7.5% BSA-coated, acid-washed (0.2 M HCl) coverslips (22 mm) and incubated at room temperature for 20 min. The coverslip was then inverted onto the Insall chamber, and excess fluid was aspirated off. Chemotaxtants (100 nM) were added to the appropriate wells: luminol (30 µl, 3 mM), isoluminol (60 µl, 3 mM), with HRP (15 µl, 6 U). After a 30 min baseline incubation period, selected wells were stimulated with PMA (25 nM) to activate PKC directly without cell-surface receptor ligation or bacterial stimuli (opsonized S. aureus and heat-killed A. actinomycetemcomitans). All assays were performed at 37°C using a Berthold Technologies microplate luminometer (LB96v).

**Plasma protein oxidation**

In addition, plasma protein carbonylation was assayed as a measure of oxidative damage, as described previously [29]. In brief, the protein content of the plasma samples was measured using bicinchoninic acid assay. Oxidized standards (prepared using sodium borohydride) and plasma samples were diluted in carbonate buffer (pH 9.6, 0.05 M) to 20 mg/ml, and 50 µl of each was pipetted in triplicate into a Nunc-Immuno plate (MaxiSorp; Nunc, Roskilde, Denmark). Samples were derivatized directly on the plate using 1 mM diinotrophenylhydrazine in 2 M HCl. Following 3 washes, the plate was incubated with blocking buffer (Tween 20 (1% v/v) in PBS) overnight at 4°C. After incubation for 2 h at 37°C with mouse anti-DNP (Sigma-Aldrich, Dorset, United Kingdom) mAb, diluted 1:2000 in 1% BSA, the bound antibody was detected with peroxidase-conjugated rat anti-mouse IgE antibody (AbD Serotec, Raleigh, NC, USA; diluted 1:5000 in blocking buffer); 50 µl citrate phosphate buffer, pH 5.0, 0.1 M, containing 20 mg tablet-solubilized methyl-β-cyclodextrin; and 10 µl 8.8 M hydrogen peroxide. The reaction was terminated by addition of 25 µl 2 M H2SO4. Absorbance was measured spectrophotometrically at 490 nm, using a BioTek plate reader (Biotek, Swindon, United Kingdom). Carbonyl content was calculated from the standard curve and expressed as nanomoles of carbonyls per milligram of protein.

**RESULTS**

Table 1 provides summary patient demographics and cathespin C gene test mutation analyses.

**Quantification of NE and LL-37 from neutrophil culture supernatants and patient plasma**

NE and LL-37 were quantified in plasma and also from cultured neutrophil supernatants, both unstimulated and following TLR and FcγR stimulation (Fig. 1A–D), and from plasma. Both proteins are activated by CTSC; NE is activated by CTSC directly, and LL-37 is activated via PR3 [30], following PR3 activation by CTSC. NE was almost completely absent and stored at −80°C until further analysis of strategically relevant cytokines, MMP9, NE, LL37, and S100A-8/9.

**Multiplex assay of neutrophil cytokine and MMP9 release**

Proinflammatory cytokine (IL-1β, IL-6, IL-8, MIP-1α, TNF-α) and MMP9 release were measured using ProcartaPlex multiplex immunoassays (Affymetrix-eBioscience, Santa Clara, CA, USA), according to the manufacturer’s instructions.
compared with control subjects; statistical signi
cification was the same or higher in patients with PLS.
both activated by CTSC, were also extremely low in patients
in patients with PLS.

PLS [32].

nitrogen oxide levels) in unstimulated neutrophils from patients with
These results add to previous work demonstrating decreased
activity. These results indicate systemic and local NSP de
processes were substantially decreased or de
proteins, NE, MPO, and CG. NETs and NET-bound
and also by colorimetric quan

Lys

Cytokine, MMP9, and S100A8/9 levels in plasma and
neutrophil culture supernatants
The proinflammatory cytokines IL-1β, IL-6, IL-8, MIP-1α, and
TNF-α were quantified from culture supernatants and plasma
samples by multiplex assay. In addition, MMP9, the major
enzyme released by neutrophils involved in the breakdown of
the ECM to facilitate movement through the tissues, and the MRPs
S100A8 and -A9 were quantified by ELISA. Figure 4A–H
(Supplemental Table 1) shows neutrophil culture supernatant
levels, and Table 2 shows plasma concentrations. Within cultured
neutrophil supernatants, there was a significant increase in IL-6,
IL-8, and MIP-1α (P < 0.0286), in the absence of stimulation,
indicating a hyperactive neutrophil phenotype. Following TLR
stimulation, cytokines IL-6, IL-8, MIP-1α, and TNF-α were all
higher in PLS than control subjects (P = 0.0286). In addition,
S100A9 was found to be elevated. The same results were achieved
with FcγR stimulation, with the exception of IL-6. The neutrophil
supernatant results demonstrate that PLS neutrophils exhibit a
hyper-reactive neutrophil phenotype. IL-8 and MMP9 levels were
higher in PLS plasma than control subjects, consistent with
neutrophil cell-culture supernatant data.

Neutrophil chemotaxis in PLS
Neutrophil directional chemotactic accuracy was measured in
PLS using the Insall chamber. Speed (overall cell movement),
velocity (movement in the direction of the chemoattractant
source), and chemotactic index (a measure of the accuracy of
cell orientation) were measured over time in the presence of
buffer or chemoattractant. Speed was significantly lower in
patients when exposed to MIP-1α (Fig. 5A), a trend that was
consistent for MIP-1α and fMLP when velocity (Fig. 5B) and
chemotactic index (Fig. 5C) were measured. Figure 5D shows the
cell paths of all tracked cells per condition, illustrating the
differences in the course of the cell movements. MIP-1α is a
target for NSPs, which deactivate MIP-1α. As NSP function is lost
in individuals with PLS, MIP-1α was used in the chemotaxis assays

Quantification of NETs and NET-bound components
The ability of patients with PLS to produce NETs was
determined by fluorometric quantification of NET DNA
and also by colorimetric quantification of the NET-associated
proteins, NE, MPO, and CG. NETs and NET-bound
proteases were substantially decreased or deficient in
patients and significantly lower (where calculated) compared
with control subjects for all receptor stimulations evaluated
(Fig. 2A–D).

The absence of, or marked reduction in, NETs produced was
confirmed by epifluorescence microscopy (Fig. 2E), providing
additional data to that recently reported in a single patient with
PLS [31]. The reduced presence of NET-bound MPO further
supports the lack of NET structures in these patients, who do
not otherwise suffer a MPO deficiency. NE and CG, which are
both activated by CTSC, were also extremely low in patients
with PLS.

Neutrophil ROS generation and plasma protein
oxidation in PLS
Total and intracellular ROS and superoxide were detected using
different chemiluminescent reagents—luminol, isoluminol, and
lucigenin, respectively (Fig. 3A–C). In all conditions, ROS
generation was the same or higher in patients with PLS
compared with control subjects; statistical significance was
reached with the use of PMA. Protein carbonylation, a ROS-
induced marker of oxidative stress, was also measured in plasma
to provide evidence of ROS hyper-responsivity ex vivo (Fig. 3D).
These results add to previous work demonstrating decreased
antioxidant defenses/increased oxidative stress (lipid hydroper-
oxide levels) in unstimulated neutrophils from patients with
PLS [32].

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All volunteers had severe prepubertal periodontitis. Mutation analysis was by direct sequencing. Patient 1 was a compound heterozygote and had the mildest clinical phenotype but also had a very low neutrophil count at the time of venepuncture and thus was used for the NET quantification assay only. Patient 2 had a Y294C mutation (maternal allele) and thus is heterozygous for this mutation. The paternal allele was not detected and is probably within the intronic region that was not subject to analyses. BMI, body mass index; ND, not detected; N/A, not applicable.
to ascertain whether neutrophils of patients with PLS chemotax differently from those of healthy control subjects and whether MIP-1α chemotaxis is at all enhanced in patients with PLS over control subjects. Neutrophils in patients with PLS showed significantly reduced speed, velocity, and chemotactic accuracy compared with those of control subjects in response to MIP-1α and also a lower chemotactic index (accuracy of movement) in response to fMLP.

**DISCUSSION**

This is, to our knowledge, the most comprehensive analysis of neutrophil function in patients with PLS compared with unaffected control subjects and the first to explore directional chemotaxis and cytokine release as part of the neutrophil repertoire. PLS neutrophils demonstrated negligible NET formation, as analyzed by assays of NET DNA, and an almost total absence of the NET-associated AMPs CG, NE, and MPO, despite the fact that PLS neutrophils are not MPO deficient, as previously reported [33]. NE and CG are both enzymes that are activated by CTSC, and in vitro studies of NSPs have demonstrated that CG and NE are able to kill *A. actino* in addition to other periodontal bacteria [34], and so their deficiency likely compromises neutrophil antimicrobial efficacy, allowing for the persistence of some pathogenic species over others in PLS. NE is necessary for NETosis, as demonstrated by the study of NE disorders, such as *ELA2*-related neutropenia, where NE activity is deficient [35], and NET formation is diminished. Conversely, in *HAX1*-related neutropenia, elastase is functional, and patients with *HAX1* neutropenia produce NETs [36].

The ROS data indicate an enhanced response to PMA in PLS, consistent with a hyper-reactive neutrophil phenotype [21] that may contribute significantly to the oxidative stress (elevated lipid hydroperoxide levels) and antioxidant compromise, reported previously in PLS [22]. The increased ROS detected in neutrophils of patients with PLS may contribute to host-mediated periodontal tissue damage at sites where neutrophils are the dominant immune cell, such as the periodontium.

Cytokine measurements demonstrated neutrophil hyperactivity in PLS, with increased release of proinflammatory cytokines (IL-6, IL-8, and MIP-1α) from unstimulated cells. This was supported further by elevated cytokine levels in patients'
Figure 2. NETs and NET-bound proteins were quantified in response to PBS (neutral control), PMA (50 nM), HOCl (0.75 mM), or bacteria (MOI 1 in 1000). These stimuli trigger NET formation in different ways; HOCl is the downstream ROS required for NET release and whose exogenous addition bypasses NADPH oxidase activation. PMA crosses the cell membrane and directly activates PKC, allowing for NET release. TLR stimulation, using heat-killed bacteria, was also used. Bacteria were selected for each assay on the basis of clinical relevance, in that they are implicated in PLS (continued on next page).
plasma relative to control subjects. In addition, MMP9 levels were elevated in the plasma of patients with PLS. Furthermore, hyper-reactivity (to a stimulus), in terms of proinflammatory cytokine release and also S100A9 concentration, was also evident from the supernatants of stimulated cultured neutrophils. Higher S100A8/9 concentrations have been reported in inflamed tissues and are known to be markers of neutrophil activation [37]. Interestingly S100A9 is a target for CG [38] to generate NIF, which has been shown to inhibit neutrophil migration and chemotaxis in vitro [39]. The action of NIF may represent 1 means of limiting the neutrophil influx into inflammatory lesions, and its absence may contribute to the nonresolving, destructive, local inflammation evident in PLS. Conversely, higher levels of S100A9 in PLS, which can form a heterodimer with S100A8, have been shown to be involved in the assembly of the NADPH oxidase complex [40], which may explain the heightened ROS levels in our PLS studies.

Neutrophil chemotaxis was shown to be defective in patients with PLS, potentially increasing neutrophil tissue transit times and further potentiating neutrophil-mediated tissue damage. NSPs de-activate MIP-1α and IL-8, and the loss of NSP function in PLS may result in relentless neutrophil recruitment to periodontal tissues by such chemokines. Neutrophil speed and periodontitis (A. actino) and PLS-related skin abscesses (S. aureus). (A) NET quantification in stimulated/unstimulated conditions (n = 5 patient; n = 4 control). (B) NET-bound elastase detected after stimulation (n = 4). (C) NET-bound MPO levels (n = 3). (D) NET-bound CG; CG was undetectable in PLS neutrophils when stimulated with HOCl (n = 3). Blue open circles and red filled circles represent samples from control and patient groups (n = 4) respectively. (E) Representative NET images in the absence of stimuli (PBS) and following the addition of PMA, highlighting the lack of NET structures in patients with PLS.
Figure 4. Cytokines IL-1β (A), IL-6 (B), IL-8 (C), MIP-1α (D), TNF-α (E), MMP9 (F), S100A8 (G), and S100A9 (H) release from patient and control neutrophils (n = 4, respectively), isolated and cultured for 16 h and incubated with RPMI 1640 (control), A. actino (TLR stimulation pathway), or opsonized S. aureus (SA; FcyR stimulation pathway). Circles are as in Fig. 1.
velocity were also reduced in response to MIP-1α relative to control subjects, and there was a significant difference in directional chemotactic accuracy of PLS neutrophils for MIP-1α and fMLP. Previous studies that identified impaired chemotaxis in PLS were performed using different assays to those presented in this study, such as the Boyden chamber [41] and the zymosan-activated serum assay [42]. The latter assays do not measure directional chemotaxis, unlike the direct visualization chamber used in our study. Indeed real-time observation of migrating cells by time-lapse video microscopy is considered the “gold standard” for investigating chemotaxis [43].

Taken together, the data generated in this study point to 2 key scenarios as mechanistic explanations for PLS periodontitis, which will be discussed in turn: 1) relentless recruitment of neutrophils into periodontal tissues, where tissue transit times are increased, and 2) compromised antimicrobial killing.

**Table 2.** Cytokine, MMP9, and S100A8 and -9 concentrations from plasma collected from patients and control groups with PLS (n = 4, respectively)

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<th>Patient, pg/ml</th>
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**Compromised antimicrobial killing**

Patients with PLS suffer modest systemic infections, with 15–20% patients reporting recurrent infections, unlike patients with CGD, characterized by the lack of a functional NADPH oxidase and the subsequent inability to produce ROS or form NETs. It is noteworthy that patients with CGD are not overtly susceptible to periodontitis [49], suggesting that the periodontal tissue destruction seen in PLS cannot be fully explained by compromised neutrophil antimicrobial defenses. PLS individuals are not systemically immunocompromised, and our reported results demonstrate that PLS neutrophils have the capacity to respond to pathogenic stimuli, thus NSPs do not constitute the primary antimicrobial defense mechanism, and the underlying cause of periodontitis in PLS cannot be explained by reduced neutrophil bacterial killing alone. We propose that the periodontal tissue damage in patients with PLS results from a complex series of events arising downstream of the CTSC deficiency and dependent on a diverse range of NSP functions that are normally active at sites of microbial challenge. Specifically, impaired bacterial killing results in persistence of pathogenic species and triggers the relentless recruitment of hyperactive and reactive neutrophils in terms of ROS and proinflammatory cytokines into the periodontium. The latter, augmented by extended tissue transit times and a failure to de-activate MIP-1α, likely drives the connective tissue damage and bone loss in PLS. Defects in neutrophil function have been shown in several studies of patients with chronic periodontitis; peripheral neutrophils from patients with chronic periodontitis exhibit hyper-reactivity with respect to ROS release [21], proinflammatory cytokine production [19] in response to a microbial challenge, and also hyperactivity in the absence of an exogenous pathogen A. actino [33]. NSP dysfunction is associated with periodontal disease in other syndromes, including Chediak–Higashi syndrome [46] and the allelic variant to PLS, Haim Munk syndrome [47]. Furthermore, neutrophils from sufferers of specific granule deficiency, characterized by defects in the packaging of azurophil and specific granules (which contain NSPs and NADPH oxidase components, respectively), have been shown to exhibit decreased phagocytosis, diminished ROS formation, and reduced chemotaxis [48].
stimulant [50]. Chemotaxis in patients with chronic periodontitis has also been found to be defective, with only partial improvement after nonsurgical therapy [27], and oxidative stress is also a strong feature of periodontitis. In PLS, failure to activate and release NSPs appears to exaggerate the above defects and offers a more plausible explanation for the aggressive periodontal tissue damage seen in PLS than one based purely on defective antimicrobial defenses.

**Conclusions**

This study has characterized, for the first time, a range of neutrophil behaviors in patients with PLS from 5 different families and provided new insights into the functional consequences of CTSC deficiency upon periodontal tissue destruction and tooth loss. Our results show that PLS neutrophils have a substantially reduced capacity for NET production, with NET visualization data confirmed by the almost-total absence of NET-related proteins CG, NE, and MPO. ROS generation was higher in PLS, and chemotactic speed and velocity to a MIP-1α stimulus were reduced, with defective directional chemotactic accuracy toward fMLP and MIP-1α. Thus, we have demonstrated a range of functional neutrophil defects in PLS, arising secondary to the CTSC deficiency and consequent failure to activate NSPs, the cumulative effects of which may conspire to destroy periodontal tissues. The failure of key neutrophil antimicrobial

![Figure 5. Average speed (A), velocity (B), and chemotactic index (C) for patient and control neutrophils. The following are definitions of the respective chemotaxis parameters: speed (overall cell movement), velocity (movement in the direction of the chemoattractant source), and chemotactic index (a measure of the accuracy of cell orientation). (D) Cell tracks overlaid for patient and control group neutrophils in the presence of RPMI 1640 (neutral control), fMLP, and MIP-1α chemoattractants to give a qualitative overview of chemotaxis. Circles are as in Fig. 2.](image-url)
activities likely maintains a stimulus for a misdirected recruitment of hyper-responsive neutrophils into periodontal tissues and provides a plausible explanation for the severe inflammation and bone loss that characterizes PLS periodontitis. Interestingly, PLS individuals do not suffer systemic infections, other than rarely reported skin abscesses; therefore, the neutrophil defects appear specifically localized to areas of the body susceptible to a direct and chronic bacterial challenge.

AUTHORSHIP

H.R. participated in research design, performed experiments, analyzed results, and prepared the manuscript. P.W. performed experiments and analyzed results. I.D. performed plasma oxidation experiments. S.M. assisted with patient recruitment. R.V. and N.T. performed patient genotyping. M.G. and I.C. supervised the experimental as well as plasma oxidation experiments. S.M. assisted with patient recruitment of hyper-responsive neutrophils into periodontal tissues and provides a plausible explanation for the severe inflammation and bone loss that characterizes PLS periodontitis. Interestingly, PLS individuals do not suffer systemic infections, other than rarely reported skin abscesses; therefore, the neutrophil defects appear specifically localized to areas of the body susceptible to a direct and chronic bacterial challenge.

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DISCLOSURES

The authors have no conflict of interest to disclose.

REFERENCES


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Characterization of neutrophil function in Papillon-Lefèvre syndrome

Helen Roberts, Phillipa White, Irundika Dias, et al.

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