

## The role of the host

Chapple, Iain; Hirschfeld, Josefine; Kantarci, Alpdogan; Wilensky, Asaf; Shapira, Lior

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## REVIEW ARTICLE

# The role of the host—Neutrophil biology

Iain L. C. Chapple<sup>1</sup>  | Josefine Hirschfeld<sup>1</sup> | Alpdogan Kantarci<sup>2</sup> | Asaf Wilensky<sup>3</sup> | Lior Shapira<sup>3</sup>

<sup>1</sup>Periodontal Research Group, School of Dentistry, Institute of Clinical Sciences, College of Medical and Dental Sciences, and Birmingham's NIHR BRC in Inflammation Research, The University of Birmingham and Birmingham Community Health Foundation Trust, Birmingham, UK

<sup>2</sup>Forsyth Institute, Cambridge, Massachusetts, USA

<sup>3</sup>Department of Periodontology, Hadassah Medical Center, Faculty of Dental Medicine, Hebrew University of Jerusalem, Jerusalem, Israel

## Correspondence

Iain L. C. Chapple, Birmingham Dental School, 5 Mill Pool Way, Edgbaston, Birmingham B5 7EG, UK.

Email: [i.l.c.chapple@bham.ac.uk](mailto:i.l.c.chapple@bham.ac.uk)

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## 1 | INTRODUCTION

Neutrophilic polymorphonuclear leukocytes (neutrophils) are terminally differentiated effector cells that comprise 50%–70% of circulating leukocytes at any one time.<sup>1</sup> Their name derives from their propensity to stain with neutral dyes, but more importantly their multilobed or polymorphic nucleus, which distinguishes them from other granulocytes, principally from eosinophils and basophils. Granulocytes differ from other cells of myeloid lineage (monocytes) due to the development of lysosomal storage granules during their differentiation from bone marrow stem cells, driven by specific transcription factors (eg, growth factor independent-1, purine rich box-1) and growth factors (eg, granulocyte colony stimulating factor).<sup>2</sup> Four different granule types appear in a specific order at various stages of neutrophil differentiation<sup>3</sup> and maturation within bone marrow (Figure 1):

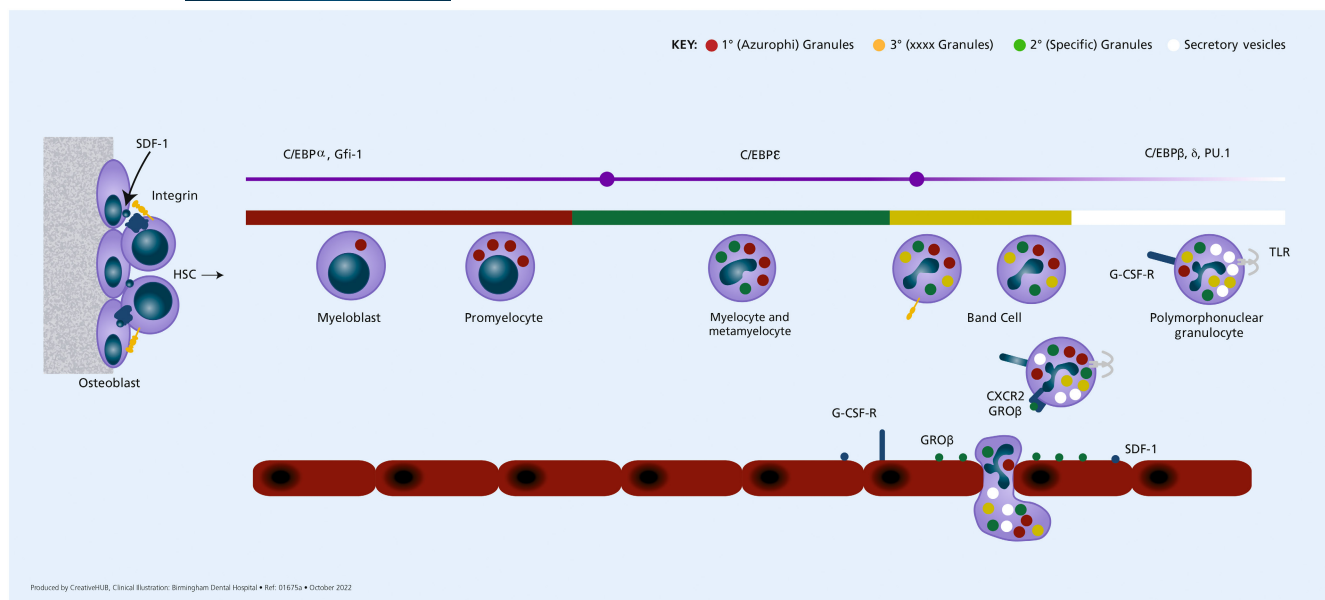
- Primary (azurophil) granules. These appear at the promyelocyte stage and are characterized by the presence of myeloperoxidase. They also contain neutrophil serine proteases (elastase, cathepsin G, proteinase-3, neutrophil serine proteinase-4),  $\alpha$ -defensins, bactericidal permeability-increasing protein, sialidase, azurocidin, and  $\beta$ -glucuronidase.<sup>4–9</sup> Azurophil granules carry cluster of differentiation surface markers CD63 and CD68 and appear to be a distinct organelle, different to other lysosomes.
- Secondary (specific) granules. These appear at the myelocyte stage and carry CD11b/CD18, CD66, CD67, and tumor necrosis

factor receptors on their surface, and the nicotinamide adenine dinucleotide phosphate oxidase components gp91phox and p22phox that are vital for reactive oxygen species generation once assembled into the full nicotinamide adenine dinucleotide phosphate oxidase complex (see later). They are peroxidase-negative granules and contain lactoferrin,<sup>4</sup> human cathelicidin-18 (a precursor of LL-37 an important antimicrobial peptide), lysozyme, collagenase, urokinase plasminogen activator, neutrophil gelatinase-associated lipocalin, vitamin B12-binding protein, lysozyme, haptoglobin, pentraxin3, secretory leukocyte protease inhibitor, orosomucoid, and  $\beta$ 2-microglobulin.<sup>10,11</sup> Specific granules fuse with the phagosome to form a phagolysosome, releasing their contents to digest the microorganisms within.

- Tertiary (gelatinase) granules. These appear at the metamyelocyte stage of granulocytopoiesis and carry the same surface markers as specific granules, but in addition they also express matrix metalloproteinase-25. They rarely fuse with the phagosome and are more readily exocytosed.<sup>12</sup> Gelatinase granules contain gelatinase, arginase-1, lysozyme,  $\beta$ 2-microglobulin and cysteine rich secretory protein-3.
- Secretory vesicles. These are the last granules to form at the band cell stage of neutrophil development and form by endocytosis rather than via the Golgi complex. They contain alkaline phosphatase and matrix metalloproteinase-25, but due to endocytosis are rich in surface receptors, expressing CD11b/CD18, CD67, gp91phox/p22phox, CD35, CD16, complement component 1q receptor, CD14, N-formylmethionine-leucyl-phenylalanine

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**FIGURE 1** Granulocytopoiesis within bone marrow. Hemopoietic stem cells (HSCs) localize to niches created by osteoblasts. CCAAT/enhancer binding protein- $\alpha$  (C/EBP $\alpha$ ) and growth factor independent-1 (Gfi-1) stimulate differentiation down a granulocyte pathway through six stages of maturation: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell (where commitment to becoming a neutrophil rather than a basophil or eosinophil occurs), and finally the mature terminally differentiated neutrophil. The different granules, primary, secondary, tertiary, and secretory vesicles form at different stages (see text). Granules form via the Golgi complex, and secretory vesicles form via endocytosis from mature neutrophils. The granules and secretory vesicles are released in the reverse order to their formation, with secretory vesicles being the most rapidly released and azurophil granules rarely being released but fusing to the phagosome to release their payload. The process of retention or egress of neutrophils from bone marrow is described in the text. CXCR2: C-X-C chemokine receptor type 2; GRO $\beta$ : growth-related oncogene  $\beta$ ; G-CSF-R: granulocyte colony stimulating factor receptor; PU.1: purine rich box-1; SDF-1: stromal derived factor-1; TLR: toll-like receptor. Figure adapted from Borregaard.<sup>2</sup>

receptor, vesicle-associated membrane protein-2, CD10, and CD13.<sup>13</sup> These receptors are rapidly mobilized to the polymorphonuclear leukocyte surface, where they bind complementary ligands to activate various downstream neutrophil processes via second messengers.

Neutrophils are critical cells to both innate and acquired (adaptive) immunity, acting for the innate immune system as first responders and in adaptive immunity as effector cells to eliminate microbes following their opsonization by immunoglobulins (Igs) produced by B-cells (plasma cells), with or without complement fixation. Neutrophils thus express multiple and diverse cell-membrane receptors, such as Fc gamma receptors (eg, Fc gamma receptor II) for IgG, and also complement receptors. Key are the pattern recognition receptors that recognize highly conserved microbe- or pathogen-associated molecular patterns on invading microorganisms. They include toll-like receptors for recognition of microbial components such as lipopolysaccharide (binds toll-like receptor 4), cytosine-phosphate-guanine motifs of bacterial deoxyribonucleic acid (DNA; binds toll-like receptor 9), and nucleotide-binding oligomerization domain-like receptors, which recognize intracellular bacterial components.<sup>14</sup> Other receptors include the following: those required for neutrophil transmigration from blood vessels to tissues (selectins, integrins); cytokine and chemokine receptors that modulate neutrophil behavior and movement; receptors for tissue damage-associated

molecular patterns, which recognize host-derived signatures to facilitate the biological debridement of wounds during tissue healing (eg, receptors for mitochondrial proteins, extracellular DNA, adenosine triphosphate); the receptor for advanced glycation end products; and bone marrow homing receptors (eg, C-X-C chemokine receptor type 4) that signal the return of neutrophils to bone marrow prior to removal of apoptotic neutrophils by stromal macrophages through phagocytosis.

This diverse neutrophil repertoire belies their terminally differentiated status; however, it is now recognized that, as well as killer cells that are preloaded and preprogrammed with a destructive antimicrobial arsenal, they are also capable of subtle synthetic processes to release cytokines and other signaling molecules, as well as pro-resolving lipid mediators that trigger the resolution of inflammation.<sup>15</sup> It is unsurprising that 10 billion neutrophils are produced per day,<sup>16</sup> circulating within blood as relatively passive cells undertaking immune surveillance in order to identify and rapidly respond to infection (via pathogen-associated molecular pattern receptors) or injury (via damage-associated molecular pattern receptors), when they engage in multiple cell-to-cell communication networks (see later).

The primary role of neutrophils is as key drivers of acute inflammation to protect the body's internal organs and tissues, prior to switching into tissue debridement mode and ultimately into a pro-resolution (of inflammation) phenotype. If acute inflammation fails to resolve then it becomes chronic in nature and leads to adverse

outcomes for the host, driving pathobiological changes that underpin most noncommunicable diseases of aging (eg, periodontitis, atherosclerosis, rheumatoid arthritis, chronic kidney disease).<sup>17-20</sup> Alternately, abscesses may form, the pus containing high numbers of dead and dying neutrophils that have failed to completely eliminate the infection (Figure 2).

Neutrophils are short-lived cells, with studies describing a life expectancy of 7-9 hours (with only a few hours present within the circulation),<sup>21</sup> up to 5.4 days when exposed to certain inflammatory stimuli that can prolong neutrophil survival.<sup>22</sup>

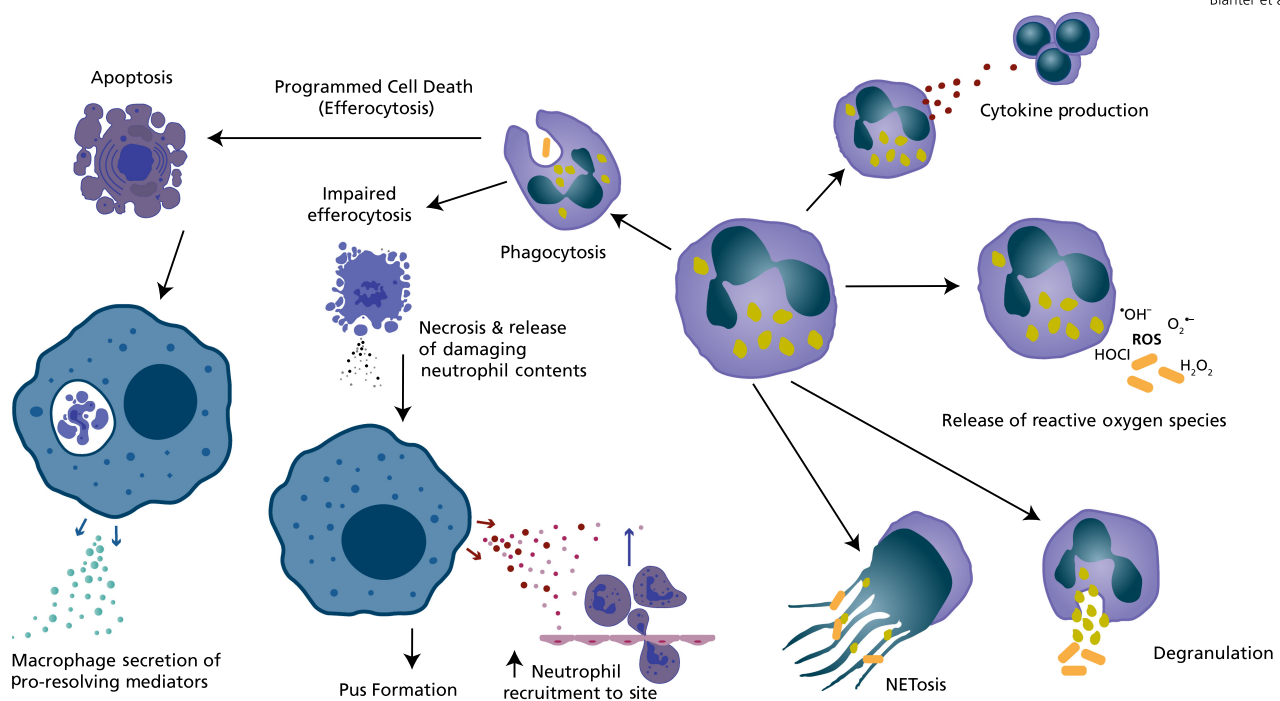
A normal adult blood neutrophil count is  $(1.5-7.5) \times 10^9/L$ , with mild neutropenia being  $(1.0-1.5) \times 10^9$  cells/L, moderate and severe neutropenia being  $(0.5-1.0) \times 10^9$  and less than  $0.5 \times 10^9$  cells/L, respectively, and neutrophilia being greater than  $8.0 \times 10^9/L$ .<sup>23</sup> Despite their primary role as phagocytes, neutrophils also contribute to the recruitment, activation, and programming of various other immune cells, via the secretion of proinflammatory and immunomodulatory cytokines and chemokines, which enhance recruitment and the

effector functions of other immune cells. Examples of both immune and nonimmune cells engaged by neutrophils include:

- dendritic cells;
- B-cells;
- CD4<sup>+</sup> T-helper cells, importantly Th17 cells (see later);
- CD8<sup>+</sup> T-cytotoxic cells;
- natural killer cells; and
- mesenchymal stem cells.

Normal neutrophil function is therefore protective against infection and injury; however, abnormal neutrophil function, whether innate due to gene mutations that impact critical physiological processes within neutrophils (eg, mutations in the cathepsin C gene in Papillon-Lefèvre syndrome,<sup>24</sup> chronic granulomatous disease,<sup>25</sup> or acquired as a result of various mediators/toxins released by microorganisms), such as antiapoptosis factors that prolong neutrophil survival and enhance the likelihood of adverse outcomes (eg,

Blanter et al 2020



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**FIGURE 2** The neutrophil antimicrobial repertoire, demonstrating key strategies employed under different environmental conditions. “Safe killing” involves phagocytosis and subsequent programmed cell death via efferocytosis and downstream apoptosis. “Unsafe killing,” where collateral host tissue damage arises following release of cytotoxic neutrophil contents, arises generally when neutrophils are overwhelmed, when phagocytosis is frustrated due to the size of the microbial challenge or by certain strategies employed by pathogens. In order to cause maximal damage to invading pathogens, neutrophils may degranulate in order to release their lysosomal contents over the offending microorganisms, coincidentally damaging adjacent host cells and tissues. Alternatively, when neutrophils are experiencing oxidative stress, they appear to direct reactive oxygen species (ROS) extracellularly as a self-preservation strategy,<sup>162</sup> preventing their necrosis but at the same time creating oxidative stress in adjacent tissues. When exposed to multiple simultaneous challenges neutrophils may undergo necrosis or a different form of programmed cell death called “NETosis”. Neutrophils also act as immune sentinels, releasing cytokines to communicate with other cells of the innate and adaptive immune systems. HOCl: hypochlorous acid; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide; <sup>•</sup>O<sub>2</sub>: superoxide; <sup>•</sup>OH: hydroxyl.

lipopolysaccharide),<sup>26</sup> or induce necrosis or NETosis (release of neutrophil extracellular traps during programmed cell death).<sup>27-29</sup>

In summary, normal neutrophil physiology involves the following:

- Their release from bone marrow to the vasculature to undertake immune surveillance.
- Entry to infected or damaged tissues by receptor-mediated transmigration across postcapillary venule walls (called diapedesis).
- Chemotaxis toward biological signals that indicate presence of infection (pathogen-associated molecular patterns, Fc-gamma receptors/complement-mediated opsonization) or injury (damage-associated molecular patterns).
- Phagocytosis of target organisms or tissue breakdown products, with or without release of neutrophil extracellular traps from viable neutrophils to trap microbes.<sup>30</sup>
- Either
  - reverse transmigration and homing to bone marrow (via C-X-C chemokine receptor type4) for removal by marrow macrophages,<sup>31</sup> or
  - apoptosis within tissues, facilitated by their surface expression of phosphatidylserine residues (so-called “eat me” signals) and efferocytosis (phagocytosis of apoptotic bodies by tissue macrophages).

Abnormal neutrophil function may arise from genetic defects or from neutrophil dysregulation induced by microbial virulence factors and, for example, glycemia/hyperglycemia<sup>32</sup> or cigarette smoking,<sup>33</sup> which can lead to catastrophic outcomes, principally:

- extracellular release of reactive oxygen species or proteolytic enzymes via exocytosis;
- NETosis, a form of programmed cell death with associated release of extracellular nuclear DNA and various other autoantigens; and
- necrosis with release of toxic granular/phagolysosomal contents.

This chapter will describe key stages in the neutrophil life cycle and function and how these may, under certain circumstances, contribute to periodontal tissue destruction and adverse systemic health outcomes.

## 2 | NEUTROPHILS AS SENTINELS OF IMMUNITY: PRODUCTION, RELEASE, AND ENTRY TO TISSUES

### 2.1 | Granulocytopoiesis and release

Granulocytopoiesis occurs in the bone marrow and involves hemopoietic stem cells that reside in niches provided by osteoblasts. The myeloid stem cells can differentiate into either monoblasts (and then monocytes) or into myeloblasts, which drive granulocyte production. The direction of differentiation is governed by a balance between two transcription factors, purine rich box-1 which drives monocyte differentiation and CCAAT/enhancer binding protein- $\alpha$

and growth factor independent-1, which together drive myelocyte/granulocyte formation (Figure 1).<sup>2</sup>

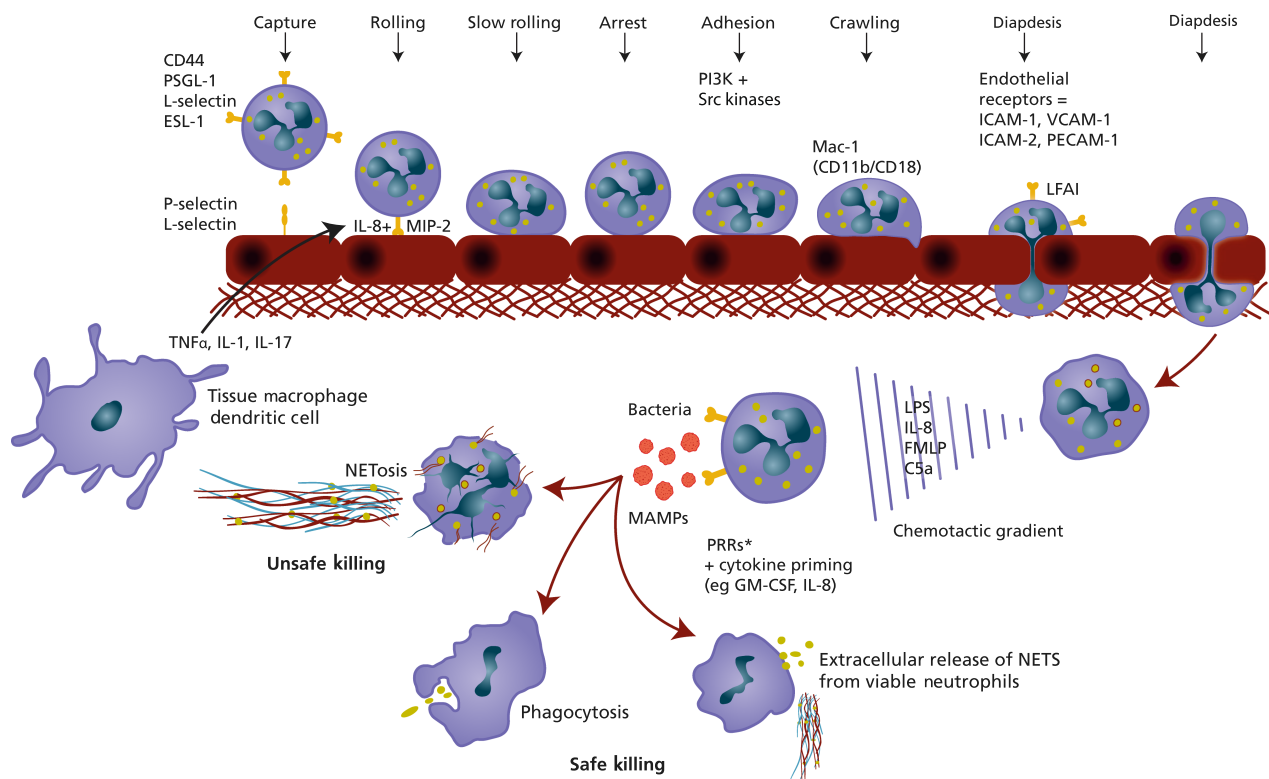
The balance between the release of neutrophils from bone marrow via marrow blood vessels and their retention within bone marrow is governed by the balance of two neutrophil surface receptors: C-X-C chemokine receptor type4, which drives retention in bone marrow, and C-X-C chemokine receptor type2, which drives release into marrow blood vessels. C-X-C chemokine receptor type4 retains neutrophils in bone marrow by binding to stromal derived factor-1 (now called C-X-C ligand 12) from bone marrow stromal cells. C-X-C chemokine receptor type2, however, binds to growth-related oncogene  $\beta$  on the vascular endothelial cells pulling neutrophils out of bone marrow and into the marrow circulation. Granulocyte colony stimulating factor works by downregulating stromal derived factor-1/C-X-C ligand12 production, thus tipping the balance from C-X-C chemokine receptor type4 (retention in marrow) to C-X-C chemokine receptor type2 expression (transmigration out of bone marrow).

The high rate of neutrophil production within bone marrow is regulated by the rate of neutrophil apoptosis within tissues<sup>2</sup> (the higher the tissue neutrophil levels the higher the apoptosis rate). As neutrophils accumulate and apoptose in tissues, macrophages/Langerhans cells reduce their production of interleukin-23, which in turn reduces interleukin-17A/F production by neutrophil regulatory T-cells.<sup>34</sup> Interleukin-17 is a key cytokine produced by Th17 lymphocytes and other immune cells that stimulates granulocyte colony stimulating factor, and hence granulocytopoiesis.<sup>35</sup> Interleukin-17 exhibits a number of functions relevant to periodontitis, including:

- increased granulocytopoiesis via elevation of granulocyte colony stimulating factor;<sup>35</sup>
- increased neutrophil recruitment via release of chemokines (C-X-C ligand 1, C-X-C ligand 2, C-X-C ligand 5, C-X-C ligand 8) by epithelial, endothelial, macrophage, and stromal cells;<sup>36</sup>
- osteoclast formation via increased osteoblast expression of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL);<sup>37</sup>
- increase in formation of epithelial cell tight junctions to increase barrier formation at exposed epithelial surfaces.<sup>38</sup>

### 2.2 | Transmigration/diapedesis

Transmigration of neutrophils across the vascular endothelium and into infected/injured tissues is a highly coordinated receptor-driven process triggered by signals that include pathogen-associated molecular patterns and damage-associated molecular patterns. These signals are detected by tissue-resident immune cells such as dendritic cells, resulting in the release of mediators such as nitric oxide, tumor necrosis factor- $\alpha$ , interleukin-1beta, and interleukin-17, which activate vascular endothelial cells, largely in the postcapillary venules of the affected tissue. This results in the first of six highly coordinated steps: capture, rolling, arrest, adhesion, crawling, and diapedesis (Figure 3).<sup>27</sup> The blood flow in postcapillary venules is slow and, therefore, supports neutrophils dropping from midstream



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**FIGURE 3** Neutrophil vascular immobilization (capture, rolling, and arrest), endothelial margination (adhesion and crawling), diapedesis, transmigration, and chemotactic trafficking into tissues in response to prokaryotic stimuli and eukaryotic “help” signals. E- and P-selectin receptors are activated on vascular endothelium by complement component 5a (C5a), leukotriene B<sub>4</sub>, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1), lipopolysaccharide (LPS, also known as endotoxin), interleukin-17 (IL-17), and histamine, and start to make contact with complementary neutrophil surface proteins such as L-selectin. This process pulls the neutrophil out of midstream blood flow, where it contacts the endothelial cells, rolls along the endothelium, and eventually arrests its motion. The adhesion is strengthened by enzymatic activity involving phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B. The neutrophil then moves between the endothelial cells mediated by binding of intergrins on its cell surface (lymphocyte function-associated antigen-1 [LFA-1/LFA1], macrophage-1 antigen [Mac-1, comprising CD11b, CD18, and complement receptor], and very late antigen-4 [VLA-4, also called intergrin  $\alpha$ 4b1, comprising CD49d and CD29]) to intercellular adhesion molecules (intercellular adhesion molecule-1 [ICAM-1] and intercellular adhesion molecule-2 [ICAM-2]) on the endothelial cells, which allow the neutrophil to move across the basement membrane and into the tissues. The neutrophil then moves along a chemotactic gradient (chemotaxins include interleukin-8 [IL-8], FMLP [N-formylmethionyl-leucine-phenylalanine], C5a, and LPS) toward the site of infection. CD: cluster of differentiation; ESL-1: E-selectin ligand-1; GM-CSF: granulocyte-macrophage colony stimulating factor; MIP-2: macrophage inflammatory protein-2; MAMPs: microbe-associated molecular patterns; MFTs: multifunctional T cells; NET: neutrophil extracellular trap; PRRs: pattern recognition receptors; PSGL-1: P-selectin glycoprotein ligand 1; PECAM-1: platelet endothelial cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1. Figure reproduced from Cooper et al.<sup>27</sup>

flow to make initial contacts with the vascular endothelial cells. This is further facilitated by nitric oxide and complement component 5a, as well as leukotriene B<sub>4</sub>, prostaglandin E<sub>2</sub>, histamine, and bradykinin, which facilitate vasodilatation (eg, nitric oxide relaxes vessel-wall muscle fibers) and increase vascular permeability, lowering blood pressure in the venules and allowing neutrophil margination to vessel walls. The cytokines released (tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , interleukin-17) stimulate upregulation of P- and E-selectins on endothelial cells. P-selectin binds complementary neutrophil ligands such as P-selectin glycoprotein ligand-1 and

L-selectin, as does E-selectin, which binds E-selectin ligand-1 and CD44. The effect is to “capture” neutrophils and marginate them against the endothelium.<sup>39</sup> These initial bonds are weak and make and break, allowing the neutrophil to “roll” along the endothelium, facilitated further by L-selectin on the neutrophil surface binding to sialyl Lewis-X glycans on the endothelial cells.<sup>40</sup> In leukocyte adhesion deficiency, which is associated with severe periodontitis, there are heritable defects in fructose transport that prevent formation of sialyl Lewis-X and therefore negatively impact neutrophil transmigration.

Rolling is followed by arrest, adhesion and crawling, which are mediated by  $\beta$ 2 integrins on the neutrophil surface, mainly leukocyte function antigen-1 and macrophage-1 antigen/complement receptor-3 (which consists of CD11b and CD18 subunits). Leukocyte function antigen-1 binds intercellular adhesion molecule-1 on vascular endothelium, whereas macrophage-1 antigen/complement receptor-3 (CD11b, CD18) binds to intercellular adhesion molecule-2 on the endothelium.<sup>41,42</sup> The interleukin-1, tumor necrosis factor- $\alpha$ , and interleukin-17 also activate endothelial cells to produce interleukin-8 (CX-C ligand8) and macrophage inflammatory protein-2, both of which are powerful mediators of neutrophil recruitment into tissues.<sup>43</sup> Such chemoattractants can “prime” neutrophils,<sup>44</sup> creating a state of alert whereby subsequent stimulation creates heightened and enhanced functions, such as reactive oxygen species production, phagocytosis, degranulation, cytokine and neutrophil extracellular trap release, and chemotaxis (see later). Priming mobilizes secretory vesicles but does not activate reactive oxygen species production by nicotinamide adenine dinucleotide phosphate oxidase, nor does it elicit degranulation.

Diapedesis is the final stage of neutrophil transmigration between endothelial cells guided by leukocyte function antigen-1 and macrophage-1 antigen binding to intercellular adhesion molecule-1 and intercellular adhesion molecule-2, respectively, and vascular cell adhesion molecule-1 and platelet endothelial adhesion molecule-1. Traversing the basement membrane is believed to be facilitated by the following:

- Release of neutrophil elastase, which degrades laminin and collagen fiber proteins to facilitate the journey.
- Polymerization reactions of the actin cytoskeleton within the neutrophil to facilitate conformational change and movement.<sup>45</sup>

The complex interactions between the various cell-adhesion molecules with the actin cytoskeleton and cadherin bonds between endothelial cells are beyond the scope of this review, but superbly summarized by Nils Borregaard in his 2010 review.<sup>2</sup>

It is important to note, however, that endogenous inhibitors of leukocyte recruitment and adhesion exist and are key regulators of neutrophil tissue influx. These include the host-expressed molecules developmental endothelial locus-1, pentraxin-3, and growth differentiation factor-15, which interfere with leukocyte function antigen-1, P-selectin, and  $\beta$ 2 integrins, respectively; and these functions have been directly observed in mice.<sup>46-49</sup> Genetic deficiencies of these regulating processes have been associated with a large number of diseases, including inflammatory conditions; however, the majority of studies have been conducted in gene-knockout animal models.<sup>50,51</sup> For developmental endothelial locus-1, aged mice exhibited a developmental endothelial locus-1 deficiency that led to enhanced neutrophil infiltration of the gingival tissues accompanied by increased periodontal bone loss.<sup>52</sup> Further studies of these potential therapeutic targets are required in humans.

In summary, neutrophils respond to alarm signals released by tissue macrophages and other immune cells, as well as directly to mediators released by bacteria, such as lipopolysaccharide and

*N*-formylmethionyl-leucyl-phenylalanine. Such mediators also activate the vascular endothelium to induce vasodilatation and reductions in blood flow rate, allowing neutrophils to fall out of midstream blood flow and contact the endothelium. Here, a sequence of receptors, initially selectins and then integrins, facilitates transmigration into tissues in a process that is tightly coregulated by endogenous inhibitors of neutrophil adhesion. The neutrophil then moves toward the site of tissue infection or injury, guided by chemotaxins, and ultimately binds to pathogen-associated molecular patterns during the initial innate immune response and then to opsonized bacteria during adaptive immunity. Ideally, phagocytosis follows, phagolysosomes form, nicotinamide adenine dinucleotide phosphate oxidase is activated, and various reactive oxygen species activate proteases released by granules into the phagosome to degrade the microbes within. The neutrophil then undergoes apoptosis and is removed safely by macrophages. The remainder of this chapter will describe in more detail the processes of chemotaxis, reactive oxygen species release, phagocytosis, protease and neutrophil extracellular trap release, and where these processes appear to become dysfunctional in periodontitis and in peri-implantitis and cause substantial collateral host tissue damage and bone loss.

### 3 | NEUTROPHIL SUBTYPES AND SURFACE MARKERS

Neutrophils are well-researched cells of the innate immune system and have been investigated for several decades. Despite this, new discoveries have been made in recent years. One of these is the notion that neutrophil populations are comprised of different subsets and phenotypes, although it had long been noted that neutrophils are heterogeneous in their behavior within the same cell population. For example, only a proportion of stimulated neutrophils from the same donor perform phagocytosis or form neutrophil extracellular traps, whereas others undergo apoptosis or appear to be nonactivated. The question has been raised as to whether distinct neutrophil subsets exist as a consequence of cell priming and activation, or as preexisting intrinsic subpopulations. A body of evidence on such subsets has been generated, and further research is underway, also in the context of periodontitis.

After transmigration into tissue, blood neutrophils undergo changes to their phenotype, depending on the presence of inflammatory triggers, cytokines, and growth factors in the tissue microenvironment. In a study by van Staveren et al<sup>53</sup> involving deep phenotyping of exercising amateur cyclists, it was shown that circulating neutrophils not only increased in their total number but also that two new subsets formed, which were characterized by changed surface expressions of CD16 and CD62L. The same group investigated these surface markers during acute, subacute, and chronic inflammation in humans and found differential release patterns of these subsets from the bone marrow, where they were present under all conditions, including homeostasis. It was concluded that, in each condition, different recruitment signals may have been the cause of this release pattern.<sup>54</sup>

Neutrophil phenotypes and subsets are currently not well understood, particularly the triggers that lead to their formation and release. Apart from inflammation, genetic factors are likely to play a role. For example, a specific gene profile was reported in systemic lupus erythematosus patients that was associated with the presence of proinflammatory low-density neutrophils and with vascular inflammation clinically.<sup>55,56</sup> Watt et al<sup>57</sup> profiled the binding of the transcription factor purine rich box-1, which is crucial in myeloid development (see earlier herein), in primary neutrophils from 93 volunteers. They reported that genetic variants associated with differential purine rich box-1 binding were associated with differences in cell count and susceptibility to autoimmune and inflammatory diseases using a biostatistical model and the human genomewide association studies catalog.<sup>57</sup>

Neutrophils not only differ interindividually, but also within the same donor in terms of maturation stage, priming or activation status, density, surface marker expression, and cell function and behavior. Primed and activated neutrophils exhibit further phenotypic differences, such as in their ability to adhere to substrates, express surface receptors, degranulate, and release reactive oxygen species, as well as to undergo apoptosis.<sup>58</sup> Depending upon the stimulus, unprimed (quiescent) neutrophils can become activated without prior priming, but generally the elicited response will be amplified in primed cells.<sup>59</sup> Priming can be understood as a continuum of different activation states, depending on the quantity and nature of the priming agent, and it can also be reversed into the quiescent state that normally characterizes circulating neutrophils.<sup>60</sup>

Recent evidence suggests that neutrophils are able to reverse-migrate back into the bloodstream from tissues, a process termed reverse diapedesis, and further back into bone marrow. In the bone marrow of a myocardial infarction model in parabiotic mice, primed and reverse-migrated neutrophils were shown to induce granulopoiesis via interleukin-1 $\beta$  release, thus establishing a direct communication pathway between damaged tissues and the bone marrow.<sup>61</sup> In humans, Buckley et al<sup>62</sup> identified a profile of cell-surface receptors (CD54<sup>high</sup>, C-X-C chemokine receptor type 1<sup>low</sup>) in those neutrophils that had performed reverse diapedesis and which was distinct from naive circulatory neutrophils and from tissue neutrophil phenotypes. They reported that 1%-2% of peripheral blood neutrophils in patients with systemic inflammation exhibited this phenotype, whereas 0.25% in healthy donors performed reverse diapedesis.

A recent study by Grieshaber-Bouyer et al<sup>63</sup> performed single-cell ribonucleic acid (RNA) sequencing in neutrophils from normal and inflamed murine tissues, aiming to answer the question of whether functional diversity of neutrophils requires discrete neutrophil subsets. The authors observed a single chronological developmental spectrum, from immature pre-neutrophils, largely in bone marrow, to mature neutrophils, predominantly in blood and spleen. Neutrophils migrating into inflamed murine tissues maintained the core signature of maturity, modified by new transcriptional activity influenced by site and stimulus. Using the Human Cell Atlas, a database sharing single-cell and spatial genomics data for all cell types under healthy conditions,<sup>64</sup> the authors further found that human

neutrophils exhibit a similar transcriptomic pattern. According to these findings, neutrophils do not exist in discrete subsets; rather, they are heterogeneous as a consequence of local and systemic signaling.

In support of this theory, neutrophils constitutively express infection-associated surface receptors, such as toll-like receptors, which are increased upon stimulation with signaling molecules such as interferon-gamma and granulocyte colony stimulating factor.<sup>65</sup> Similarly, expression of Fc-gamma receptor types present on neutrophils and complement component receptors are upregulated by cytokines and antigens such as *Staphylococcus aureus* and the bacterial product *N*-formylmethionyl-leucyl-phenylalanine.<sup>66,67</sup> Also, exogenous noxae, such as cigarette smoke and electronic cigarette smoke, were shown to induce release of interleukin-8, a strongly chemotactic factor for other neutrophils.<sup>68</sup>

In preclinical infection models, circadian differences in neutrophil behavior were also observed, with neutrophils showing enhanced tissue infiltration and microbial clearance during nighttime.<sup>69,70</sup> Furthermore, a morning peak in neutrophil aging has been observed in both mice and humans, favoring clearance from the circulatory system and egress of newly matured neutrophils from the bone marrow during the morning and daytime.<sup>69,71</sup> This would suggest that a fraction of systemic neutrophils is similarly matured, possibly reducing heterogeneity. To date, the majority of circadian neutrophil studies have been conducted in murine models, and human studies are currently scarce.

Another time-related factor affecting neutrophil function is age. Immune response deterioration with increasing age (immunosenescence) is thought to be a key mechanism of chronic inflammatory disease onset. Research has shown that most aspects of neutrophil function are decreased in aged humans, including responses to priming agents, chemotaxis, phagocytosis, and possibly the generation of reactive oxygen species.<sup>71,72</sup> These defects in neutrophil function may arise from changes to the cell membrane and/or to proximal events in receptor signaling. For example, neutrophil priming and activation in response to a range of ligands is decreased with age, including *N*-formylmethionyl-leucyl-phenylalanine, granulocyte colony stimulating factor, and lipopolysaccharide.<sup>73</sup> Age reportedly also impairs neutrophil responses to toll-like receptor 2 and toll-like receptor 4 agonists,<sup>74</sup> though their expression was not found to be decreased,<sup>75</sup> supporting the theory of dysfunctional signaling pathways.

Neutrophils also possess surface markers with varying expression patterns and, so far, unknown roles, such as olfactomedin-4 and CD177.<sup>76</sup> The latter is expressed on the plasma and granule membranes of neutrophils with an interdonor variability of 0%-100%. Whereas the CD177-null phenotype is caused by a single-nucleotide polymorphism, the mechanism underlying the bimodal (sometimes even trimodal) CD177 expression is not completely understood.<sup>77</sup> Equally, the function of CD177 remains elusive, although it was found that CD177 may be a receptor for platelet endothelial cell adhesion molecule-1.<sup>78</sup> As CD177 is located on both intracellular granule membranes and the plasma membrane of neutrophils, it can



be further upregulated on the cell surface upon activation and degranulation.<sup>79</sup> However, this mechanism does not fully explain the diversity in isolated circulating neutrophils from healthy donors.

Neutrophils have long been perceived as a proinflammatory cell type once activated, whereas proinflammatory (M1) and anti-inflammatory (M2) macrophages have been regarded as distinct subsets since the 1990s.<sup>80</sup> More recently, similar neutrophil phenotypes have been discovered, termed N1 and N2 neutrophils, which were initially found to be associated with tumor growth and, therefore, termed tumor-associated neutrophils.<sup>81</sup> For head and neck cancers, the increased presence of tumor-associated neutrophils was shown to be a negative independent prognostic factor for tumor recurrence, as well as for overall survival.<sup>82</sup>

N1 and N2 phenotypes are known to feature high and low intracellular density, respectively. Functionally, N1 phenotypes are proinflammatory, whereas N2 cells have more anti-inflammatory properties.<sup>83</sup> These neutrophils are now thought to be present in inflamed tissues other than tumors and to be part of the innate immune response. However, very few studies have been conducted to investigate N1 and N2 phenotypes in noncancer inflammation, the majority of these being in preclinical models. In infected mice, resting neutrophils and N1 and N2 neutrophils were also reported to exhibit morphological differences of their nuclei (round, multilobular, and ring-shaped, respectively), as well as distinct cytokine and chemokine expression signatures.<sup>84</sup> For example, interleukin-4 and interleukin-10 were found to be expressed by N2, whereas interleukin-12 was found in N1, and resting neutrophils did not express either.

In the context of periodontitis, the existence of different neutrophil phenotypes has been confirmed in both circulating and oral neutrophils. Fine et al<sup>85</sup> reported three distinct oral neutrophil subsets: resting/naive circulatory neutrophils, para-inflammatory neutrophils found in the healthy oral cavity, and proinflammatory oral neutrophils found in periodontitis. Para-inflammatory neutrophils were defined as intermediately activated, as opposed to highly activated neutrophils, which also appeared as two distinct subpopulations with different size, granularity, and expression of specific CD markers. Though the periodontal and oral environments certainly contribute to neutrophil priming and activation due to the presence of microbial antigens, host inflammatory mediators, and unfavorable osmotic conditions, it is possible that certain phenotypes are preferentially recruited to inflamed periodontal tissues.

A recent study showed higher expression of CD177 on neutrophils isolated from gingival crevicular fluid compared with blood neutrophils from periodontitis patients.<sup>86</sup> This significant increase was found in all patients tested ( $n = 13$ ), but not in the gingival crevicular fluid neutrophils from periodontally healthy subjects. Nevertheless, a higher proportion of CD177<sup>+</sup> blood neutrophils was still found in periodontitis patients than in healthy subjects. In a second control group, synovial fluid from joints with inflammatory arthritis was analyzed for CD177<sup>+</sup> neutrophils, showing no increase of this surface marker compared with blood neutrophils. These results could be explained by a different arsenal of chemotactic factors being

released locally, depending on the presence of pathogenic or health-associated communities in the oral cavities, or in sterile inflammation as seen in inflammatory arthritis. These findings further point toward an unknown role of CD177 in infection-mediated chemotaxis.

Functional neutrophil phenotypes have been analyzed in periodontitis since the 1970s, mostly in blood neutrophils isolated from periodontitis patients versus healthy individuals.<sup>87</sup> Chemotaxis, phagocytosis, reactive oxygen species, and cytokine release, as well as neutrophil extracellular trap forming capabilities of neutrophils, have been quantified, and the results of these studies are summarized in the appropriate sections of this chapter. Whether these functionally different neutrophils in periodontal health and disease constitute distinct phenotypes, however, is currently not known.

Many of the neutrophil phenotypes or possible subsets described in this section have not been investigated in the context of periodontitis, such as N1, N2, and circadian subsets. Studies are needed that further compare different neutrophil phenotypes isolated from the circulation, periodontal tissues, and gingival crevicular fluid from the same individuals in both health and disease. Importantly, neutrophil marker expression, cell morphology, and functional aspects, such as reactive oxygen species and cytokine release, as well as cell death pathways, need to be coinvestigated. Such studies may not only provide more insight into the reasons underlying the vast nature of neutrophil heterogeneity but also into neutrophil-microbe interaction pathways, with the aim of identifying new therapeutic targets.

## 4 | NEUTROPHIL CHEMOTAXIS IN HEALTH AND DISEASE

Neutrophil chemotaxis is the directional migration at a velocity of 4–14  $\mu\text{m}/\text{min}$  in vivo, as assessed by intravital microscopy in animal models.<sup>88,89</sup> Neutrophils migrate toward the highest concentration of a chemotactic agent, which can be host or microbial derived. Examples are the bacterial peptide *N*-formylmethionyl-leucyl-phenylalanine, chemokines interleukin-8/C-X-C ligand8, macrophage inflammatory protein-1 $\alpha$  (C-C motif chemokine ligand3), granulocyte chemotactic protein2 (C-X-C ligand6) and epithelial cell-derived neutrophil-activating peptide-78 (C-X-C ligand5), complement component 5a, and lipid mediators like leukotriene B4.<sup>90</sup> Neutrophils follow a chemotactic gradient through its specific receptors for each agent, usually heptahelical G protein-coupled receptors, and the chemotactic response is mainly regulated by the Rho family of GTPases.

Circulating neutrophils, when encountering a chemotactic gradient in the small blood vessels, enter a cascade of events enabling them to leave the blood vessel and enter the tissue. Tethering and rolling of neutrophils on the endothelial surface is the first step, followed by activation of integrin and the firm adhesion of neutrophils to the blood vessel wall. This activation is mediated by local chemokines or proinflammatory signals. Next, neutrophils crawl and migrate through the vascular endothelium, a process termed diapedesis. The molecular mechanisms of neutrophil trans-endothelial migration

have been extensively reviewed elsewhere<sup>91</sup> and are discussed earlier in the chapter.

Leukocyte function antigen-1 integrin (CD11a/CD18) and macrophage-1 antigen (CD11b/CD18), are central beta-2 integrins of this process. The most prominent integrin on neutrophils is CD11b/CD18, which has a binding capacity to more than 40 known ligands, including intercellular adhesion molecules, complement fragment iC3b, fibrinogen, and other extracellular matrix proteins.<sup>92</sup> During selectin-mediated rolling and slow rolling, neutrophils engage with chemokines and cytokines presented on the surface of vascular endothelial cells with several different receptors.<sup>93</sup> Neutrophils can cross the endothelial barrier via two distinct routes, either between two endothelial cells via cell-to-cell junctions (paracellular route) or directly through them (transcellular route).<sup>91</sup> Each route requires the engagement of several further adhesion molecules.

Neutrophils are able to migrate through very tight spaces within the endothelial layer and connective tissue, requiring extensive cytoplasmic and nuclear deformation through cytoskeleton rearrangements,<sup>94</sup> of which actin microfilaments are particularly well researched in this context. Two poles are formed during migration: the leading-edge cell protrusion named a pseudopod, and the rear end, termed the uropod. Actin stability is polarized, with dynamic F-actin concentrated in the pseudopod and stable F-actin concentrated at the uropod, where there is high actomyosin contractility.<sup>95</sup>

During movement, neutrophils shed extracellular vesicles derived from uropod extensions, resulting from surface adhesion and generation of submicrometer trails. These contain cytoplasmic material and enhance cell-to-cell communication in health and disease.<sup>95</sup> The vesicles are distinct from neutrophil-derived membrane microvesicles, which are generated by activated neutrophils at the site of inflammation. Microvesicles derived from uropods have the function to guide subsequent immune cells, including other neutrophils, toward the inflammatory focus through the mechanism of slowly releasing chemokines into the surrounding tissues.<sup>96</sup>

The pseudopod and uropod are dynamic structures that can alter their location rapidly, allowing the neutrophil to change direction and migrate efficiently toward the target.<sup>97</sup> Directional changes are necessary either due to sensing of physical obstacles or due to changes in the chemotactic gradient.<sup>83,98</sup> Interestingly, neutrophil-like HL60 cells migrating in restrictive three-dimensional collagen matrices in vitro were shown to retain overall chemotactic migration despite turning more frequently as matrix density increased in order to circumvent obstacles rather than remodel the matrix.<sup>99</sup> However, neutrophil-like cells have also been criticized as a model system, as they do not resemble primary neutrophils sufficiently. For example, they display impaired reactive oxygen species and neutrophil extracellular trap formation compared with blood neutrophils<sup>100,101</sup> and exhibit different chemotactic behavior,<sup>102</sup> whereas degranulation or enzyme release has barely been described in the literature. However, these functions have an important role in intentional and pathognomonic tissue remodeling.

Indeed, neutrophil-mediated degradation of host tissue components, such as collagens, and peroxidation of host proteins and lipids

by reactive oxygen species have been demonstrated in vitro.<sup>103-106</sup> This has been proposed as a mechanism of the tissue damage seen in periodontitis, particularly if neutrophils have impaired chemotactic abilities and, therefore, longer tissue transit times. In both blood and oral neutrophils from periodontitis patients, neutrophils were found to have significantly decreased chemotactic accuracy compared with healthy individuals by several independent studies. For example, already in 1980, Van Dyke et al<sup>107</sup> reported that the majority of young subjects with rapidly progressing periodontitis showed depressed neutrophil chemotaxis both before and after periodontal therapy, indicating that an intrinsic neutrophil-associated defect affecting chemotaxis existed.

Individuals with this form of periodontitis are also known to harbor the gram-negative pathogen *Aggregatibacter actinomycetemcomitans* in their periodontal pockets more commonly than patients with periodontitis starting later in life. Neutrophils exposed to low levels of leukotoxin produced by this bacterium, and able to penetrate cells and tissues, caused marked activation of neutrophils while inducing nondirectional migration in vitro.<sup>108</sup> High concentrations of leukotoxin (100 ng/mL) strongly induced NETosis and subsequent cell death and was also associated with reduced neutrophil speed, and velocity toward chemoattractants.<sup>108</sup> Together, these outcomes are likely to lead to significant collateral tissue damage, if they arise in vivo. Also, environmental factors like cigarette smoke, a well-established risk factor for periodontitis, was shown to impact on neutrophil chemotactic accuracy.<sup>109</sup> For neutrophils present in oral tissues, this may be particularly relevant, as toxic tobacco smoke components are absorbed by the oral mucosa.<sup>110</sup>

The central role of activated neutrophils with dysfunctional chemotaxis in periodontal tissue damage is further supported by a study investigating chemotaxis in neutrophils from Papillon-Lefèvre syndrome patients.<sup>24</sup> This is a rare autosomal recessive disorder characterized by severe prepubertal periodontitis and tooth loss, caused by a mutation in the cathepsinC gene leading to complete loss of enzyme activity. The lack of this serine protease resulted in a reduced ability of neutrophils to undergo chemotaxis efficiently, while releasing higher levels of reactive oxygen species and proinflammatory cytokines than healthy controls do.

Because neutrophil proteases like elastase, cathepsinG, and matrix metalloproteinases are also necessary for tissue remodeling during basal membrane and extracellular matrix transmigration of neutrophils,<sup>111</sup> studies are needed to investigate the imbalance between physiological degradation and tissue regeneration that leads to pathologic sequelae. This may require the use of comprehensive multicellular in-vitro models. Thus far, neutrophil chemotaxis and migration in the context of periodontitis have mostly been researched using two-dimensional migration chambers, such as Boyden, Zigmond, and Insall chambers.<sup>24,112,113</sup> These have been used to assess directional accuracy, speed, and velocity of neutrophils. Simpler trans-well plate migration assays have been employed to examine the proportion of neutrophils crossing a membrane toward a chemotactic agent.<sup>114</sup> However, two-dimensional migration assays do not capture all aspects of in-vivo neutrophil migration.

Therefore, efforts have been made to create three-dimensional models using hydrogels, which mimic tissue conditions in terms of density, stiffness, and provision of adhesion substrates for neutrophils.<sup>115,116</sup> This is important, as neutrophil adhesion, spreading, and speed are affected by substrate stiffness, as neutrophils have mechanosensing abilities to help them to adapt to their environment.<sup>117,118</sup> In vivo, spinning-disk confocal intravital microscopy has been employed in zebrafish and mice, which can visualize neutrophil trafficking in capillary networks, trans-endothelial migration, and chemotaxis.<sup>119,120</sup> In periodontal research, the use of in-vitro three-dimensional tissue models in particular has the potential to provide researchers with a better and more detailed understanding of the role of neutrophil trafficking and impaired chemotaxis in disease onset and progression.

## 5 | PHAGOCYTOSIS IN HEALTH AND DISEASE

Phagocytosis, the intracellular uptake and breakdown of ingested microorganisms, is a critically important neutrophil antimicrobial strategy. Primed neutrophils perform enhanced phagocytosis compared with unprimed cells.<sup>58</sup> Depending on particle size, up to 50 microbial cells can be ingested via endocytosis.<sup>121,122</sup> The resulting phagosome fuses with primary and secondary granules containing myeloperoxidase, serine proteases, gelatinase, lysozyme, defensins, and bactericidal permeability-increasing protein<sup>123</sup> (see earlier). Phagocytosis is initiated through activation of a range of receptors, including Fc-gamma receptors, complement receptors, and nonopsonic pattern recognition receptors, such as Dectin-1, Mincle and toll-like receptors, that directly recognize conserved pathogen-associated molecular patterns.<sup>124</sup>

The generation of reactive oxygen species is a central step of phagocytosis and requires assembly of the nicotinamide adenine dinucleotide phosphate oxidase complex in activated cells. For assembly, the cytosolic components p47phox, p67phox, and p40phox associate with the membrane-bound gp91phox (nicotinamide adenine dinucleotide phosphate oxidase 2) and p22phox, which are mainly present in specific granules, and to a lesser extent in the plasma membrane and in secretory vesicles.<sup>123</sup> Although reactive oxygen species normally lead to acidification, neutrophils have regulation mechanisms that achieve a transient change in phagosomal pH from neutral to alkaline, in contrast to other phagocytic cells. This may provide activity niches for different granule enzymes to function, as their optimal pH range is from pH 6 to 10.<sup>125</sup> Neutrophils use passive leakage of protons, active proton pumps (vacuolar-type ATPases), ion transport, and exchange systems such as the sodium ion/proton exchanger, and possible bicarbonate buffering to regulate their phagosomal pH. Transporters and channels, such as chloride transport mechanisms, are used to achieve vesicle membrane charge compensation.<sup>123</sup>

Phagocytosis as a mechanism of microbial killing is considered safe for the surrounding tissues, as degranulation and

reactive oxygen species release occur mainly within the phagosome. However, when neutrophils encounter microbial biofilms like those found in the oral cavity, a phenomenon named frustrated phagocytosis occurs, as neutrophils are unable to engulf biofilm-embedded bacteria.<sup>126</sup> Nonetheless, the biofilm pathogens are recognized by neutrophils, which induces extracellular release of enzymes and other toxic compounds, causing damage to surrounding host cells and tissue components such as collagens. These, then, not only become additional substrate for the biofilm community to expand, but also enhance inflammation by releasing damage-associated molecular patterns.<sup>127,128</sup>

In addition, pathogens have evolved a number of mechanisms to survive phagocytosis. Examples are inhibition of the oxidative burst in the phagosome, inhibition of vacuolar-type ATPase activity, and blocking fusion of neutrophil granules with the phagosome. Additionally, some pathogens use catalase to disrupt the generation of oxidative metabolites, or release pore-forming enzymes that may be used to escape from the phagosome membrane into the cytosol.<sup>123,129</sup> A specific example is *Helicobacter pylori*, which can divert specific granules to the plasma membrane, leading to the reduced nicotinamide adenine dinucleotide phosphate complex being unavailable at the phagosome and instead to large amounts of extracellular reactive oxygen species being produced.<sup>130</sup> Such microbial defense strategies have also been investigated in some periodontal pathogens: *Treponema denticola* and *Porphyromonas gingivalis* efficiently block neutrophil phagocytic events by interfering with cytoskeleton pathways, providing protection to other bacteria in the oral cavity also. Furthermore, *T. denticola* and *P. gingivalis* proteases can degrade complement components and thus escape opsonization and phagocytosis.<sup>131</sup>

Both impaired and enhanced neutrophil phagocytosis have been described by several studies in patients with rapidly progressing periodontitis, formerly termed juvenile, aggressive, or refractory periodontitis.<sup>132-137</sup> At the same time, neutrophils in these patients released excess reactive oxygen species and granule contents extracellularly, as reported in some studies. This combination may, in part, explain the rapid tissue loss seen in these forms of periodontitis. However, the contradictory reports on phagocytic function highlight the need for further well-designed mechanistic studies of neutrophils from periodontitis patients. Genetic studies of neutrophils relating to phagocytosis have thus far been carried out mainly on polymorphisms in the Fc-gamma receptor genes, but the majority of these did not find a clear association.<sup>138</sup> One study investigated single-nucleotide polymorphisms in the neutrophil formyl peptide receptor 1, which indirectly enhances phagocytosis via upregulation of Fc-gamma and complement receptors, and found to be associated with "aggressive periodontitis" in an African American population.<sup>139</sup>

There are multiple challenges in conducting mechanistic phagocytosis studies. Most neutrophil defense mechanisms are closely related. For example, cytoskeleton reformation is required for all defense mechanisms including chemotaxis, whereas reactive oxygen species production is also required for phagocytosis and neutrophil extracellular trap formation. Moreover, different responses

can be provoked through activation of the same receptors, such as Fc-gamma receptors and toll-like receptors.<sup>140</sup> Hence, using agonists and antagonists often leads to unwanted side effects in neutrophils and may affect the many components of the molecular machinery involved in phagocytosis, including receptor signaling cascades, cytoskeleton reformation, reactive oxygen species production, cytosolic trafficking, and membrane fusion processes.

Phagocytosis experiments are usually conducted under in-vitro conditions, which lack the multitude of signaling factors present in vivo. A recent study showed that neutrophil phagocytosis of periodontal pathogens was more effective when platelets and plasma factors were present.<sup>141</sup> This emphasizes that neutrophils in vivo interact with other host cells, mediators, and bacteria, all of which are likely to modify neutrophil responses. Carefully designing experiments and coinvestigating neutrophil functions in well-defined patient populations and controls might give clues about specific dysfunctional signaling in phagocytosis.

## 6 | NEUTROPHIL REACTIVE OXYGEN SPECIES RELEASE IN HEALTH AND DISEASE

### 6.1 | Physiologic neutrophil microbial killing

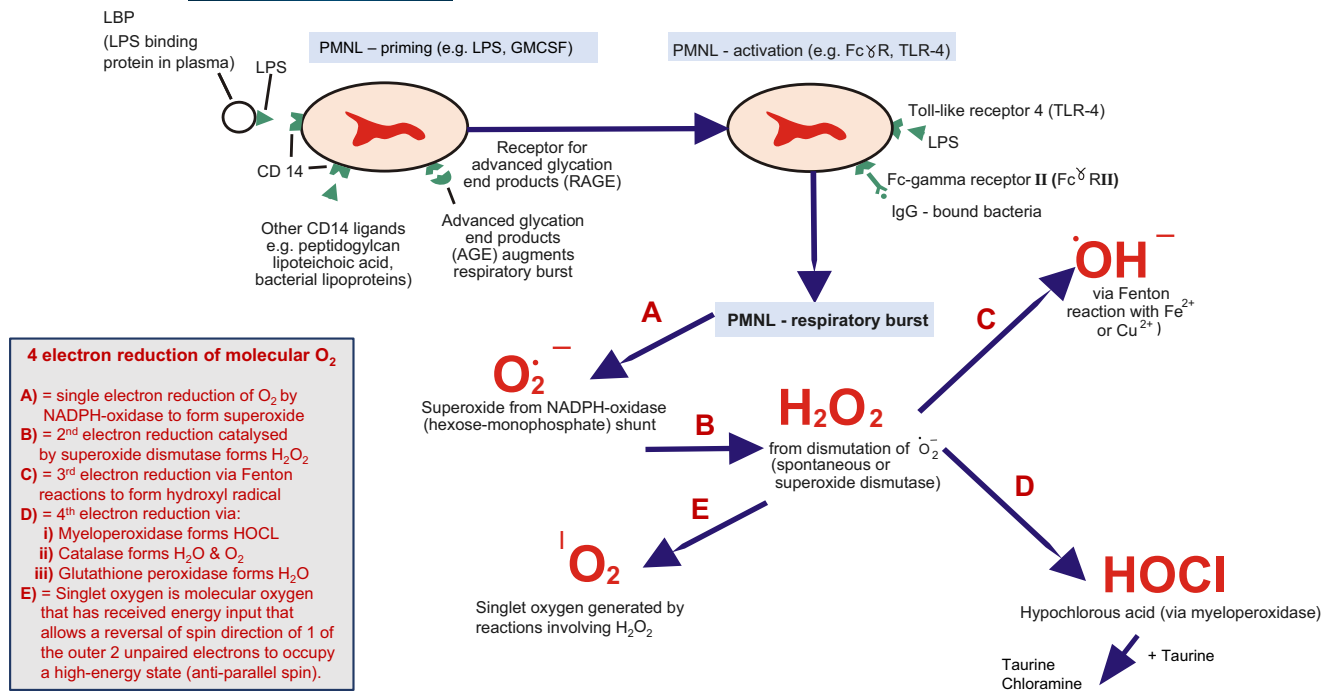
The generation of reactive oxygen species by neutrophils, also termed the “respiratory burst,” is a physiologic response to invading pathogens and is primarily protective. The respiratory burst generates a cascade of reactive oxygen species achieved by a four-electron reduction of molecular oxygen mediated by a series of enzymes (Figure 4). Neutrophil surface receptor ligation by diverse microbial and nonmicrobial stimuli triggers various signal transduction pathways, which act via second messengers to assemble the membrane-bound and cytosolic components of the nicotinamide adenine dinucleotide phosphate oxidase, ideally on the phagosome membrane but also on the plasma membrane, leading to extracellular reactive oxygen species release when neutrophils exhibit internal oxidative stress (reviewed in detail by Chapple and Matthews).<sup>142</sup> The oxidase is assembled from three membrane subunits (gp-91phox/nicotinamide adenine dinucleotide phosphate oxidase2, p22phox, and Ras related protein 1A) and four cytosolic proteins (p47phox, p67phox, p40phox, and Ras-related C3 botulinum toxin substrate2). The phosphorylation of the cytosolic nicotinamide adenine dinucleotide phosphate oxidase components is necessary for their translocation to the plasma/phagosomal membrane. Once assembled, the complete oxidase initially generates the superoxide anion, which either spontaneously dismutates to hydrogen peroxide or the dismutation is catalyzed by the enzyme superoxide dismutase (Figure 4). Hydrogen peroxide can diffuse across the phagosomal and outer plasma membranes and can itself be converted to the potent hydroxyl radical via Fenton or Haber-Weiss reactions with divalent iron or divalent copper cations, respectively. The hydroxyl radical is highly destructive and can oxidize vital cellular structures, including proteins, lipids, and nucleic acids. Superoxide can also

react with nitric oxide radical to form the equally damaging peroxynitrite anion. Hydrogen peroxide can be removed by a variety of intracellular enzymes, including catalase (forming water and molecular oxygen), glutathione peroxidase (forming oxidized glutathione from its reduced form), or myeloperoxidase, to form hypochlorous acid, as depicted in Figure 4.

The safe killing of microbial pathogens occurs within the phagocytic vacuole (phagosome) where following fusion of lysosomal granules with the phagosome to form a phagolysosome, the reactive oxygen species change the pH of the phagolysosome and activate proenzymes (released by the neutrophil granules) to destroy the pathogenic contents within. Following degradation of the phagolysosomal contents, the neutrophil undergoes apoptosis, expressing phosphatidylserine residues on its surface to facilitate efferocytosis by macrophages (Figure 2). In this manner, collateral host tissue damage is prevented. However, if excess reactive oxygen species are produced or extracellular reactive oxygen species are released, tissue damage results through the aforementioned oxidation reactions. Recent data emerging from an in-depth study of the gram-positive anaerobic rod *Filifactor alocis*, which is reported in high numbers in periodontitis, demonstrated an inhibition of neutrophil apoptosis by *F. alocis*. Mechanisms involved reduced activation of caspases 3, 8, and 9 and upregulation of important antiapoptotic proteins. Engagement of toll-like receptors 2 and 6 by the organisms appeared to be involved in prolonging the neutrophil lifespan, which, given the potential for neutrophil-mediated collateral tissue damage, may have implications for a pathogenic role for *F. alocis*.<sup>143</sup>

### 6.2 | Neutrophil reactive oxygen species hyperresponsiveness in periodontitis: Work from the Karolinska Institute

The generation of significant reactive oxygen species by neutrophils requires a pH of 7.0-7.5 and a minimum oxygen tension of approximately 1%.<sup>144,145</sup> Such conditions are found in periodontal pockets,<sup>146,147</sup> demonstrating the potential for excess reactive oxygen species production at such an important site of periodontal tissue damage. Mendes et al<sup>148</sup> investigated the role of *Fusobacterium nucleatum* in actively inducing low oxygen tension, which may modulate angiogenesis and endothelial cell activity through expression of hypoxia-inducible factors, known to modulate the expression of nitric oxide synthases, vascular endothelial growth factor, and cytokine release as adaptive responses to hypoxia, but which can drive periodontal tissue destruction.<sup>149</sup> They demonstrated that *F. nucleatum* actively depleted oxygen and that the resulting hypoxia significantly increased several hypoxia-inducible factor isoforms. Inducible nitric oxide synthase increased, but nitric oxide remained unchanged, with increases in vascular endothelial growth factor release at 4 hours and vascular endothelial growth factor receptor 1 at 12 hours. Changes in interleukin-1alpha and tumor necrosis factor- $\alpha$  release were time dependent, with an initial reduction at 4 hours and an increase by 24 hours. Indeed, hypoxia has been shown to result in



**FIGURE 4** The four-electron reduction of molecular oxygen is initiated by ligand binding to various neutrophil cell surface receptors, thus activating via second messengers (such as protein kinase C) the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex to assemble and generate superoxide radicals (first electron reduction). Various downstream chemical reactions arise spontaneously or are catalyzed by specific enzymes to effect the second electron reduction (creating hydrogen peroxide [H<sub>2</sub>O<sub>2</sub>]), the third reduction (creating the potent hydroxyl radical [OH<sup>-</sup>]) and the final reduction which generates either hypochlorous acid (HOCl), water (H<sub>2</sub>O) and oxygen (O<sub>2</sub>) dependent upon the specific catalyst (myeloperoxidase, catalase, or glutathione peroxidase). In “safe” neutrophil killing the various neutrophil antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) ensure oxygen radicals and reactive oxygen species, which are not true radicals but remain highly reactive, are removed to liberate water and molecular oxygen. GMCSF: granulocyte-macrophage colony stimulating factor; IgG: immunoglobulin G; LPS: lipopolysaccharide; PMNL: polymorphonuclear leukocyte.

a destructive “hypersecretory” neutrophil phenotype in chronic obstructive pulmonary disease, similar to that described in periodontitis, which is associated with an enhanced capacity for endothelial injury, and a corresponding elastase signature consistent with neutrophil degranulation.<sup>150</sup>

A number of studies undertaken in the 1980s and 1990s by various groups investigated the release of reactive oxygen species by neutrophils from patients with juvenile periodontitis, and what was termed “chronic periodontitis” at the time. These are summarized in Table 1, which has been updated from our review in 2007<sup>142</sup> to the present day. There was a general trend toward excess reactive oxygen species release that emerged in both juvenile and chronic periodontitis; however, overall data interpretation was complicated due to the use of a wide variety of substrates employed to determine reactive oxygen species release. However, clarity started to emerge following a series of carefully planned and executed studies by Gustafsson and coworkers in Sweden. Using the chemiluminescent substrate luminol to detect total reactive oxygen species production, they demonstrated small but consistently significantly higher levels of reactive oxygen species production by Fc gamma receptor-stimulated peripheral blood neutrophils from periodontitis patients relative to age- and gender-matched periodontitis-free controls.<sup>151-154</sup> It was notable that, when isoluminol was employed as the substrate, only very low levels of Fc gamma

receptor-stimulated reactive oxygen species could be detected in the extracellular space, and a similar difference was found between patients and controls.<sup>154</sup> At the time, the biological basis for the reported hyperreactive neutrophil phenotype in periodontitis was unclear, but it was not associated with the method of blood neutrophil preparation,<sup>153</sup> or with a reduced expression of diacylglycerol kinase,<sup>155</sup> the presence of polymorphisms in the Fc gamma receptor,<sup>154,156</sup> or the expression of adhesion molecules.<sup>151</sup> The seminal work of the Karolinska team deserves special mention, and it created the momentum for further ex-vivo studies by our own group, where we employed divalent cations and glucose in the neutrophil suspension media in order to replicate, as closely as possible, the in-vivo environment.

### 6.3 | Studies of dysfunctional neutrophil reactive oxygen species generation and oxidative stress: Work from Birmingham, UK

#### 6.3.1 | Peripheral blood neutrophil hyperresponsivity in periodontitis patients

In 2007, our group took forward the work of Asman, Bergström, and Gustafsson, using different reactive oxygen species substrates

**TABLE 1** Studies investigating reactive oxygen species production from in vitro-activated peripheral neutrophils (updated from ref 142).

Stimulants and result (periodontal disease vs health)									
Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formylmethionyl-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Asman et al (1984) <sup>331</sup>	Juvenile periodontitis: 8 males, 6 females with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> using Hank's balanced salt solution Peak signal	100:1	Latex ↔ <i>Staphylococcus aureus</i> ↑					
Ellegaard et al (1984) <sup>332</sup>	Juvenile periodontitis: 12 Chronic periodontitis: 10 Controls: 22, age/sex matched	Cytochrome c reduction using Hank's balanced salt solution			Zymosan ↔ Zymosan ↔			↔	Not detected
Henry et al (1984) <sup>333</sup>	Juvenile periodontitis: <sup>c</sup> 26 Asian subjects with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> using Hank's balanced salt solution Peak signal	5 mg/10 <sup>6</sup> cells (1.1 mg/mL)	Zymosan ↔ Zymosan <sup>d</sup> ↑	Zymosan ↔				
Asman et al (1986) <sup>334</sup>	Juvenile periodontitis: 5 males, 8 females with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> Peak signal	200:1	<i>Staphylococcus aureus</i> ↑		<i>Staphylococcus aureus</i> ↑			
Van Dyke et al (1986) <sup>135</sup>	Localized juvenile periodontitis: 14 patients and 17 controls	Cytochrome c reduction	6.7 mg/mL		Zymosan ↔				
Åsman (1988) <sup>335</sup>	Juvenile periodontitis: 6 males, 6 females (after successful treatment) with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> using Hank's balanced salt solution in N-formylmethionyl-leucyl-phenylalanine assay only Percentage chemiluminescence based on peak signal	200:1 12.5 nM N-formylmethionyl-leucyl-phenylalanine	<i>Staphylococcus aureus</i> ↑		<i>Staphylococcus aureus</i> ↑	↔		

(Continues)

TABLE 1 (Continued)

Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs health)						
				Autologous serum opsonized	Nonautologous serum opsonized	Immuno-globulin or histone II* opsonized	N-Formyl(methionyl)-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 1,2,13-dibutyrate*	None	
Asman et al (1988) <sup>336</sup>	Juvenile periodontitis: 3 males, 7 females with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> using Hank's balanced salt solution Peak signal	200:1	<i>Staphylococcus aureus</i> ↑						
Whyte et al (1989) <sup>337</sup>	Young chronic periodontitis: n = 8 with age-matched controls Old chronic periodontitis: 8 patients with age-matched controls	Luminol-dependent chemiluminescence using Hank's balanced salt solution Peak signal Subject polymorphonuclear leukocyte assayed individually with polymorphonuclear leukocytes from a "standard" donor	100 μL suspension (of optical density at 625nm of 1.0)/10 <sup>6</sup> cells	<i>Fusobacterium nucleatum</i> ↑ <i>Escherichia coli</i> ↑ <i>Fusobacterium nucleatum</i> ↓ <i>Escherichia coli</i> ↓	<i>Fusobacterium nucleatum</i> ↑ <i>Escherichia coli</i> ↑ <i>Fusobacterium nucleatum</i> ↓ <i>Escherichia coli</i> ↓					
Mattout et al (1990) <sup>338</sup>	Juvenile periodontitis <sup>6</sup>	Not known	Not known				↓	↔		
Guarnieri et al (1991) <sup>339</sup>	Chronic periodontitis: 14 patients with 16 controls Gingival crevicular fluid polymorphonuclear leukocytes Peripheral polymorphonuclear leukocytes	Cytochrome c reduction	Not known					↑ ↓	↔ ↑	
Shapira et al (1991) <sup>340</sup>	Rapidly progressing periodontitis: 4 males, 1 female with age-matched controls	Cytochrome c reduction using Hank's balanced salt solution Luminol-dependent chemiluminescence using Hank's balanced salt solution Counts per minute at 5 min	0.5-1 μg/mL phorbol-myristate-acetate Bacteria not known						<i>Streptococcus</i> ↑* <i>Streptococcus</i> ↑*	↔ ↔

TABLE 1 (Continued)

		Stimulants and result (periodontal disease vs health)							
Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formylmethionyl-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Zafropoulos et al (1991) <sup>341</sup>	Rapidly progressing periodontitis: 19 patients Juvenile periodontitis: 10 patients Chronic periodontitis: 10 patients All with age/sex-matched controls	Luminol-dependent chemiluminescence Peak signal	0.35 mg/1.4 × 10 <sup>5</sup> cells (0.35 mg/mL)	Zymosan ↔ Zymosan ↔ Zymosan ↔	Zymosan ↔ Zymosan ↔ Zymosan ↔				
Asman and Bergström (1992) <sup>342</sup>	Juvenile periodontitis: 4 patients with controls Juvenile periodontitis: 3 patients with controls	Luminol-dependent chemiluminescence <sup>b</sup> Percentage chemiluminescence based on peak signal	200:1	Staphylococcus aureus (↑) Staphylococcus aureus or Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis (↑)	Staphylococcus aureus (↑)	Staphylococcus aureus or Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis (↑)			
Kimura et al (1993) <sup>343</sup>	Localized juvenile periodontitis: 15 patients Generalized juvenile periodontitis: 13 patients Chronic periodontitis: 52 patients Unmatched controls: 30, age = 30.5 ± 0.9 y	Dichlorofluorescein-diacetate oxidation and flow cytometry with whole blood	100 ng/mL				(↑) (↑) (↑)		↔ ↔ ↔
Gomez et al (1994) <sup>344</sup>	Rapidly progressing periodontitis: 3 or 5 patients Juvenile periodontitis: 3 or 4 patients Chronic periodontitis: 3 or 4 patients All with age/sex-matched controls	Luminol-dependent chemiluminescence <sup>b</sup> using Hank's balanced salt solution Peak signal and total chemiluminescence	0.32 mg/mL 5 × 10 <sup>-6</sup> M phorbol 12,13-dibutyrate	Zymosan ↔ Zymosan ↔ Zymosan ↔	Zymosan ↔ Zymosan ↔ Zymosan ↔			↔* ↔* ↔*	
Leino et al (1994) <sup>345</sup>	Localized juvenile periodontitis: 3 males, 6 females with age/sex-matched controls	Luminol-dependent chemiluminescence using calcium/magnesium ion-free Hank's balanced salt solution and leukocytes. Peak signal	50 μg/10 <sup>5</sup> cells (100 μg/mL) 76 nM N-formylmethionyl-leucyl-phenylalanine 80 nM phorbol-myristate-acetate	Unopsonized zymosan ↑ Zymosan (↑) Zymosan (↑) (females only)	Zymosan (↑) Zymosan (↑) (females only)	Zymosan (↑) Zymosan ↑ (females only)	↑	(↑)	

(Continues)



TABLE 1 (Continued)

Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs health)				
				Autologous serum opsonized	Nonautologous serum opsonized	II* opsonized	Immuglobulin or histone	N-Formylmethionyl-leucyl-phenylalanine
Mouyret et al (1994) <sup>346</sup>	Gingivitis: 8 patients Chronic periodontitis: 8 patients Early onset periodontitis: 17 patients Unmatched controls: 7	Luminol-dependent chemiluminescence on polymorphonuclear leukocyte and whole blood Peak signal and chemiluminescence index	Not known	Zymosan ↔ Zymosan ↔ Zymosan ↔	↔ ↔ ↔			
Gustafsson and Asman (1996) <sup>151</sup>	Chronic periodontitis: 9 males, 5 females with age/sex-matched controls	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin Peak signal expressed as ratio to paired control	300:1		<i>Staphylococcus aureus</i> ↑			
Gustafsson et al (1997) <sup>347</sup>	As for Gustafsson and Asman (1996) <sup>151</sup>	As for Gustafsson and Asman (1996) <sup>151</sup>	300:1, as for Gustafsson and Asman (1996) <sup>151</sup> with priming <sup>a</sup>		<i>Staphylococcus aureus</i> ↑ after priming			
Fredriksson et al (1998) <sup>152</sup>	Chronic periodontitis: <sup>b</sup> 8 males, 9 females with age/sex-matched controls	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin Peak signal Dichlorofluorescein-diacetate oxidation and flow cytometry	300:1 ± priming with tumor necrosis factor-α and lipopolysaccharide		<i>Staphylococcus aureus</i> ↑ ± priming <i>Staphylococcus aureus</i> ↔ ± priming			
Hurtta et al (1998) <sup>348</sup>	Localized juvenile periodontitis: 5 females (4 in maintenance treatment phase) with age/sex-matched controls	Luminol-dependent chemiluminescence using calcium/magnesium ion-free Hank's balanced salt solution Peak signal	50 μg/10 <sup>5</sup> cells (100 μg/mL) Unopsonized zymosan (↑)					

TABLE 1 (Continued)

		Stimulants and result (periodontal disease vs health)							
Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Autologous serum opsonized	Nonautologous serum opsonized	Immuno-globulin or histone II* opsonized	N-Formyl(methionyl)-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Biasi et al (1999) <sup>349</sup>	Generalized early onset periodontitis: 6 males, 9 females with age/sex-matched controls	Cytochrome c reduction in Hank's balanced salt solution with 0.2% human albumin plus calcium/magnesium ions	0.1 mg/mL 10 <sup>-7</sup> M N-formyl(methionyl)-leucyl-phenylalanine 10 ng/mL phorbol-myristate-acetate	Zymosan ↔			↓	↔	↔
Fredriksson et al (1999) <sup>350</sup>	Chronic periodontitis: 8 males, 9 females with age/sex-matched controls	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin Peak signal	300:1		<i>Staphylococcus aureus</i> ↑				
Fredriksson et al (1999) <sup>350</sup>	Chronic periodontitis, non-smoking: 21, and 29 controls Chronic periodontitis, smoking: 19, and 14 controls	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin Peak signal	300:1		<i>Staphylococcus aureus</i> ↑ <i>Staphylococcus aureus</i> (↑)				
Gainet et al (1999) <sup>351</sup>	Rapidly progressing periodontitis: 6 males, 4 females Localized juvenile periodontitis: 4 males, 4 females Chronic periodontitis: 5 males, 3 females Unmatched controls: 14	Dichlorofluorescein-diacetate oxidation and flow cytometry using whole blood	10 <sup>-6</sup> M N-formyl(methionyl)-leucyl-phenylalanine				↑ ↑ ↓ ↔ ↔		↑ ↔ ↔
Gustafsson et al (2000) <sup>352</sup>	Chronic periodontitis: 20 (8 smokers, 12 nonsmokers) with 18 unmatched controls (6 smokers, 12 nonsmokers)	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin using leukocytes Peak signal	300:1		<i>Staphylococcus aureus</i> (↑)				

(Continues)

TABLE 1 (Continued)

Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs health)					
				Autologous serum opsonized	Nonautologous serum opsonized	Immunoglobulin or histone II* opsonized	N-Formylmethionyl-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Fredriksson et al (2003) <sup>154</sup>	Chronic periodontitis (successfully treated, periodontally healthy), nonsmoking: 10 males, 5 females with age/sex-matched controls	Luminol-dependent chemiluminescence using phosphate-buffered saline with 0.25% human albumin Isoluminol-dependent chemiluminescence using Hank's balanced salt solution with divalent cations and 0.25% albumin Peak signal	300:1 <i>Staphylococcus aureus</i> 2 mg/mL zymosan 2 pmol/mL phorbol-myristate-acetate	Autologous serum opsonized	Zymosan <sup>†</sup> (‡)	<i>Staphylococcus aureus</i> † <i>Staphylococcus aureus</i> †	N-Formylmethionyl-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Gronert et al (2004) <sup>155</sup>	Localized aggressive periodontitis: 4 Family members without localized aggressive periodontitis: 4 Chronic periodontitis: 6 Unmatched controls: 10	Cytochrome c reduction, no experimental details given	Leukotriene B <sub>4</sub> , interleukin-8, N-formylmethionyl-leucyl-phenylalanine, phorbol-myristate-acetate Amounts not known				↑ ↔ ↔	↑ ↔ ↔	
Zekonis and Zekonis (2004) <sup>353</sup>	Chronic periodontitis (untreated?); 9 males, 13 females with 16 unmatched controls	Luminol-dependent chemiluminescence using buffy coat cells and Hank's balanced salt solution. Cell number assayed variable; chemiluminescence data retrospectively adjusted for cell number	0.2 ng/mL lipopolysaccharide <sup>†</sup> 6 × 10 <sup>6</sup> <i>Escherichia coli</i> /mL	<i>Escherichia coli</i> lipopolysaccharide † <i>Escherichia coli</i> †					↑
Sadzeviciene et al (2005) <sup>354</sup>	Type 1 diabetes and severe periodontitis: 38, with 27 unmatched controls	Lucigenin-dependent chemiluminescence using buffy coat cells and Hank's balanced salt solution. Cell number assayed variable; chemiluminescence data retrospectively adjusted for cell number	6 × 10 <sup>6</sup> <i>Staphylococcus aureus</i> or <i>Escherichia coli</i> /mL	<i>Staphylococcus aureus</i> † <i>Escherichia coli</i> †					

TABLE 1 (Continued)

Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs health)					
				Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formyl(methionyl)-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
Matthews et al (2007) <sup>157</sup>	Chronic periodontitis: 18 Matched controls: 18	Luminol chemiluminescence for total reactive oxygen species peak signal Isoluminol chemiluminescence for extracellular reactive oxygen species, with 6U horseradish peroxidase, peak signal	100 μL 1 × 10 <sup>6</sup> cells/mL (1 × 10 <sup>5</sup> cells) Opsonized <i>Staphylococcus aureus</i> (Fc gamma receptor II) 300:1 ± priming <i>Fusobacterium nucleatum</i> 100:1 ± priming	Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formyl(methionyl)-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
				Total reactive oxygen species: opsonize <i>Staphylococcus aureus</i> ↑ <i>Fusobacterium nucleatum</i> ↔ Extracellular reactive oxygen species: Baseline ↑ <sup>m</sup>	Total reactive oxygen species: opsonize <i>Staphylococcus aureus</i> ↑ <i>Fusobacterium nucleatum</i> ↔ Extracellular reactive oxygen species: Baseline ↑ <sup>m</sup>	Total reactive oxygen species: Baseline ↔ Extracellular reactive oxygen species: ↑	Total reactive oxygen species: Baseline ↔ Extracellular reactive oxygen species: ↑	Total reactive oxygen species: Baseline ↔ Extracellular reactive oxygen species: ↑	Total reactive oxygen species: Baseline ↔ Extracellular reactive oxygen species: ↑
Matthews et al (2007) <sup>158</sup>	Chronic periodontitis: 19 Matched controls: 19	Luminol chemiluminescence for total reactive oxygen species—peak signal Isoluminol chemiluminescence for extracellular reactive oxygen species, with 6U horseradish peroxidase—peak signal	25 μL opsonized <i>Staphylococcus aureus</i> (Fc gamma receptor II) 300:1 ± priming <sup>15</sup>	Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formyl(methionyl)-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 12,13-dibutyrate*	None
				Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔	Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔	Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔	Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔	Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔	Baseline stimulated: Opsonized <i>Staphylococcus aureus</i> Total reactive oxygen species: ↑ Extracellular reactive oxygen species: ↔

(Continues)

TABLE 1 (Continued)

Reference	Disease status and numbers	Assay method <sup>a</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs health)				
				Autologous serum opsonized	Nonautologous serum opsonized	Immunglobulin or histone II* opsonized	N-Formylmethionyl-leucyl-phenylalanine	Phorbol-myristate-acetate or phorbol 1,2,13-dibutyrate*
Ling et al (2016) <sup>164</sup>	Chronic periodontitis: 20 Matched controls: 20	Lucigenin-dependent chemiluminescence in phosphate-buffered saline supplemented with glucose and cations <sup>b</sup> Peak signal	100 µL of 1 × 10 <sup>6</sup> cells/ mL (1 × 10 <sup>5</sup> cells)	Baseline stimulated: <i>Fusobacterium nucleatum</i> ↔ <i>Porphyromonas gingivalis</i> ↔ Posttherapy: <i>Fusobacterium nucleatum</i> ↔ <i>Porphyromonas gingivalis</i> ↔	Baseline ↑	Baseline ↑	Baseline unstimulated ↑	Posttherapy ↔

Note: Response compared with control cells: ↑ and ↓, large and small increase; ↓ and ↓, large and small decrease; ↔, no difference. Arrows in parentheses indicate trends that were not statistically significant or based on small sample numbers.

Abbreviations: CP, chronic periodontitis; G, gingivitis; GEOP, generalised early onset periodontitis; GJP, generalised JP; JP, juvenile periodontitis; LAP, localised aggressive periodontitis; LJP, localised JP; ND, not detected; RPP, rapidly progressing periodontitis.

<sup>a</sup>Using isolated peripheral blood polymorphonuclear leukocyte and phosphate-buffered saline or other buffer without divalent cations unless stated. Unmodified Hank's balanced salt solution usually contains calcium and magnesium ions. Divalent cations can affect luminol-dependent chemiluminescence<sup>154</sup> and interactions between zymosan and complement receptor-3.<sup>345</sup>

<sup>b</sup>Patient and matched control cell pairs assayed at the same time.

<sup>c</sup>Young patients (18–35y) with localized and generalized disease.

<sup>d</sup>Opsonized with heat-inactivated serum (56°C for 30 min).

<sup>e</sup>Data taken from Fredriksson et al.<sup>154</sup>

<sup>f</sup>Zymosan opsonized with complement using immunoglobulin-depleted serum.

<sup>g</sup>Priming agents used were tumor necrosis factor-α, retina-derived growth supplement, lipopolysaccharide, and N-formylmethionyl-leucyl-phenylalanine at levels that did not cause activation.

<sup>h</sup>Treated patients that had ≥5 mm attachment loss at more than six sites.

<sup>i</sup>Hydrogen peroxide production after treatment with interleukin-8 (50 ng/mL) followed by N-formylmethionyl-leucyl-phenylalanine; interleukin-8 alone had no effect. Other studies demonstrated that hydrogen peroxide production did not differ between any of the patient groups and controls after treatment with tumor necrosis factor-α (100 U/mL) or lipopolysaccharide (5 µg/mL) with/without N-formylmethionyl-leucyl-phenylalanine.

<sup>j</sup>Zymosan opsonized with guinea pig complement.

<sup>k</sup>Similar results found with interleukin-8 and leukotriene B4 treatment.

<sup>l</sup>Compared with controls, patient's cells showed a significant more than fourfold increase in response to *Escherichia coli* lipopolysaccharide.

<sup>m</sup>Priming by 10 mL of *Escherichia coli* lipopolysaccharide (5 mg/mL, equivalent to 50 ng/10<sup>5</sup> cells), granulocyte-macrophage colony stimulating factor (1.25 ng/mL, equivalent to 12.5 pg/10<sup>5</sup> cells). Lipopolysaccharide priming had no impact. granulocyte-macrophage colony stimulating factor did increase reactive oxygen species production, implying granulocyte-macrophage colony stimulating factor may prime peripheral blood neutrophils in periodontitis patients in vivo. Phox gene expression (*p22*, *p47*, *p67*, *gp91*) was no different in patient and control cells at baseline and stimulation with *Fusobacterium nucleatum* (3h) consistently reduced *gp91PHOX* transcripts.

<sup>n</sup>Priming utilized 10 µL of phosphate-buffered saline (unprimed control) or heat-killed *Porphyromonas gingivalis-Fusobacterium nucleatum* mixture (equivalent to 0.75 *Porphyromonas gingivalis* + 1.5 *Fusobacterium nucleatum* per neutrophil) and 100 µL of isolated cells.

<sup>o</sup>Superoxide production following phorbol-myristate-acetate stimulation remained significantly elevated in patient and control neutrophils versus no stimulation.

and supplementing culture media with glucose and cations in order to reproduce in-vivo physiology. We conducted a program of studies and demonstrated that neutrophils isolated from the peripheral blood of periodontitis patients generated significantly greater total reactive oxygen species (measured by luminol chemiluminescence) and extracellular reactive oxygen species (measured by isoluminol) following Fc gamma receptor stimulation with opsonized *S.aureus* than neutrophils from age-, sex-, and smoking-matched healthy nonperiodontitis controls did.<sup>157</sup> The outcome was independent of priming with granulocyte-macrophage colony stimulating factor and was termed "hyperreactivity." Interestingly, periodontitis patients' neutrophils also exhibited significantly higher baseline unstimulated reactive oxygen species release than controls did, irrespective of priming with lipopolysaccharide (but not for priming with granulocyte-macrophage colony stimulating factor), which we termed "hyperactivity." This hyperactive/reactive neutrophil phenotype appeared to be independent of elevations in phagocytic oxidase-gene expression (*p22*, *p47*, *p67*, *gp91*), which was determined by reverse transcription polymerase chain reaction. We then investigated the impact of successful periodontal therapy upon the peripheral blood neutrophil hyperresponsive phenotype, 3-months postperiodontal treatment and following Fc gamma receptor stimulation with/without priming with heat-killed *P.gingivalis* and *F.nucleatum*. The elevated pretreatment total reactive oxygen species production by patients' neutrophils remained following successful therapy, albeit at a slightly lower magnitude. Whereas the total reactive oxygen species release from unstimulated neutrophils was no different between patients and unaffected controls pre- or posttherapy, extracellular reactive oxygen species generation by unstimulated patient neutrophils was significantly elevated pre- and posttherapy relative to healthy controls, irrespective of priming.<sup>158</sup> We concluded that both reactive and constitutive mechanisms underpinned neutrophil hyperresponsiveness in periodontitis patients.

### 6.3.2 | Molecular basis for neutrophil hyperresponsiveness in periodontitis patients

Gene expression analysis using microarrays demonstrated differential expression of 163 genes between patients' and control neutrophils, with 149 upregulated messenger RNA (mRNA) transcripts and 14 downregulated transcripts in patients.<sup>159</sup> A significant number of upregulated genes in patients' neutrophils were interferon-responsive genes, and use of interferon-alpha as a priming agent for control patient neutrophils replicated the enhanced Fc gamma receptor-stimulated reactive oxygen species production seen in periodontitis-patient neutrophils, whereas lipopolysaccharide exposure did not. The same outcome was demonstrated for mRNA profiles, and subsequent serological investigations revealed significantly elevated levels of interferon-alpha in patients' plasma relative to unaffected controls. Plasma interferon-alpha levels, as well as neutrophil interferon-responsive gene expression profiles, decreased with successful treatment to the levels found in controls. We concluded

that elevations in interferon-alpha levels in the plasma of periodontitis patients may be one determinant of the distinct molecular phenotype and hyperresponsivity exhibited by patients' peripheral blood neutrophils. Our subsequent studies employing specific neutralizing antibodies against plasma interleukin-8, interferon-alpha, and granulocyte-macrophage colony stimulating factor in blood from periodontitis patients and healthy controls demonstrated that periodontitis patients' plasma contained sufficient interleukin-8, interferon-alpha, and granulocyte-macrophage colony stimulating factor to stimulate the excessive reactive oxygen species release seen with periodontitis patients' neutrophils,<sup>160</sup> explaining in part the hyperactivity previously reported. Moreover, interferon-alpha also appeared to act as a priming agent for a subsequent Fc gamma receptor stimulus associated with neutrophil hyperreactivity.

We have reported that extracellular reactive oxygen species release by periodontitis-patient neutrophils is stronger following exposure to *F.nucleatum* (ATCC 10953) and opsonized *S.aureus* than following exposure to *P.gingivalis* and *Escherichia coli* lipopolysaccharide,<sup>161</sup> consistent with a potentially pathogenic role for *F.nuceatum* upstream of *P.gingivalis*. Gene expression studies of neutrophils exposed to heat-killed *F.nucleatum* (ATCC 10953) indicated that 208 of the 5680 genes identified as present in periodontitis and control patient neutrophils were more than twofold greater differentially expressed between *F.nucleatum*-stimulated and unstimulated control neutrophils. Of these, 165 were more highly expressed in neutrophils stimulated by *F.nucleatum* and 43 transcripts were more abundant in the unstimulated cells. Notably, 14% were reactive oxygen species response genes that exhibited at least 2.5-fold changes, with a predominance of antioxidant genes such as hemoxygenase-1 and superoxide dismutase-2, as well as heat shock protein-40 (HSP40), transcripts being significantly elevated in stimulated neutrophils. Comparisons of transcriptional responses following *F.nucleatum* stimulation of periodontitis-patient neutrophils and unaffected controls demonstrated that C-X-C ligand 3, hemoxygenase-1, heat shock protein-40, and superoxide dismutase-2 transcript levels were significantly elevated periodontitis-patient neutrophils, whereas only C-X-C ligand 3 and superoxide dismutase-2 transcripts were significantly increased in stimulated control cells. Overall, the data imply a neutrophilic response in periodontitis patients to their own reactive oxygen species production, generating antioxidant responses consistent with those reported by Dias et al.<sup>162</sup> Moreover, the heat shock proteins act as molecular chaperones stabilizing proteins that have been denatured by reactive oxygen species activity,<sup>163</sup> adding further strength to a reactive oxygen species-protective gene expression signature in pathogen-stimulated periodontitis neutrophils.

### 6.3.3 | Superoxide hyperactivity and hyperreactivity in periodontitis-patient neutrophils

We then investigated superoxide release by peripheral blood neutrophils in periodontitis patients and reported excessive extracellular superoxide release both in the absence of periodontal bacteria (hyperactivity) and in their presence (hyperreactivity), but

this hyperresponsive superoxide-release phenotype was eliminated by successful periodontal treatment. Interestingly, C-reactive protein concentrations in patients' plasma were higher than in control patient plasma, and periodontal treatment resulted in a significant lowering of plasma C-reactive protein in patients. Pre-therapy unstimulated neutrophil superoxide release correlated significantly with plasma C-reactive protein concentrations. We concluded that periodontitis was characterized by peripheral blood neutrophils exhibiting superoxide hyperactivity and hyperreactivity to periodontal pathogens, which was not a constitutive feature of periodontitis patients.<sup>164</sup> The positive relationship between unstimulated neutrophil superoxide release and plasma C-reactive protein prior to therapy suggests a protective role for C-reactive protein in reducing oxidative stress and systemic inflammation *in vivo*.

### 6.3.4 | Compromised antioxidant capacity and oxidative stress

Consistent with periodontitis patients demonstrating enhanced reactive oxygen species release from peripheral blood neutrophils, we then studied antioxidant defenses, both peripherally in serum and locally in gingival crevicular fluid of periodontitis patients versus unaffected controls. Patients had significantly lower total antioxidant capacity in gingival crevicular fluid relative to controls, consistent with oxidative stress within the periodontal tissues.<sup>165</sup> Plasma total antioxidant defense was also lower in patients than in unaffected controls, which is likely to result in low-grade systemic inflammation resulting from the hosts' immune-inflammatory response to the periodontal bacteremia. It was unclear at the time whether the compromised total antioxidant defenses within gingival crevicular fluid predisposed to or resulted from reactive oxygen species-mediated damage. A subsequent periodontal treatment intervention study demonstrated that successful therapy resulted in elevations of gingival crevicular fluid total antioxidant defenses to control patient levels, suggesting that the pretreatment antioxidant compromise was the result of excess reactive oxygen species activity rather than predisposing to it.<sup>166</sup>

Reduced glutathione is a critical chain-breaking antioxidant and a master regulator of innate immune function, acting by controlling the activation of redox-regulated proinflammatory gene transcription pathways, such as the nuclear factor  $\kappa$ B pathway.<sup>167</sup> Unsurprisingly, concentrations of reduced glutathione within the gingival crevicular fluid of periodontitis patients prior to periodontal treatment were significantly lower than in healthy controls, consistent with the compromised total antioxidant activity previously reported. Periodontal treatment, however, did not restore concentrations of reduced glutathione but did result in reductions in oxidized glutathione, thus restoring the reduced glutathione/oxidized glutathione ratio to one, consistent with a reducing environment, which in turn protects against redox-regulated inflammatory gene transcription events.<sup>168</sup> We subsequently investigated reduced glutathione/oxidized glutathione ratios within the cytoplasm of peripheral blood neutrophils from periodontitis patients versus unaffected controls and modeled the activation of the antioxidant response element of nuclear DNA by the

redox-regulated anti-inflammatory gene transcription factor nuclear factor erythroid-2-related factor 2, a master regulator of the antioxidant response, using differentiated HL60 cells as a neutrophil model. We demonstrated that extracellular superoxide release was enhanced by depletion of intracellular reduced glutathione. This appeared to be due to upregulation of acid sphingomyelinase-driven lipid raft formation. We demonstrated reduced levels of reduced glutathione in periodontitis neutrophils that were hyperreactive to microbial stimuli. Moreover, nuclear factor erythroid-2-related factor 2 activity levels in circulating neutrophils from periodontitis patients were impaired, resulting in reduced synthesis of reduced glutathione due to reduced nuclear factor erythroid-2-related factor 2-mediated transcription of glutamate cysteine ligase catalytic subunit and glutamate cysteine ligase modifier subunit mRNAs, compared with periodontally healthy subjects' neutrophils. The latter produce  $\gamma$ -glutamyl synthase, the rate-limiting enzyme in synthesis of reduced glutathione. Pretreatment of both HL60 cells and primary neutrophils from periodontitis patients with sulforaphane increased expression of the glutamate cysteine ligase catalytic subunit and glutamate cysteine ligase modifier subunit, improved intracellular ratios of reduced glutathione/oxidized glutathione, and also reduced agonist-activated extracellular superoxide release.<sup>162</sup> These data collectively suggested that periodontitis-patient neutrophils exhibit a deficiency in nuclear factor erythroid-2-related factor 2 pathways that may drive extracellular superoxide hyperactivity in peripheral blood neutrophils in periodontitis patients.

In summary, the body of evidence from studies at the Karolinska Institute by Anders Gustafsson and colleagues and from our own studies demonstrate that peripheral blood neutrophils of periodontitis patients are both hyperactive and hyperreactive with respect to reactive oxygen species release. The hyperreactivity is in part due to priming within the circulation by interferon-alpha, and also direct activation by other mediators such as granulocyte-macrophage colony stimulating factor and interleukin-8 released in response to bacterial stimuli. Both the total and the extracellular reactive oxygen species release are exaggerated in periodontitis-patient neutrophils, and extracellular superoxide production, as measured by lucigenin chemiluminescence, is enhanced, leading to oxidative stress within periodontal tissues, and also within the systemic circulation. Successful periodontal treatment abrogates the hyperreactivity of total reactive oxygen species production; however, extracellular superoxide release from unstimulated neutrophils remains elevated, despite the potentially protective role played by C-reactive protein. There is, therefore, evidence for both microbe-inducible reactive oxygen species hyperreactivity and also constitutive hyperactivity, the latter in part being associated with a compromise in the anti-inflammatory gene transcription factor nuclear factor erythroid-2-related factor 2.

### 6.3.5 | Impact of cigarette smoke extract on neutrophil reactive oxygen species release

We also undertook studies investigating the impact of cigarette smoke extract on blood neutrophil reactive oxygen species release. Cigarette smoke extract lowered the ability of neutrophils to

generate reactive oxygen species following challenge by *F. nucleatum* and IgG-opsonized *S. aureus*. However, the response was bimodal, with high cigarette smoke extract concentrations stimulating extracellular reactive oxygen species generation, suggesting a differential effect of cigarette smoke extract on neutrophil function, generally preventing elimination of periodontal pathogens but, in heavy smokers, also stimulating reactive oxygen species release and oxidative stress-mediated tissue damage.<sup>33</sup> Further work demonstrated that water-soluble components of cigarette smoke were able to directly induce the production of superoxide by otherwise unstimulated neutrophils. These cigarette smoke extract components also dampened superoxide release from neutrophils exposed to pathologic challenge, suggesting that smoking may initiate and maintain oxidative stress via neutrophil-mediated activity at periodontally healthy sites and also participate in disease progression by reducing innate immune responses.<sup>169</sup>

#### 6.4 | Neutrophil-mediated oxidative stress as a mechanistic link between periodontitis and systemic noncommunicable diseases

In a large (n = 770) 10-year cohort of patients with chronic kidney disease established in Birmingham, periodontitis prevalence was over 90% compared with levels in the general population of 45%–50%.<sup>20</sup> Periodontitis, when expressed as mean or cumulative probing pocket depth, or as percentage sites bleeding on probing, or as clinical attachment loss or periodontal inflamed surface area score, was an independent risk factor for poorer renal function (expressed as estimated glomerular filtration rate) and stiffer arteries (assessed as pulse wave velocity). We also analyzed oxidative stress by measuring serum levels of protein carbonyls (oxidized proteins) and F2-isoprostanes (oxidized lipids) and demonstrated significant correlations between the aforementioned periodontal exposures and oxidized plasma proteins and lipids.<sup>20</sup> Structured equation modeling was undertaken to analyze the potential for a causal relationship between the periodontal wound area (periodontal inflamed surface area score) and poorer renal function (estimated glomerular filtration rate) and demonstrated a causal relationship between periodontal inflamed surface area as an exposure and estimated glomerular filtration rate as the outcome, mediated via oxidative stress.

In a separate diabetes cohort, we demonstrated a reduced plasma antioxidant capacity and elevated plasma protein carbonyls in patients with type 2 diabetes and comorbid periodontitis compared with diabetes patients without periodontitis. Periodontitis was also associated with significantly higher hemoglobin A1c, higher fasting glucose levels, and with a reduced pancreatic  $\beta$ -cell function in diabetes patients. Type 2 diabetes patients with periodontitis also exhibited elevated plasma C-reactive protein levels relative to those without periodontitis and also demonstrated the lowest levels of high-density lipoprotein cholesterol of all groups.<sup>32</sup>

It appears, therefore, that the neutrophil-mediated systemic oxidative stress generated in periodontitis patients' blood may be

causally related to poorer outcomes in systemic comorbidities such as type 2 diabetes and chronic kidney disease.

#### 6.5 | Neutrophil cytokine hyperreactivity in periodontitis patients

The establishment of reactive and constitutive mechanisms underpinning neutrophil reactive oxygen species hyperresponsiveness in periodontitis patients led to the hypothesis that a similar phenotype may manifest in terms of cytokine and chemokine production. Only one study had previously examined cytokine production by peripheral blood neutrophils in patients with a noncommunicable disease. Hatanaka et al<sup>170</sup> had demonstrated peripheral blood neutrophil cytokine hyperreactivity in diabetes patients. Given the similarities in immune-inflammatory dysfunction in myeloid cells in diabetes and periodontitis, we undertook a series of studies to attempt to answer this hypothesis. We measured the production of interleukin-8, interleukin-1beta, tumor necrosis factor- $\alpha$ , and interleukin-6 by conventional enzyme-linked immunosorbent assay in peripheral blood neutrophils purified from 20 periodontitis patients and 20 unaffected and matched (age, sex, never-smokers) unaffected controls.<sup>171</sup> Peripheral blood neutrophils were cultured for 18 hours in the presence of *E. coli* lipopolysaccharide, heat-killed *F. nucleatum* (ATCC 10953), and *P. gingivalis* (ATCC 33277) and IgG-opsonized *S. aureus* (NCTC 6571) at 37°C and 5% carbon dioxide alongside an RPMI medium control, in order to stimulate toll-like receptor 2 and toll-like receptor 4, as well as Fc gamma receptor II. Periodontitis patients were then treated to target within a 4-week period by supra- and subgingival professional mechanical plaque removal of all sites greater than 4 mm in depth, and the experiment repeated. There was no difference between unstimulated patient peripheral blood neutrophils at baseline relative to posttherapy, or for controls, with the exception of tumor necrosis factor- $\alpha$ , whose levels were higher following treatment in patients' peripheral blood neutrophils. However, patient peripheral blood neutrophils released higher levels of respective cytokines than controls did, and this reached significance for interleukin-6 at the posttherapy review appointment.

Importantly, stimulation of the evaluated neutrophil surface receptors elicited a highly significant positive cytokine response at baseline and following treatment for both patient and control peripheral blood neutrophils for all stimuli, but the combined (interleukin-8, interleukin-1beta, tumor necrosis factor- $\alpha$ , interleukin-6) cytokine release was significantly higher for patients' neutrophils than in controls. Individual cytokine levels were also significantly elevated by all stimuli in patient and control peripheral blood neutrophils, with a clear order of magnitude of interleukin-8 > interleukin-6 > tumor necrosis factor- $\alpha$  = interleukin-1beta, and patients' neutrophils released significantly more of each individual cytokine than controls did, both before and after successful periodontal therapy.

The data from this study demonstrated that peripheral blood neutrophils from periodontitis patients generate excess



proinflammatory cytokines relative to nonperiodontitis controls; and importantly, unlike reactive oxygen species release, successful periodontal treatment was unable to correct the cytokine hyperreactivity. The results were significant, in that, taken together, they demonstrated for the first time that peripheral neutrophils in periodontitis patients are constitutively hyperreactive for cytokine release. The alternate explanation, that they are in a primed state following exposure to periodontal bacteria within peripheral blood (or their virulence factors), did not explain the retention of the dysregulated hyperreactive phenotype 2 months following successful treatment. Although a follow-up period of 2 months may appear short, the life cycle of neutrophils within peripheral blood is a maximum of 5 days; therefore, the data strongly implied the training of neutrophil granulocytes in periodontitis patients within the bone marrow during myelopoiesis.

Immunological memory is a well-established and critical component of adaptive immunity, enabling a rapid mobilization of the immune response to a previously encountered pathogen. However, such memory responses are not traditionally associated with innate immune cells such as neutrophils, yet recent research has demonstrated that inflammatory or microbial challenges can imprint a form of memory in innate immune cells.<sup>172</sup> The term "trained immunity" is employed to describe the state of hyper-preparedness that results from both metabolic and epigenetic changes within the bone marrow during myelopoiesis, as opposed to the permanent genetic changes that are associated with development of classical immunological memory of the adaptive immune response. Such training has been shown to take place within hematopoietic stem cells within bone marrow, following inflammation-induced epigenetic, metabolic, and transcriptomic alterations to myeloid cell populations,<sup>173</sup> and can lead to both protective and destructive immune cell phenotypes. For example, maladaptively trained immune cells have been associated with increased severity of periodontitis as well as with rheumatoid arthritis in an experimental study employing a mouse model.<sup>174</sup> Following induction of periodontitis in these animals, the trained immune cell phenotype was transmissible by hematopoietic stem cell transplantation to naive recipients, which subsequently exhibited increased inflammatory responsiveness, including enhanced proinflammatory cytokine release by neutrophils. Such a maladaptive response may also explain the failure of successful periodontal therapy to reverse the peripheral blood neutrophil cytokine hyperreactivity found in periodontitis patients to nonperiodontitis patient levels.<sup>171</sup> Hence, it is important to differentiate between maladaptive trained immunity and protective trained immunity. An example of the latter, again demonstrated *in vitro* and *in vivo* using murine models, is the ability of neutrophils to protect against tumor growth through reactive oxygen species production.<sup>175</sup> Given that neutrophils express cytokine receptors that, when stimulated, can activate the nicotinamide adenine dinucleotide phosphate oxidase for reactive oxygen species production, such innate immune training may not only contribute to the peripheral blood neutrophil reactive oxygen species hyperactivity and reactivity displayed by periodontitis patients' neutrophils but may also contribute to the systemic

oxidative stress demonstrated in noncommunicable diseases, such as chronic kidney disease and type 2 diabetes (see earlier discussion). Moreover, the release of excess interleukin-8 along with other neutrophil-enabling cytokines, such as interleukin-17, is likely to increase granulocytogenesis via elevations in granulocyte colony stimulating factor, further potentiating autoimmune or inflammatory diseases in which granulocytes are implicated in their pathogenesis.

## 7 | NEUTROPHIL PROTEASE RELEASE IN HEALTH AND DISEASE

Neutrophil granules contain multifunctional proteins in bilayer membranes and are described in detail in Section 1. Neutrophil proteases lyse pathogens, degrade intracellular proteins and other cells, and also facilitate neutrophil movement through host tissues to their target location. Neutrophil serine proteases from primary granules, such as elastase, can be rapidly deployed in response to microbial challenge and degrade internalized microbes, and external microbes when released following degranulation of activated neutrophils. Serine proteases are essential contributors to the physiologic response to infection, functioning as antimicrobial agents as well as immunomodulators. Uncontrolled and non-inhibitor-bound (free) neutrophil elastase is a major cause of tissue loss and degeneration in periodontitis, similar to that seen in chronic lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease. In wounds, proteolysis induced by host-derived enzymes performs a similar role in reversing or halting tissue regenerative processes and degrading growth factors.<sup>176</sup> These factors further increase the total protease activity within the wound and exacerbate host tissue damage. Elastase thus impedes keratinocyte migration, causing delayed wound healing. European researchers have contributed significantly to data in this area. For example, peripheral blood neutrophils in periodontitis patients have been shown to release more active elastase after *in vitro* activation compared with healthy controls;<sup>177</sup> also, neutrophil serine protease deficiencies in Papillon-Lefèvre syndrome patients secondary to a lack of cathepsin C required to activate the neutrophil serine proteases led to a reduced chemotactic ability of neutrophils and an inability to generate neutrophil extracellular traps, as well as elevated levels of macrophage inflammatory protein-1 $\alpha$  and C-X-C ligand 8 (interleukin-8). Such defects were proposed to lead to the severe periodontal tissue destruction due to a relentless recruitment and retention of hyperresponsive neutrophils with increased tissue transit times into the periodontium alongside compromised antimicrobial capacity.<sup>24</sup>

### 7.1 | Matrix metalloproteinases

Matrix metalloproteinases function as transmembrane, membrane-anchored, or free enzymes released into the extracellular space. Matrix metalloproteinases are responsible for the turnover and degradation of extracellular matrix proteins. There are over 23

mammalian matrix metalloproteinases that degrade or cleave various collagens or other extracellular matrix proteins. Matrix metalloproteinases also act on nonmatrix proteins, such as cytokines, chemokines, antimicrobial peptides, surface proteins, receptors, and adhesive and junctional proteins. About 10% of the human genome encodes for proteins with a signal peptide, leading to an extensive array of potential extracellular matrix metalloproteinase substrates. When combined, several matrix metalloproteinases can potentially act on over 600 substrates.

Matrix metalloproteinase-7 (matrilysin) is an epithelial matrix metalloproteinase induced in response to a wide range of insults and controls neutrophil egress. This was demonstrated by preclinical studies of matrix metalloproteinase-7<sup>-/-</sup> mice, where the transepithelial migration of neutrophils was halted in response to lung or colon injury and was associated with markedly lower levels of C-X-C ligand 1 in the luminal space. When secreted by injured mucosal epithelium, C-X-C ligand 1 becomes bound to the glycosaminoglycan chains of syndecan-1, a type I transmembrane proteoglycan on the basolateral surface of epithelial cells. In response to epithelial damage, matrix metalloproteinase-7 sheds syndecan-1, liberating an intact ectodomain with the chemokine cargo. The release of this complex allows neutrophils both to migrate through the mucosal barrier and their subsequent activation. Although matrix metalloproteinase-7 shedding of syndecan-1/C-X-C ligand 1 complexes appears to generate a chemokine gradient that neutrophils follow into the luminal space, these granulocytes would, of course, be moving against the gradient. Thus, the matrix metalloproteinase-7 shedding of syndecan-1/C-X-C ligand 1 complexes appears to control another neutrophil process, which may limit neutrophil activation at the mucosal interface. Syndecan-1/C-X-C ligand 1 restricts neutrophil activation and prevents reactive oxygen species-mediated host tissue damage, especially around a damaged epithelial layer.

Neutrophils express matrix metalloproteinase-8 (collagenase-2), which cleaves C-X-C ligand 8 family chemokines to increase their chemotactic potency. In addition to neutrophil chemotaxis, matrix metalloproteinase-8 mediates a neutrophil-controlled feed-forward mechanism to orchestrate the initial inflammatory response and promote responsiveness to lipopolysaccharide stimulation. Different mechanisms of matrix metalloproteinase-8-mediated processing appear to be disease- or cell-type-specific. Neutrophil-derived matrix metalloproteinase-8 is the predominant collagenase in normally healing wounds.<sup>178</sup> Overexpression and activation of matrix metalloproteinase-8 is involved in the pathogenesis of nonhealing chronic leg ulcers in people with diabetes. Likewise, matrix metalloproteinase-8 is a critical regulator of periodontal tissue homeostasis and is released mainly by neutrophils during periodontal disease progression. Excessive collagenolytic activity in chronic wounds arises due to the reduced levels of tissue inhibitor metalloproteinase-1.<sup>179</sup>

Matrix metalloproteinase-9 is a major proteolytic enzyme produced by neutrophils and degrades tissue matrix.<sup>180</sup> Matrix metalloproteinase-9 also activates vascular endothelial growth factor, promoting revascularization at an injured site and leading to the release of certain growth factors. In addition to degrading collagen, fibronectin, and other extracellular matrix components, matrix

metalloproteinase-9 can degrade intracellular proteins such as actin, tubulin, and high mobility group box 1, because these intracellular damage-associated molecular patterns released from necrotic cells perpetuate the inflammatory response. Indeed, studies have suggested that matrix metalloproteinase-9 may serve a protective function to limit the injury caused by dying cells. In most cells, except neutrophils, matrix metalloproteinase-9 is regulated at the transcriptional level by cytokines and growth factors such as interleukin-13, tumor necrosis factor- $\alpha$ , transforming growth factor beta, vascular endothelial growth factor, and via epigenetic mechanisms (histone modification, DNA methylation, and noncoding RNA). Matrix metalloproteinase-9 is primarily regulated in neutrophils at the posttranslational level, since preformed matrix metalloproteinase-9 is stored in gelatinase granules and released upon neutrophil activation by inflammatory signals.<sup>181</sup> Matrix metalloproteinase-9 also plays a role in inflammation resolution. Matrix metalloproteinase-9 stimulates inflammation by inhibiting neutrophil apoptosis and macrophage phagocytosis through the CD36 receptor.<sup>182</sup> Intact CD36 stimulates neutrophil apoptosis and macrophage phagocytosis. Matrix metalloproteinase-9 also cleaves platelet glycoprotein-4 into several fragments that inhibit neutrophil apoptosis and macrophage phagocytosis. Increased concentrations of neutrophil-derived proteases, such as elastase, matrix metalloproteinase-8, and matrix metalloproteinase-9 are therefore characteristic of nonhealing wounds in people with diabetes and periodontal and peri-implant diseases.<sup>183</sup>

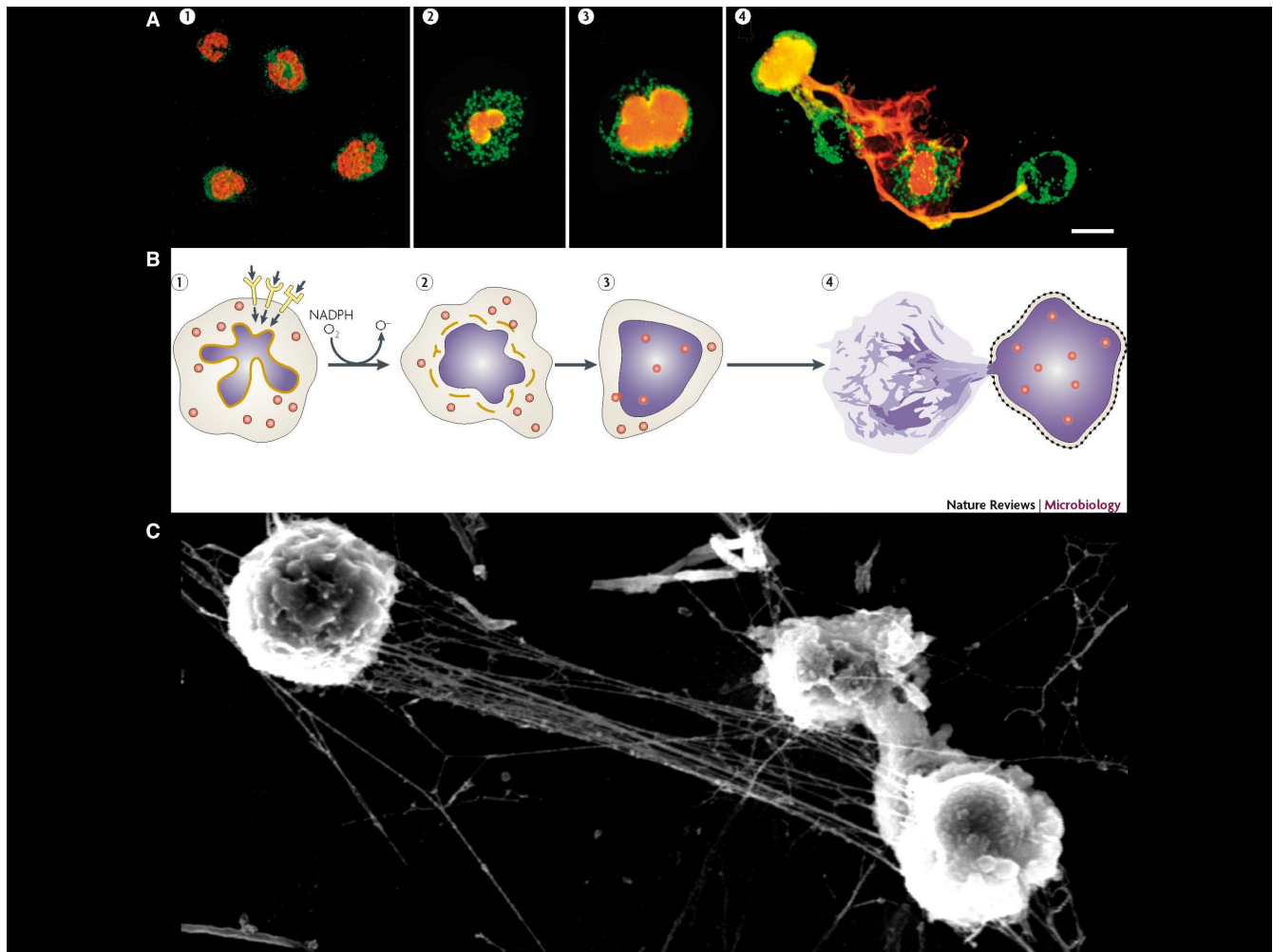
## 8 | NEUTROPHIL EXTRACELLULAR TRAPS IN HEALTH AND DISEASE

DNA carries the genetic code for living organisms, which when translated into proteins defines the phenotype and biological functions of all living organisms. However, DNA also possesses powerful antimicrobial properties, demonstrated to be highly conserved throughout nature, for example, in protecting the root tip of plants such as sweet peas from invasion by soil fungi.<sup>184,185</sup> One would imagine, therefore, that the presence of DNA within human tissues may be beneficial as an antimicrobial strategy; however, it is also associated with various detrimental effects, such as autoimmune diseases like systemic lupus erythematosus and small-vessel vasculitis, by virtue of being autoantigenic in the form in which it is released into tissues.<sup>186,187</sup> Blood plasma and tissue fluids thus carry deoxyribonucleases to remove DNA from tissues in order to prevent the persistence of such autoantigens and the potential for associated breaks in immune tolerance that may lead to autoimmune disorders like rheumatoid arthritis. A major source of tissue DNA release is neutrophil extracellular traps, for which detailed reviews are available.<sup>27,29</sup> This section will headline the nature of neutrophil extracellular traps, the requirements and mechanisms of production, the beneficial and detrimental impacts of neutrophil extracellular traps in humans, and their potential role in the pathophysiology of periodontitis and associated systemic comorbidities, such as rheumatoid arthritis and coronavirus disease 2019 lung disease.

## 8.1 | Discovery of neutrophil extracellular traps and mode of release

Originally believed to be artifacts when visualized during electron microscopy studies of neutrophils, Brinkman and colleagues from the Max Planck Institute demonstrated, in an elegant series of studies, that neutrophils, when exposed to multiple stimuli, underwent a novel form of programmed cell death called “NETosis.”<sup>188,189</sup> They demonstrated using ex-vivo methods that heavy stimulation of neutrophil surface receptors triggered a series of events initially involving activation of the nicotinamide adenine dinucleotide phosphate oxidase, activation and nuclear translocation of the enzyme peptidyl arginine deiminase-4, and release of various other cytosolic, granular, and cytoskeletal components of neutrophils, including cytosolic calprotectin and its S100A8 and S100A9 subunits;<sup>190</sup> neutrophil serine proteases (eg, neutrophil elastase, cathepsin G, proteinase-3), myeloperoxidase, and defensins from azurophilic granules; cathelicidin (human cathelicidin-18/LL-37) from specific granules; and activation

of the actin cytoskeleton. Peptidyl arginine deiminase-4 was shown to hypercitrullinate the histone backbone of nuclear chromatin, replacing the positively charged amino acid arginine with the neutrally charged citrulline, thus facilitating wholesale de-condensation of the nuclear DNA double helix structure from its histone protein backbone. In parallel with this, the nuclear membrane formed vesicles between its outer and inner membranes, prior to disintegrating and releasing the DNA and associated histones (eg, histone A3) into the cytoplasm of the neutrophil; there, it co-localized with the various lysosomal granular antimicrobial peptides such as myeloperoxidase, human neutrophil elastase, and proteinase-3, forming a toxic cocktail of DNA, histones, and antimicrobial peptides. The final steps of NETosis involved disintegration of the outer plasma membrane, contraction of the actin cytoskeleton and forceful ejection of the DNA/antimicrobial peptide cocktail into the extracellular milieu, where it formed a weblike structure. The neutrophil extracellular traps were subsequently shown to trap invading microorganisms, which adhered to its positively charged areas via electrostatic forces. **Figure 5**



**FIGURE 5** Sequence of events leading to, and following, neutrophil extracellular trap release. A, Fluorescence photomicrographs. B, Schematic maps of the sequence of these events. C, Scanning electron micrograph from our own work demonstrating the events at higher magnification. Stages 1-4 are described in detail in the main text. The exquisite fluorescence micrographs shown in A were reproduced with kind permission from Brinkmann and Zychlinsky<sup>189</sup> and combined with our own scanning electron microscope images to form the figure reproduced from Cooper et al<sup>27</sup> with permission from Wiley Publishing.

is reproduced from our earlier publication in this journal and demonstrates the classical NETosis process.

## 8.2 | Triggers for NETosis

A variety of stimuli have been shown to trigger NETosis following the ligation of multiple neutrophil receptors and downstream signal transduction.<sup>191</sup> These are summarized by White et al,<sup>29</sup> and include the following:

- bacteria (eg, *Streptococcus pneumoniae*, *E.coli*, *Mycobacterium tuberculosis*, *Serratia marcescens*);
- bacterial-derived components (eg, lipopolysaccharide, N-formylmethionyl-leucyl-phenylalanine, glucose oxidase, *S.aureus* leukotoxin);
- periodontal bacteria (eg, *F.nucleatum*, *P.gingivalis*, *Streptococcus* sp, *A.actinomycetemcomitans*);
- fungi, parasites, protozoa (eg, *Candida albicans*, *Aspergillus* sp, *Cryptococcus* sp);
- viruses (eg, *Influenza A*, *Human immunodeficiency virus 1*, *Severe acute respiratory syndrome-related coronavirus*);
- host-derived mediators (eg, platelets, platelet activating factor, nitric oxide, antibodies, interleukin-1beta, granulocyte macrophage colony stimulating factor + complement 5a, LL-37).<sup>27,192,193</sup>

The wide diversity of neutrophil extracellular trap stimuli demonstrates the complexity of this neutrophil defense strategy which, though targeted at protecting the host against microbial infection, is also activated by host immune-inflammatory factors, damage-associated molecular patterns,<sup>194</sup> and, importantly, platelets.<sup>195</sup> Indeed, though lipopolysaccharide can directly activate NETosis, it is also able to indirectly achieve the same via platelets, which bind to and activate toll-like receptor 4.<sup>196</sup> The significance of this mechanism is that with low-grade endotoxemia<sup>197</sup> and platelet activation<sup>198</sup> being features of periodontitis, together they provide multiple mechanisms of neutrophil activation associated with intravascular thrombosis in cardiovascular disease as well as pulmonary complications of severe acute respiratory syndrome coronavirus 2 secondary to neutrophil extracellular trap aggregation in the small vessels of the pulmonary vascular tree, thereby providing plausible mechanistic explanations for the links between periodontitis and the two former diseases.<sup>197,199</sup> Vitkov et al<sup>200</sup> provide a focused review of the potential role for neutrophil extracellular traps as a link between severe periodontitis and the higher rates of mortality, intensive care admissions, and ventilator-assisted breathing<sup>201-203</sup> in coronavirus disease 2019, consistent with the oral-vascular-pulmonary hypothesis for severe acute respiratory syndrome coronavirus 2 lung disease.<sup>204</sup>

The original thesis of Brinkmann et al<sup>188</sup> was hydrogen peroxide was the trigger for NETosis following activation of the nicotinamide adenine dinucleotide phosphate oxidase, a process taking approximately 4 hours. However, our own studies employing various inhibitors of the nicotinamide adenine dinucleotide phosphate

oxidase and those enzymes involved in the reactive oxygen species cascade demonstrated that hypochlorous acid, formed downstream of hydrogen peroxide, was the true trigger.<sup>205</sup> We were able to restore neutrophil extracellular trap production by neutrophils from chronic granulomatous disease patients—where the nicotinamide adenine dinucleotide phosphate oxidase is defective and neutrophil extracellular traps are not normally produced—by exposing chronic granulomatous disease-patient neutrophils to hypochlorous acid, and neutrophil extracellular trap release occurred in less than an hour.

## 8.3 | Vital neutrophil extracellular trap release

Interestingly, neutrophil extracellular traps are also released by viable neutrophils when subjected to a less potent stimulation over shorter time periods. Under these circumstances the constituent DNA is mitochondrially derived rather than nuclear DNA; hence, the cells remain viable after neutrophil extracellular trap release.<sup>30</sup> This discovery led to the hypothesis that mitochondrial neutrophil extracellular trap release may represent a rapid response antimicrobial strategy, whereas the stronger and more protracted neutrophil stimulation that stimulates NETosis is a more catastrophic event, leading to cell death, and is more likely to cause collateral host tissue damage directly or via autoimmune mechanisms.<sup>27</sup> As with many biological processes, neutrophil extracellular trap production is yet more complex, with evidence for oxidant-independent mechanisms of neutrophil extracellular trap release, demonstrated using *S.aureus* stimulation for as little as 10 minutes. This rapid mechanism involved the release of intact secretory vesicles and arose without fusion of the nuclear and plasma membrane to extrude the neutrophil extracellular traps fusion, implying a dependence upon vesicular exocytosis for neutrophil extracellular trap release rather than nicotinamide adenine dinucleotide phosphate oxidase activation.<sup>206</sup>

The ability of neutrophil extracellular traps to directly kill pathogens is open to question. Palmer et al<sup>207</sup> demonstrated neutrophil extracellular trap evasion strategies employed by several periodontal bacteria, such as membrane-bound and secreted deoxyribonuclease production, enabling evasion of neutrophil extracellular trap killing via disassembly of neutrophil extracellular trap DNA and associated antimicrobial peptides.

## 8.4 | The role of neutrophil extracellular traps in periodontitis

The role of neutrophil extracellular traps in the pathogenesis of periodontitis has received scant attention. Vitkov et al<sup>208,209</sup> demonstrated neutrophil extracellular traps in pus collected from periodontal pockets and also associated with the pocket epithelium. They proposed that the neutrophil extracellular traps may form a protective shield within the pocket to prevent microbial invasion of the epithelial lining, a thesis based upon the observation that

bacterial numbers within the purulent gingival fluid they had sampled were inconsistent, implying that phagocytosis may have been overwhelmed, resulting in NETosis as a backup strategy. The first demonstration of neutrophil extracellular traps within periodontal tissues was by Cooper et al,<sup>27</sup> who used confocal microscopy and co-labeling of DNA and myeloperoxidase and demonstrated substantially more neutrophil extracellular traps in inflamed than in healthy periodontal tissues. Consistent with this was the demonstration of substantially higher levels of citrullinated peptides in the stromal tissues of periodontitis patients than in unaffected controls,<sup>210</sup> implying a pathogenic role for inflammation-induced citrullination within periodontal connective tissues. Conversely, citrullination within the epithelial lining of the crevice/pocket was present in health and gingivitis, implying a physiologic role in epithelial cells.

Given that peripheral blood neutrophils in periodontitis patients are hyperactive and hyperreactive in terms of reactive oxygen species production, and that the nicotinamide adenine dinucleotide phosphate oxidase plays a critical role in traditional NETosis, we hypothesized that peripheral blood neutrophils from periodontitis patients would release more neutrophil extracellular traps when stimulated by phorbol-myristate-acetate and hypochlorous acid than nonperiodontitis controls would. However, there were no differences in NETosis between patients and controls for either stimulus. Treatment did significantly reduce neutrophil extracellular trap production in periodontitis patients, but more significant was a reduced ability for periodontitis-patient plasma to degrade neutrophil extracellular traps than plasma from nonperiodontitis controls. Moreover, deoxyribonuclease levels in plasma from periodontitis patients were significantly lower than in healthy controls. The potential implications of the reported data are that neutrophil extracellular trap DNA removal from periodontitis tissues may be reduced, potentially creating a source of autoantigens within an inflamed periodontal "citrullinome."<sup>211</sup>

## 8.5 | Neutrophil extracellular trap degradation and consequences of suboptimal removal

Efficient removal of neutrophil extracellular traps from tissues is important to prevent autoimmune diseases and represents a highly orchestrated series of events, commencing with degradation by deoxyribonucleases. Following degradation, neutrophil extracellular trap components are opsonized by complement component 1q in order to facilitate phagocytosis by macrophages. The process is believed to be immunologically silent, similar to apoptosis, whereby macrophages express a nonphlogistic phenotype, biologically debriding without releasing proinflammatory mediators, thus preventing tissue damage.<sup>212</sup> Compromised neutrophil extracellular trap clearance is associated with several autoimmune diseases, given that neutrophil extracellular traps not only comprise DNA, but also immunogenic antimicrobial peptides such as myeloperoxidase and proteinase-3 and high levels of citrullinated proteins such as histones. Antinuclear antibodies characterize systemic lupus erythematosus,

specifically antibodies to double-stranded DNA, ribonuclear proteins (anti-Smith antibodies), and Sjögren syndrome-related antibodies A and B,<sup>213</sup> and neutrophil extracellular traps are one source of such immunogens.<sup>214,215</sup> Reports suggest compromised DNA degradation results from elevated serum levels of deoxyribonuclease-1 inhibitors<sup>214</sup> or anti-neutrophil extracellular trap antibodies or complement component 1q binding, which may physically block neutrophil extracellular trap degradation sites.<sup>186</sup> Several vasculitides are also associated with autoantibodies to proteinase-3 and myeloperoxidase, collectively known as antineutrophil cytoplasmic antibodies, the most well documented being granulomatosis with polyangiitis (formerly Wegener granulomatosis), where 95% of patients are antineutrophil cytoplasmic antibody positive at diagnosis, with proteinase-3 being the target antigen in the majority of cases.

Perhaps the most common autoimmune disease in which neutrophil extracellular trap components serve as autoantigens is rheumatoid arthritis, which is also independently associated with periodontitis. In rheumatoid arthritis, antibodies are generated against citrullinated proteins antigens (anti-citrullinated protein antibodies), such as those present in neutrophil extracellular traps, and are 88%-98% specific for rheumatoid arthritis.<sup>216</sup> One hypothesis for the etiopathogenesis of periodontitis-linked rheumatoid arthritis is the so-called "two-hit model,"<sup>217</sup> whereby an initial inflammatory stimulus results in the generation of extra-articular autoantigens and a break in immune tolerance arises that results in anti-citrullinated protein antibody generation. Once seropositive for anti-citrullinated protein antibodies, clinical signs and symptoms of rheumatoid arthritis develop on average 10 years following the development of initial seropositivity and are proposed to result from a second inflammatory or infectious trigger, more likely localized to the joint. One potential source of citrullinated proteins within the periodontal tissues is *P. gingivalis*, which produces its own peptidyl arginine deiminase that has been shown to auto-citrullinate *P. gingivalis* proteins as well as citrullinate host proteins. A second and more likely source is neutrophil extracellular trap release by neutrophils during periodontal inflammation. Such a relationship deserves a full review in its own right, and that is available from Bartold and Lopez-Oliva.<sup>218</sup> If periodontitis is causally related to rheumatoid arthritis, one would expect to find anti-citrullinated protein antibodies within the serum of periodontitis patients prior to rheumatoid arthritis being manifest. de Pablo et al<sup>219</sup> reported exactly that finding in their 2012 study, in which they found a higher prevalence and elevated titers of anti-citrullinated protein antibodies as well as antibodies to uncitrullinated proteins in periodontitis patients who did not exhibit rheumatoid arthritis, relative to nonperiodontitis controls. They concluded that the dominant antibody response in periodontitis was to uncitrullinated proteins of the rheumatoid arthritis autoantigens and proposed that the loss of immune tolerance may cause epitope spreading to citrullinated epitopes, thereby linking the autoimmune response in periodontitis to presymptomatic rheumatoid arthritis. Spengler et al<sup>220</sup> subsequently demonstrated higher levels of extracellular DNA in synovial fluid from rheumatoid

arthritis patients than from osteoarthritis and psoriatic arthritis controls, which correlated with peptidyl arginine deiminase activity and neutrophil counts within the joint fluid. They also demonstrated that NETosis associated significantly with DNA release and demonstrated citrullinated proteins, active peptidyl arginine deiminase-4, and peptidyl arginine deiminase-2 attached to neutrophil extracellular traps within the fluid, creating further links to neutrophil activity, periodontitis, and systemic diseases.

## 9 | NEUTROPHILS IN WOUND HEALING

Wound healing is a highly regulated process involving sequential steps, any one of which can subvert optimal clinical outcomes if less than 100% efficient. The wound healing process broadly involves an inflammation step initially, which is followed by a resolution step.<sup>180</sup> Acute inflammation represents a reversible response to injury, which can be elicited by infectious agents (eg, bacteria, viruses), chemical agents (eg, acids, bases), trauma, or external stimuli (eg, heat). When the stimulus persists and the inflammatory process fails to resolve, chronic inflammation results and leads to extended exposure of the host tissues to additional stimuli; under these circumstances, healing becomes more complex.<sup>221</sup> Thus, there is a critical connection between the temporality of inflammation and optimal wound healing. In mammals without systemic diseases, acute wound healing is predictable and highly efficient in the oral cavity and elsewhere. As in every form of the inflammatory response, it starts with vascular hemostasis, continues with the recruitment of phagocytes, and resolves with stromal cell proliferation and tissue remodeling.<sup>222</sup> “Homeostatic healing” of tissues requires a well-orchestrated sequence of events, and any disruption to this process or prolonged exposure to infectious or physical irritants leads to chronic and nonresolving inflammation. A chronic wound arises where inflammation remains in an active phase at the same time that the body is attempting to heal. It therefore involves phases of repair and tissue degradation in sequence or simultaneously, resulting in fibrosis and destruction. In aseptic wounds, the healing is predictable and follows clear sequential and highly coordinated steps. In contrast, in septic wounds, such as those arising within the oral cavity, the wound-healing process is further complicated by continuous exposure to microorganisms and their products, even in systemically healthy individuals.

### 9.1 | Neutrophils in early wound healing

Neutrophils are key players in the regulation of wound healing from its very early stages. Their numbers are low in healthy tissues but increase rapidly at the site of injury. As vasodilation and extravasation occur, platelets interact with collagen and the extracellular matrix, triggering the clotting cascade.<sup>111</sup> Hemostasis is accomplished with clotting factors, growth factors, and cytokines. Platelet-induced wound healing is also critical

in recruiting neutrophils and resolving the acute inflammatory process by producing pro-resolving lipid mediators, such as lipoxins, by lipoxygenase-mediated cell-cell interactions.<sup>223</sup> Thus, neutrophils are the first immune cells that enter the wound area, and neutrophil-mediated phagocytosis is crucial for removing damaged tissue, bacteria, and foreign material, assisted by macrophages.<sup>182</sup> Neutrophils release cytokines that are involved both in the activation and resolution of the inflammatory process during wound healing.<sup>224</sup> Though their function was thought to be limited to phagocytic removal of dead cells, bacteria, and other microorganisms, neutrophils are also critical for wound debridement. Studies have demonstrated that tissue pathology worsens when neutrophils are depleted. In humans, neutrophil defects are associated with extensive tissue damage where periodontal tissues are involved.<sup>225</sup> Neutrophil migration leads to the resolution of acute inflammation where a homeostatic balance is established, and tissue integrity is accomplished by fibroblast-mediated extracellular matrix formation and remodeling. This efficient and highly controlled repair process requires optimal neutrophil function that essentially culminates in apoptotic clearance of neutrophils by macrophages, ensuring that neutrophils do not release any cytotoxic contents that could damage the healing tissues and trigger chronic inflammation via production of damage-associated molecular patterns<sup>182</sup> (Figure 2).

### 9.2 | Neutrophils in chronic wounds

In chronic inflammation, neutrophils play a very different role.<sup>226-228</sup> In pathologic conditions, such as nonhealing pressure or diabetic ulcers, healing is disrupted. Chronic inflammation requires a continuous crosstalk between neutrophils and monocytes, leading to monocytic recruitment to the inflamed site.<sup>229</sup> This process is mediated by azurocidin, LL-37, and cathepsin G, which are released from neutrophil granules (see Section 1). These proteins regulate monocyte recruitment by formyl peptide receptors, leading to the slowing of rolling monocytes and their extravasation into the affected tissues.<sup>230</sup> Neutrophils also produce interleukin-6, which activates endothelial cells, a process regulated by gp130, which results in the expression of adhesion molecules on endothelial cells. Another critical function of neutrophils is the release of “find me” signals by expression of phosphatidylserine residues on their outer surface membrane. These signals attract monocytes that enter the tissues as macrophages and subsequently phagocytose apoptotic neutrophils and promote their own removal through a process termed efferocytosis<sup>231,232</sup> (Figure 2: efferocytosis). In addition to working as a disposal mechanism of apoptotic neutrophils and debris to prevent neutrophil-mediated destruction of host tissues, efferocytosis is an active mechanism through which resolution of inflammation is regulated, increasing the biosynthesis of specialized pro-resolving mediators (eg, D-series resolvins and maresins).<sup>233</sup> Specialized pro-resolving mediators limit excessive polymorphonuclear neutrophil recruitment and stimulate the resolution-phase macrophages

working as autocoids.<sup>234</sup> In hypoxic environments, efferocytosis is accelerated by locally produced resolvins.<sup>235</sup> Disrupted efferocytosis has been linked to a defective neutrophil function in leukocyte adhesion deficiency-1-associated severe periodontitis.<sup>236</sup> Thus, neutrophils contribute to the resolution of inflammation, promote healing, and facilitate tissue repair through a close interaction with the macrophages.

Periodontitis lesions are characterized by chronic inflammation with a dominant plasma cell presence and associated antibody production, attracting an extensive neutrophil infiltration with associated reactive oxygen species production and release of destructive proteolytic enzymes.<sup>237</sup> Wound healing is therefore impaired, and lesions exhibit episodes of tissue degradation and fibrosis, resulting in further neutrophil recruitment, thus prolonging their presence at sites of inflammation. Platelets also promote the extravasation of neutrophils from the vasculature.<sup>238</sup> In chronic inflammatory diseases that mirror periodontitis in their pathophysiology, such as rheumatoid arthritis, platelets are the most abundant cell type in inflamed joints and recruit neutrophils to the joint. This process is facilitated by P-selectin and platelet-derived neutrophil-attracting chemokine heterodimers C-C motif chemokine ligand5 and C-X-C ligand4. Neutrophils also recruit further neutrophils via interleukin-17, leukotriene B4, and chemokine release.

Meanwhile, epithelial cells, hepatocytes, and fibroblasts use pathogen detection mechanisms for neutrophil recruitment, while macrophages and mast cells initiate neutrophil recruitment by controlling and inducing blood vessel permeability and chemokine production. Pathogen-associated molecular patterns and/or damage-associated molecular patterns released by infectious organisms activate neutrophils, macrophages, mast cells, epithelial cells, T and B cells, and dendritic cells via pattern recognition receptor binding (eg, toll-like receptors, nucleotide-binding oligomerization domain-like receptors, C-type lectin receptors, and retinoic acid-inducible gene1-like receptors). This mechanism is crucial for infectious inflammation.

### 9.3 | Dysregulated periodontal wound healing in comorbid systemic diseases

Several oral and systemic diseases, including periodontitis, can cause hypersensitive neutrophil function, whereby neutrophils mediate self-tissue destruction within the host. One classic example of this phenomenon is the Stage III, Grade C molar/incisor pattern periodontitis (previously known as localized aggressive periodontitis), where neutrophils are hyperactivated. Likewise, diabetes can also lead to hyperglycemia-mediated hyperactive neutrophils that are self-destructive.<sup>239</sup> In the oral cavity, hyperglycemia-induced hyperactive neutrophils exacerbate periodontal inflammation,<sup>239</sup> elsewhere, the same pathologic outcome is observed in nonhealing foot ulcers.<sup>240</sup> Thus, neutrophil apoptosis and efferocytosis and balanced neutrophil function

are critical for preventing self-damage to host tissues (Figure 2). In physiologic conditions, in addition to apoptotic clearance, neutrophils are cleared from the circulation in the liver, spleen, and bone marrow. Increased C-X-C chemokine receptor4 expression in aged neutrophils is thought to help direct neutrophils back to the bone marrow, where they are eliminated. As previously discussed (see Section 2.1), C-X-C chemokine receptor type4 negatively regulates the release of newly formed neutrophils from the bone marrow.

Neutrophil terminal trafficking into the intestinal tract is critical for regulating the commensal microbiome, a process yet to be explored in the oral cavity. Senescent or infection-associated neutrophils can also die within the vasculature. In this case, Kupffer cells in the liver or dendritic cells regulated by the interleukin-23-interleukin-17-granulocyte colony stimulating factor axis remove the necrotic neutrophils.<sup>241</sup> This was also demonstrated in a severe form of periodontitis in leukocyte adhesion deficiency-1.<sup>242</sup>

A key molecule emerging in recent years is sirtuin. Sirtuins are a vast family of histone deacetylases, oxidized nicotinamide adenine dinucleotide-dependent enzymes, consisting of seven members. Sirtuin 1, sirtuin 6, and sirtuin 7 are located within the nucleus, sirtuin 2 in the cytoplasm, and sirtuin 3, sirtuin 4, and sirtuin 5 are found within the mitochondria. Sirtuins regulate age-related metabolic disorders, inflammation, response to stress, and cardiovascular and neuronal functions. Although understanding of this family of molecules is limited at present, it has been shown that sirtuin 1 deficiency reduces the recruitment of fibroblasts, macrophages, mast cells, and neutrophils to wound sites and delays wound healing. The mechanism of sirtuin 1-induced wound healing is thought to be mediated by downregulation of matrix metalloproteinase-9 and involves the Forkhead box O-c-Myc pathway in neutrophils.

Experiments in zebrafish have allowed the visualization of dual oxidase 1-derived hydrogen peroxide tissue gradients and their crosstalk with neutrophil infiltration into inflamed tissues.<sup>243</sup> Hydrogen peroxide directly recruits neutrophils via the Src-family tyrosine kinase Lyn, and indirectly by signaling pathways mediated by nuclear factor  $\kappa$ B, mitogen-activated kinases, and activator protein-1. Hydrogen peroxide induces the expression of C-X-C ligand8 (interleukin-8) by facilitating the accessibility of transcription factors to its promoter through covalent histone modifications, a critical gene for neutrophil chemoattraction.

Inflammation develops in response to pathogen-associated molecular patterns released into the tissues during infection and to damage-associated molecular patterns generated in response to a sterile injury (eg, burn, hypoxia, or chemical stimuli). During sterile thermal injury, neutrophils use formyl peptide receptor 1 to follow a gradient of formylated peptides, which are released from the mitochondria of damaged cells at the injured site.<sup>230</sup> The same formyl peptide receptor is used to recognize bacterial formylated peptides. Although neutrophil recruitment during infection is critical for protection, formyl peptide receptor-mediated recruitment during a sterile injury is detrimental due to the associated reactive

oxygen species, protease, and antimicrobial peptide release by neutrophils. For example, neutrophil-mediated inflammation in obesity results from the release of obesity-associated damage-associated molecular patterns, which activate the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome, integrins, increased adhesion of neutrophils, and vascular dysfunction. Whether the neutrophils found in tissues during obesity-associated inflammation differ from those isolated from blood during distinct inflammatory responses remains to be determined. Nevertheless, both decreased and increased proinflammatory responses have been documented in obese patients.

## 9.4 | Inflammasomes in wound healing

Inflammasomes are a major component of the innate immune system, with crucial roles in protection against microbial infections, cancer, and inflammatory disorders. They are multimeric protein platforms that lead to inflammatory protease zymogen caspase-1 activation, which drives proteolytic maturation of proinflammatory cytokines interleukin-1 $\beta$  and interleukin-18. In parallel, active caspase-1 induces the cleavage and activation of the pore-forming protein gasdermin D that causes rupture of the cell membrane and cytokine and damage-associated molecular pattern release. This is termed “pyroptotic cell death” and is crucial for the innate immune response to control infection, tumor progression, or inflammatory disorders. To date, multiple innate sensors with the potential to assemble inflammasome complexes have been identified, including the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 1b, nucleotide-binding oligomerization domain-like receptor family CARD domain-containing protein 4, nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3, absent in melanoma-2 receptor, and pyrin receptor, and also the most recent nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 6 and -9.<sup>244</sup> Nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasomes are expressed in phagocytes in chronic wounds and are activated by two principal mechanisms, termed canonical and noncanonical activation. The canonical nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome activation requires two signals: (1) a priming signal to promote the expression of the inflammasome components, such as nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3, as well as the immature inflammatory cytokines pro-interleukin-1 $\beta$  or pro-interleukin-18; and (2) a subsequent activation signal, such as extracellular adenosine triphosphate or bacterial toxins, to promote the oligomerization and activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3.<sup>244</sup> The non-canonical nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome activation occurs upon gram-negative bacterial infection or circulating lipopolysaccharide. Mechanistically, lipopolysaccharide binding to caspase-11

induces oligomerization and proteolytic cleavage of the gasdermin D protein. Neutrophil extracellular trap overproduction in diabetic wounds is associated with an activated nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome and with induced interleukin-1 $\beta$  release in macrophages. In streptozotocin-induced diabetes in rats, neutrophil extracellular trap degradation (via topical deoxyribonuclease I) reduces the activation of the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome and leads to accelerated wound healing, suggesting that neutrophil extracellular traps might further amplify the inflammatory state in nonhealing wounds.

Metabolic diseases associated with nonhealing skin wounds seem to drive neutrophils toward mediating inflammation by upregulating inflammatory genes; this, in turn, may compromise the ability of neutrophils to directly and/or indirectly (via communication/effects on macrophages) promote healing.<sup>245</sup> Importantly, the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome is reported to be overexpressed in the gingiva of periodontitis patients.<sup>246</sup> Studies in a murine ligature-induced periodontitis model, using ligatures impregnated with *P.gingivalis* or not (control), in wild type or nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 knockout mice, suggested that nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 is involved in regulatory pathways that limit periodontitis. *P.gingivalis* enhanced the targeting of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3-positive neutrophils to the alveolar bone crest, suggesting a role for this subpopulation in bone loss. In addition, in nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 knockout mice, mature interleukin-1 $\beta$  expression was lower, and almost no neutrophils were mobilized, shedding new light on the role of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 in periodontitis by highlighting the ambiguous role of neutrophils, and *P.gingivalis*, which impacts nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 functions.

## 9.5 | Role of neutrophils in fibrosis

Neutrophils may also be involved with fibrotic changes in tissues through epithelial-mesenchymal transition. Elastase is secreted by neutrophils into the microenvironment, increasing interleukin-8 expression in surrounding cells. Interleukin-8 is responsible for additional leukocyte recruitment and may trigger epithelial-mesenchymal transition and increase the survival and proliferation of endothelial-derived fibroblasts/myofibroblasts, leading to fibrosis.<sup>247</sup> Elastase also cleaves the interleukin-8 receptor C-X-C chemokine receptor type 1, interfering with neutrophil functions and antibacterial properties, thereby prolonging inflammation and increasing fibrosis-based changes.<sup>248</sup> Prolonged inflammation may also arise through elastase-mediated degradation of complement, releasing the neutrophil chemoattractant,



C5a. The neutrophil-derived oxidative burst leads to the formation of hypochlorous acid from hydrogen peroxide catalyzed by myeloperoxidase and induces injury to epithelial cells, thereby implicating the switch to fibrotic tissue deposition. Though less studied in oral fibrotic lesions, the impact of neutrophils on fibrosis has been observed in pulmonary fibrotic disorders, as these cells transmigrate to pulmonary fluids (such as bronchoalveolar lavage fluid) and recruit other leukocytes.

Neutrophils are required for healthy mucosal tissues in the oral cavity and elsewhere.<sup>225,249</sup> Leukocyte adhesion deficiency-1, caused by mutations of the gene encoding CD18 or integrin B2, leads to extensive periodontal inflammation and periodontitis associated with excessive interleukin-17-mediated inflammation that would normally be suppressed by the infiltrating neutrophils.<sup>242</sup> Mucosal neutrophils play a vital homeostatic role in preventing pathological inflammation by actively preventing both (1) microbial infection and (2) overactivation of mucosal lymphocytes.<sup>90,250</sup> The lung is a mucosal organ similar to the oral mucosa, housing neutrophil populations that possess unique phenotypes, behaviors, and functions during homeostasis. At steady state, neutrophils can be found accumulating intravascularly within the pulmonary microvasculature. The process is termed margination (Figure 3), and this pool of neutrophils exhibits a prolonged transit time through the lung microvasculature. Intravascular neutrophils are programmed to function as immune sentinels. In addition to their antimicrobial role, neutrophils in the lung possess an angiogenic transcriptomic signature that includes the expression of angiogenic factors like apelin and vascular endothelial growth factor A.

## 10 | NEUTROPHILS AS A CONDUIT FOR PERIO-SYSTEMIC DISEASE LINKS

Neutrophils can play a significant role in connecting distant organs. The oral-systemic disease connection has been thought to be mediated by three mechanisms: (a) oral bacteremia to reach distant sites; (b) spillover of inflammatory cytokines and other mediators produced by oral inflammation amplifying the pathological process elsewhere in the body; (3) neutrophil-mediated posttranslational changes to proteins within the inflamed periodontium, such as citrullination, creating autoantigens for the generation of anti-citrullinated protein antibodies, which cause rheumatoid arthritis in susceptible people.

It is clear that oral inflammation impacts and exacerbates systemic inflammatory burden, where neutrophils and macrophages generate substantial levels of inflammatory mediators that are released from local tissues to distant organs. In contrast, a lack of consistent and sustained systemic bacteremia or sepsis due to oral infections, including the most severe forms of periodontitis, suggested additional mechanisms could be involved in the systemic transport of bacteria.<sup>251</sup> Though a clear consensus is yet to be reached, there are two potential and intertwined pathways through which neutrophils could play a role in the dissemination of oral and specifically periodontal bacteria: (1) reverse migration, and (2) Trojan horse. Some suggest that the Trojan horse concept results from reverse transmigration,

whereby neutrophils loaded with infectious microorganisms reenter the circulation and deliver the microbial cargo and even live bacteria to a distant site, during which the microorganism is protected from immune surveillance and clearance.

### 10.1 | Forward and reverse migration

Under sterile inflammatory conditions, extravasated neutrophils were shown to reenter the vasculature through reverse migration, which is highly relevant to infectious environments and pathologies. As opposed to “forward migration,” “reverse migration” follows a different sequence of events. In forward migration, neutrophils sense, prioritize, and integrate the chemotactic stimuli into a migration response toward the damage. Forward migration (Figure 3) occurs in several stages. Early neutrophil recruitment is followed by amplification of the response. There are more than 30 distinct receptors (eg, G protein-coupled receptors, Fc receptors, adhesion receptors, cytokine receptors, and pattern recognition receptors) on neutrophils, which can respond to proinflammatory mediators and mediate neutrophil migration, function, behavior, and polarization. In addition, the signaling pathways induced by these receptors regulate the transcriptional activity, phagocytosis, apoptosis, degranulation, and reactive oxygen species production through phosphoinositide 3-kinase- $\gamma$  and phospholipase C. *N*-formyl peptides, such as *N*-formylmethionyl-leucyl-phenylalanine are derived from bacterial proteins and mitochondria after tissue damage to activate neutrophils.<sup>230</sup> Thus, an injured and infected site, such as the periodontal pocket, acts as a niche for neutrophil activation. In this environment, leukotriene B4 is produced from arachidonic acid by 5-lipoxygenase to induce neutrophil polarization and migration, playing a significant role early after wounding to recruit neutrophils and in the amplification stage of neutrophil recruitment. In addition to being a site of injury, the periodontal pocket (and, therefore, the peri-implant pocket) presents with a gradient of hypoxia, which creates an anaerobic milieu. The microbial communities differ depending on the oxygen content of periodontal pocket. Earlier publications demonstrated that the anaerobiosis increases microbial resistance to neutrophil-mediated killing.<sup>252</sup> Cathepsin G may be a critical antimicrobial mechanism through which neutrophils kill periodontal bacteria.<sup>253</sup> Although anaerobiosis may be a strategy to resist the neutrophil-mediated killing by the periodontal pathogens in a hypoxic periodontal pocket environment,<sup>131</sup> limited data also associated increased neutrophil infiltration and hypoxia around peri-implant tissues.<sup>254</sup> A very recent study used mathematical modeling to demonstrate the interactions between neutrophils and biofilm during which virulent species synergistically resist neutrophil-mediated killing under hypoxic conditions.<sup>255</sup>

Not all recruited neutrophils die at the injury site. Neutrophils that leave a wound can return to the vasculature through reverse transmigration to distal sites.<sup>256</sup> Previously unknown and unappreciated, this process is now recognized, and more than 90% of human neutrophils can reverse migrate. Reverse migration may contribute

to a distant inflammatory process in another organ, leading to damage, and spreading such reactions throughout the body may lead to systemic and/or chronic inflammation.

Reverse migration may arise secondary to competition between chemoattractant sources. A neutrophil may prefer competing signals, or some signals may act as a chemorepellent to induce neutrophil exit from damaged tissues. For example, hypoxia-inducible factor 1 $\alpha$  activation increases neutrophil retention at the site of tissue injury, whereas C-X-C ligand8 can act as a chemorepellent in higher concentrations. Pro-resolving lipid mediators can also promote neutrophil reverse migration during the resolution of inflammation.<sup>257</sup> Leukotriene B4 is a neutrophil chemoattractant; its production pathway can be altered to produce pro-resolution mediators such as lipoxin A4 after lipid-mediator class switching, whereas resolvins and protectins stop the neutrophil influx.<sup>223</sup> Reverse transmigration is also thought to be mediated by junctional adhesion molecule-C downregulation, a junctional adhesion protein that usually prevents reverse transmigration. In humans, this process has been observed in patients with rheumatoid arthritis. Reverse-transmigrating neutrophils are resistant to apoptosis. Neutrophils can leave inflamed or infected sites, travel through the lymphatics, and relocate to the lymph nodes or bone marrow, where it is believed they can influence the training of pro-myeloid cells via epigenetic and metabolic mechanisms, to mature into neutrophils that exhibit a hyperreactive phenotype (see Section 6.5).

Excessive and persistent neutrophil infiltration is pathologic in multiple inflammatory diseases, including periodontitis, rheumatoid arthritis, diabetes, cardiovascular diseases, pulmonary fibrosis, and multiple organ failure. Resolvins and protectins resolve inflammation by promoting the removal of leukocytes from injured tissue sites and may decrease chronic inflammation by promoting neutrophil reverse migration from locally damaged sites. Phagocytes secrete resolvins, protectins, lipoxins, and maresins. These specialized pro-resolving mediators from omega-3 fatty acids limit the recruitment of neutrophils and their ingestion by macrophages. Pro-resolving mediators upregulate the expression of C-C motif chemokine receptor 5 (a receptor for inflammatory chemokines such as C-C motif chemokine ligand3 and C-C motif chemokine ligand5) by senescent neutrophils and activated T cells.<sup>258</sup> Thus, C-C motif chemokine receptor 5-positive apoptotic leukocytes sequester inflammatory chemokines and act as terminators of their signaling during the resolution of inflammation in periodontitis and peritonitis. Resolvins and lipoxins have also been shown to control the macrophage polarization induced after a chitosan scaffold implantation. Lipoxins or resolvins shifted the polarization balance toward an anti-inflammatory phenotype in a murine air pouch model.

## 10.2 | Specialized pro-resolving mediators of inflammation

Resolvins primarily comprise E-resolvins (18-hydroxyecosapentaenoic acid, resolvin E1, resolvin E2, resolvin E3) derived from ecosapentaenoic

acid, and D-resolvins (resolvin D2-D6) derived from docosahexaenoic acid. Such specialized pro-resolving mediators of inflammation inhibit neutrophil infiltration and eventual apoptosis, inhibit further proinflammatory mediator production, stimulate chemokine identification, aid in microbial clearance, and reduce pain signaling. Lipoxins are synthesized from arachidonic acid in platelets, neutrophils, erythrocytes, and reticulocytes. Lipoxins regulate innate and adaptive immune responses and modulate the function of immune cells, including neutrophils, macrophages, and T- and B-cells. Additionally, lipoxins regulate the expression of many inflammatory genes and transcription factors, including nuclear factor  $\kappa$ B, activator protein-1, nerve growth factor, and peroxisome proliferator-activated receptor- $\gamma$ . Their binding mediates the anti-inflammatory and pro-resolution effects of lipoxins to G protein-coupled N-formyl peptide receptor2 (ALX), G protein-coupled receptor32, estrogen receptor, high-affinity cysteinyl leukotriene receptor, and aryl hydrocarbon receptors. Lipoxins help recruit neutrophils at the site of inflammation and later help clear neutrophils from the site of inflammation, roles that help transform the inflammatory phase into the resolution phase. Resolvin E1 resolves inflammation by decreasing neutrophil infiltration, inhibiting neutrophil transmigration, downregulating leukocyte adhesive molecules (CD11/CD18), activating adenosine diphosphate-dependent platelets, suppressing nuclear factor  $\kappa$ B activation, and promoting polymorphonuclear neutrophil apoptosis.<sup>238,259</sup> The pro-resolving effects of resolvin E2 and resolvin E3 are mediated by decreased neutrophil infiltration, and the pro-resolving effects of resolvin D1 are mediated by reduced infiltration of neutrophils, transmigration of neutrophils, and increased phagocytic activity of macrophages. The pro-resolving effects of resolvin D2 are mediated by reduced neutrophil infiltration, decreased adipose tissue macrophages, and improved insulin sensitivity. Specialized pro-resolving mediators have a context-dependent activity mediated by distinct cellular targets and specific receptors.

Neutrophils can also provide niches within which pathogens can survive and replicate. Neutrophil infiltration in response to early cell death of recruited neutrophils is pronounced in infections altered by pathogens that carry virulence factors. A significant variable in infection is the duration of damage, especially if the pathogen is not cleared. This leads to the broader recruitment of immune cells such as macrophages, dendritic cells, mast cells, and T cells. Early-arriving neutrophils phagocytose the bacteria and activate the inflammasome by interleukin-1 $\beta$  secretion, which, in turn, stimulates nonimmune tissue cells to produce proinflammatory cytokines and chemokines.

The host must distinguish whether or not a tissue injury is due to an infection to determine the strength and length of the appropriate response. Indeed, neutrophils migrate differently to host-produced versus pathogen-produced signals defining their phenotype. Systemic diseases also provide favorable sites for neutrophils to migrate. Impairment of neutrophil function and the creation of inflammatory attraction have been demonstrated for obesity, diabetes, and cardiovascular diseases, where the tissue environment can dysregulate neutrophil functions.<sup>260</sup> Smoking also induces neutrophil dysfunction. Specifically, diseases and conditions associated

with increased oxidative stress, increased glucose concentrations, and the activation of endothelial cells (leading to increased basal neutrophil-endothelial cell interactions), as well as with reduced nitric oxide concentrations, can induce greater susceptibility to thrombosis in vessels and, independently of each other, induce a proinflammatory and prothrombotic phenotype of the microvasculature, thus resulting in enhanced damage following ischemic insults. In metabolic diseases, hyperinflammatory neutrophils are not adequately cleared from injured tissues with altered function, further exacerbating tissue injury.

## 11 | ORAL NEUTROPHILS IN HEALTH AND DISEASE

Neutrophils, together with salivary antimicrobial peptides and antibodies, mediate oral mucosal defense. They are present in both the healthy and inflamed oral mucosa, suggesting they actively carry out innate immune surveillance.<sup>261</sup> Neutrophils are the main leukocyte able to transit the junctional and pocket epithelia and to enter the oral cavity. In orally healthy and fully dentate subjects, approximately 30000 neutrophils enter the oral cavity per minute.<sup>262</sup> In individuals with gingivitis and periodontitis, the number of neutrophils emerging in the gingival crevice significantly increases.<sup>263,264</sup> This can be explained by both the increased presence of chemotactic factors and the enlarged surface area of inflamed tissues within periodontal pockets.

Neutrophils are also present in the oral cavity of edentulous people, suggesting that they transmigrate oral epithelium, too.<sup>265</sup> In the salivary glands, neutrophils enter the excretory duct system, which is likely triggered by the presence of ascending bacteria and chemoattractants.<sup>266</sup> Oral neutrophils have been isolated by using isotonic mouth rinses or by aspirating gingival crevicular fluid, which yields lower numbers of neutrophils and is technically demanding.<sup>86</sup> Hence, neutrophils obtained from mouth rinses contain gingival crevicular fluid neutrophils as well as those that have entered the oral cavity via the oral epithelium and salivary ducts.

When neutrophils reach the oral cavity, they have migrated through vascular endothelium, connective tissue, and epithelial layers and have encountered antigens and activating factors. Furthermore, they are instantly challenged by a higher osmotic pressure present in the saliva environment, causing water influx and neutrophil swelling.<sup>267</sup> As a consequence, oral neutrophils are not long lived, and it is plausible that they display a phenotype different from that of circulatory neutrophils.<sup>268</sup> In comparison with blood neutrophils, they were reported to display decreased chemotactic abilities and have been referred to as "terminally migrated neutrophils."<sup>269</sup>

Oral neutrophils have been investigated regarding their surface markers and functions, such as chemotaxis, phagocytosis, and NETosis, with heterogeneous and sometimes conflicting results. For example, two independent studies reported impaired phagocytosis compared with blood neutrophils,<sup>270,271</sup> whereas two other studies found increased phagocytosis, though with reduced killing

capacity.<sup>85,269</sup> Similarly, analysis of the same surface markers by different research groups has led to contradicting outcomes.<sup>272</sup> This may be explained by the fact that each of these studies investigating oral neutrophils employed different test and control groups, experimental conditions, and marker panels measured. A substantial body of research on oral neutrophil phenotypes has been accomplished by Glogauer and coworkers, and this work has been summarized elsewhere.<sup>272</sup>

Rijkschroeff et al<sup>265</sup> compared oral neutrophils from edentate to dentate individuals and found, with the exception of CD16, opposing patterns of surface marker expression of CD11b, CD63, and CD66. This may be attributed to an altered oral microbiome in edentulous patients and strengthens the notion that neutrophil phenotype is influenced by the microbial challenge. However, it may also point toward epithelial transmigration versus junctional epithelium transmigration as an influencing factor. Junctional epithelium contains fewer tight junctions than the oral gingival epithelium, well-developed gap junctions, and has wider intercellular spaces facilitating neutrophil transmigration.<sup>273</sup> Although surface marker expression in relation to tissue properties such as density and elasticity has not been investigated to date, it is known that neutrophils change their morphology and adapt their intracellular signaling in response to these properties.<sup>118</sup>

Neutrophil extracellular trap formation in oral neutrophils has also been investigated. Moonen et al<sup>269</sup> reported that these neutrophils from orally healthy donors showed enhanced unstimulated neutrophil extracellular trap release compared with blood neutrophils. Vitkov et al,<sup>209</sup> in their descriptive microscopic study of gingival crevice neutrophils, found that gingival crevicular fluid was interwoven by neutrophil extracellular traps with entrapped bacterial cells. Neutrophil extracellular trap formation with entrapped bacteria was also seen on the epithelial surface of periodontal tissues.<sup>208</sup> Other studies confirmed these findings by showing the presence of H3 citrulline, a neutrophil extracellular trap activation marker, in gingivitis and periodontitis tissue biopsies and in supragingival biofilm samples.<sup>27,193,274</sup>

NETosis is considered a distinct programmed cell death pathway,<sup>275</sup> and according to previous studies, oral neutrophils are prone to neutrophil extracellular trap formation. However, it is not known what proportion undergoes this form of cell death. A transcriptome study of oral neutrophils by Lakschevitz et al<sup>276</sup> found upregulation of pro-survival members of the B-cell lymphoma2 family of apoptosis regulators and downregulation of proapoptosis members in patients with generalized periodontitis, compared with healthy subjects. Additional functional analysis confirmed that the percentages of viable neutrophils were significantly increased in the oral cavity of periodontitis patients. They concluded that a pro-survival neutrophil phenotype was predominant in periodontitis patients, contributing to chronic inflammation.

Although this finding appears to contradict the pro-NETosis phenotype, it is possible that these phenotypes exist alongside each other, or that downregulation of apoptosis may be a facilitating condition for neutrophil extracellular trap formation, as described in cystic fibrosis. In contrast, a study by Nicu et al<sup>277</sup> also investigated

apoptosis in oral neutrophils from periodontitis patients versus healthy controls and found higher apoptosis rates in periodontitis patients. This study utilized a more specific method to detect apoptosis than that of Lakschevitz et al, and both studies used different case definitions for their patient and control groups. These differences may explain the contradictory outcomes and highlight the importance of employing recognized definitions for periodontal health and disease as well as the issue of limited comparability of studies using different methodologies.

Because oral neutrophils can be obtained noninvasively and at low cost, and because they reflect activation of the immune system, efforts have been made to utilize oral neutrophil phenotypes as diagnostic and prognostic markers for oral diseases, including periodontitis and oral cancer.<sup>278</sup> The orogranulocytic migratory rate was investigated as a marker for oral health as early as the 1960s<sup>279</sup> and was subsequently confirmed to be a useful indicator of oral inflammation.<sup>280</sup> Only recently, more comprehensive analyses of oral neutrophil function and CD marker expression have been carried out and indicate that these cells remain partially functional, though with unknown survival times in the natural oral environment. Further research is needed to understand whether these cells effectively contribute to host defense once in the oral cavity, and whether certain phenotypes have diagnostic or prognostic value.

## 12 | NEUTROPHILS IN CELL-TO-CELL COMMUNICATIONS

Though neutrophils are first responders to external stimuli, their interactions with other cells within the inflammatory environment may determine whether the inflammation switches to a resolution pathway or to a chronic state. Neutrophils can amplify or suppress the activity of other cell types through their ability to induce cellular crosstalk, and those interactions may be crucial in health and disease.

### 12.1 | Interactions of neutrophils with other immune cells

#### 12.1.1 | B-cells

In vitro studies had demonstrated that neutrophils secrete cytokines that can affect B-cells. Neutrophils were found to support the expansion of B-cells and their differentiation to plasma cells through the secretion of several growth factors, such as B-cell activating factor (a member of the tumor necrosis factor superfamily) and a proliferation-inducing ligand.<sup>281</sup> B-cell activating factor is produced and stored inside the neutrophils and is expressed on their membranes.<sup>282,283</sup> In the reverse direction, activated B-cells secrete chemotactic factors and induce the migration of more neutrophils into the inflammatory site, as has been demonstrated in a rheumatoid arthritis model.<sup>284</sup> An interesting discovery was the existence

of a subpopulation of neutrophils with an enhanced ability to support B-cells, the co-called B-cell-helper neutrophils.<sup>285</sup> These cells secrete additional factors with a direct effect on B-cells (B-cell activating factor, CD40L, interleukin-21, and neutrophil extracellular traps). They also support the activation of T-cell-independent antibody production and secretion.

In contrast, neutrophils were also reported to contribute to B-cell inhibition and downregulation of antibody production. Human neutrophils were found to suppress B-cell activities through contact-dependent and -independent pathways and lead to B-cell apoptosis.<sup>286,287</sup> The ability of neutrophils to up- or downregulate B-cell activities exemplify their role in the control of the immune response.

#### 12.1.2 | T-cells

T-cells and neutrophils are able to bidirectionally modulate their recruitment to the inflammatory environment. The recruitment of Th1 and Th17 cells, is induced by the secretion of chemotactic factors, such as C-C motif chemokine ligand 2, C-X-C ligand 9 and 10 by activated neutrophils, and Th1 cells can in return recruit neutrophils.<sup>288</sup> Besides recruitment, neutrophils and T-cells can also modulate their functions reciprocally. Activated T-cells secrete growth factors that control the expression of activation markers as well as their survival,<sup>288</sup> and neutrophils can promote the differentiation of Th1 and Th17 cells.<sup>289</sup> In contrast, some subpopulations of neutrophils were found to have a suppressive effect on CD4+ and CD8+ T-cells.<sup>290</sup> Those neutrophils can downregulate T-cell proliferation and interferon-gamma secretion. In addition, neutrophils were found to increase the migration of T-regulatory cells via the secretion of lipoxin A4, an anti-inflammatory lipid mediator.<sup>291</sup>

#### 12.1.3 | Monocytes/macrophages

Monocytes/macrophages and neutrophils are both professional phagocytes, arising from common precursors, and are expected to communicate in the inflammatory environment. They work together to eliminate pathogens and to build an effective immune response. Tissue macrophages respond to infective stimuli by the secretion of a variety of chemotactic molecules (C-X-C ligand 1 and 2, interleukin-1 alpha, monocyte chemoattractant protein-1) that attract neutrophils and amplify the inflammatory process.<sup>292,293</sup> They also support their viability within tissues via the release of growth factors.<sup>294</sup> Following recruitment, neutrophils are able to support a cycle of monocyte recruitment from blood to the tissues<sup>295</sup> and to support the polarization of macrophages into the proinflammatory M1 phenotype.

To avoid an exaggerated response that may induce collateral tissue damage, these two cells can collaborate in the resolution phase of inflammation. M1 macrophages express tumor necrosis factor on their surface, which induces neutrophil apoptosis.<sup>296</sup> The apoptotic neutrophils are cleared by macrophages via phagocytosis,<sup>297</sup>

via the toll-like receptor 2/4-myeloid differentiation primary response 88-dependent pathway, and via the activation of nicotinamide adenine dinucleotide phosphate oxidase.<sup>298</sup> This process induces the transformation of macrophages from a proinflammatory M1 to the anti-inflammatory M2 phenotype.<sup>241,299</sup> Failure to induce such a change induces a chronic inflammatory response and pathological tissue breakdown.

## 12.2 | Bone resorption

Apart from their classic role as tissue protectors and immune modulators, neutrophils have the ability to interact with bone cells and to effect inflammatory bone resorption. Activated neutrophils express membrane-bound RANKL, which can induce osteoclastogenesis via its receptor on osteoclasts and via a nuclear factor  $\kappa$ B-dependent pathway.<sup>300-304</sup> The membrane-bound RANKL acts on osteoclasts and their precursors, converting them to mature and active bone-resorbing cells. Only activated neutrophils have been reported to express receptor activator of nuclear factor- $\kappa$ B, the receptor for RANKL.<sup>50,305</sup> Owing to the large number of neutrophils in periodontitis and peri-implantitis lesions, these cells may have a substantial impact on the bone resorption process in the two aforementioned inflammatory diseases.

## 12.3 | Epithelial and stromal cells

The mucosal barrier around the tooth (ie, the gingiva comprising a few layers of epithelial cells) and stromal cells are the cellular constituents of the connective tissue underlying the epithelium. These cells are the majority of the cells in the periodontal environment, and their role in the regulation of immunity is sometimes underestimated. Recently, their role in the regulation of inflammation was explored using advanced molecular techniques.<sup>261</sup> This detailed study revealed that immune-related pathways are upregulated in stromal and epithelial cells in periodontal health and disease, and most of them are related to the recruitment of neutrophils. The investigators identified populations of stromal cells that promote neutrophil migration, which were found to have an active role in the recruitment of neutrophils to periodontitis sites. The stromal cell genes involved include CXCL1, -2 and -8 and CSF3.

## 13 | ROLE OF NEUTROPHILS IN PERI-IMPLANT DISEASES

Dental implants are an effective and widely used treatment modality for the prosthetic rehabilitation of missing teeth. However, dental implants create a unique niche that is different in many aspects from the niche surrounding teeth. This distinctive environment presents an exceptional immunological challenge for the oral immune system. The accumulation of dental plaque on implant surfaces initiates

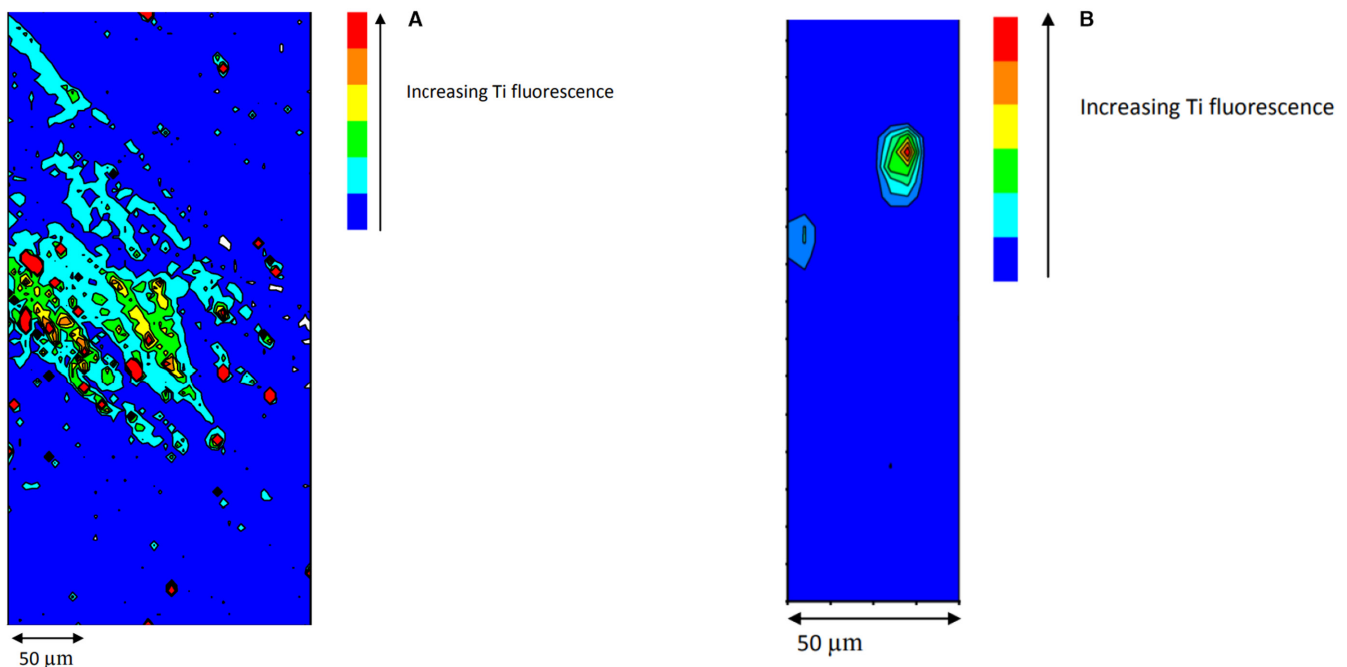
an inflammatory response in the peri-implant mucosa, similar to that which occurs around teeth. The constant and complex host-bacterial interactions shape local immune responses to maintain mucosal homeostasis.<sup>306</sup> This delicate balance may become dysregulated, resulting in an uncontrolled immune response that may lead to oral pathologies such as peri-implantitis.<sup>307,308</sup> Peri-implantitis was defined at the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions as a plaque-associated pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and progressive loss of the supporting bone.<sup>309</sup>

Since a microbial biofilm forms on implant surfaces,<sup>310</sup> it is reasonable to assume that neutrophils, which constitute the first line of host response against pathogens, play a significant role in the immune mechanisms involved in peri-implantitis. Indeed, using a model of murine experimental peri-implantitis, Heyman et al<sup>308</sup> demonstrated that the frequency of neutrophils doubled in peri-implantitis when compared with control mice and was 25 times higher in peri-implantitis than in periodontitis, suggesting a more aggressive and uncontrolled host response in peri-implantitis. These findings agree with numerous earlier and recent studies that characterized the inflammatory response around implants in human and animal models and revealed significantly higher percentages and densities of neutrophils in peri-implantitis than in periodontitis.<sup>311-315</sup> A common finding among these studies was that neutrophils were detected in various compartments of the peri-implant mucosa, such as in the pocket epithelium, connective tissue, central areas of the infiltrated connective tissue, and in the vicinity of the implants. Furthermore, neutrophils were found within the endothelium and were observed transmigrating through the endothelium.<sup>312,315</sup> Taken together, the wide distribution of neutrophils within peri-implant tissues and the increased neutrophil percentages and densities that arise in peri-implantitis suggest they play a key role in this disease. In addition to the aforementioned data, the functionality of neutrophils is of major importance. To cope with dental biofilm, neutrophils are equipped with several previously described oxidative defense systems that allow eradication of microorganisms and include the generation of reactive oxygen species via the nicotinamide adenine dinucleotide phosphate oxidase (respiratory burst),<sup>316</sup> neutrophil extracellular trap release,<sup>29,317</sup> and activation of nitric oxide synthases that catalyzes the production of nitric oxide.<sup>318</sup> To evaluate the cellular expression of reactive oxygen/nitrogen species, Dionigi et al<sup>319</sup> collected human samples from periodontitis and peri-implantitis lesions and found that the infiltrated connective tissue in peri-implantitis specimens was considerably larger and contained significantly larger areas populated with higher densities of neutrophils and inducible nitric oxide synthase-positive cells than those in periodontitis samples. They also observed that the noninfiltrated connective tissue area lateral to the infiltrated connective tissue contained significantly greater proportions and densities of neutrophils, inducible nitric oxide synthase-, nicotinamide adenine dinucleotide phosphate oxidase 2-, and neutrophil extracellular trap-positive cells in peri-implantitis than in periodontitis sites. The accumulation of reactive

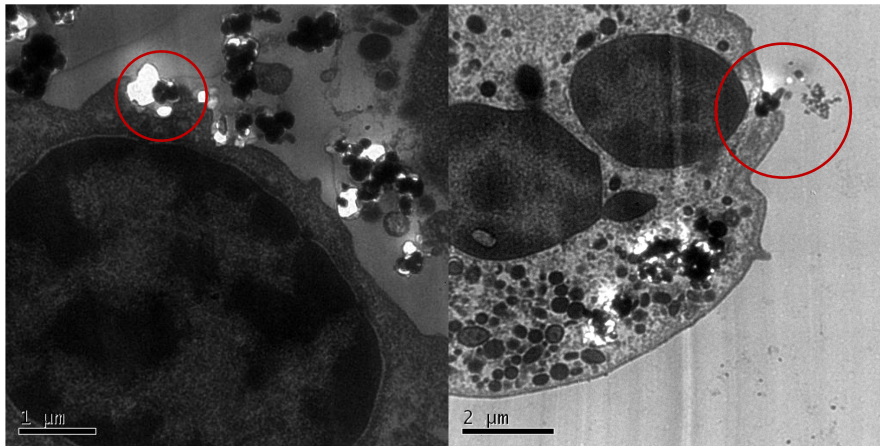
oxygen species in the tissue disrupts the oxidant-antioxidant balance, resulting in oxidative stress that may facilitate cytotoxicity and genotoxicity. Bressan et al<sup>320</sup> demonstrated that elevated reactive oxygen species facilitated the influx of neutrophils, leading to the production of large amounts of matrix metalloproteinases, ultimately resulting in the degradation of collagen, and Mijiritsky et al<sup>321</sup> reported that bone loss may be closely associated with high levels of reactive oxygen species in an inflammatory environment. Overall, high levels of reactive oxygen species, leading to an unbalanced oxidative state, can drive the activation of the inflammatory response and have been suggested to contribute to the progression of periodontitis<sup>142,322</sup> and possibly of peri-implantitis.

Although bacterial plaque is considered the main etiologic factor in peri-implantitis, recently published data suggest that titanium particles and ions emanating from the implant also activate the inflammatory response in the peri-implant mucosa and might be the primary cause of peri-implantitis. The release of titanium particles into the tissues surrounding the implant was demonstrated in preclinical and human biopsies<sup>323-326</sup> albeit with a lack of direct evidence for their role in peri-implantitis. With regard to neutrophils, in-vivo and in-vitro studies demonstrated that titanium particles may stimulate the production of high reactive oxygen species levels and recruit abnormal quantities of neutrophils that produce elevated levels of metalloproteinase.<sup>320,327</sup> Titanium particles, which are approximately 1-3 $\mu\text{m}$ , can be engulfed by neutrophils and were associated with an increase in superoxide anions, morphological changes, secretion of tumor necrosis factor- $\alpha$ , and stimulation of the inflammatory response.<sup>328</sup> To dissect the role of

the microbiota and titanium in the pathogenesis of peri-implantitis, Heyman et al<sup>307,308,329</sup> administered broad-spectrum antibiotics and antifungal treatment to mice in order to eradicate the microbiome. Notably, the treatment prevented bone loss around teeth but not around implants, emphasizing that bone loss in each specific niche involves distinct microbial and immunological mechanisms. Interestingly, whereas the antibiotic treatment considerably reduced all major immune cell subpopulations in the gingiva and peri-implant mucosa, the frequency of neutrophils in the peri-implant mucosa was not altered by the antibiotic treatment, suggesting they play a major role in bone loss around implants.<sup>307</sup> We have demonstrated co-localization of titanium breakdown products in peri-implantitis tissues with neutrophils, adjacent to the tissue microcirculation, using synchrotron X-ray fluorescence mapping of thin (4-6 $\mu\text{m}$ ) peri-implant tissues recovered from regions adjacent to percutaneous commercial pure titanium (implants, including dental implants (Figure 6). Moreover, transmission electron microscope imaging after 1 hour of stimulation with titanium oxide as anatase (45 nm or less) dispersed in phosphate-buffered saline at a concentration of 2000 ppm also demonstrated the presence of membrane-bound titanium nanoparticles within the neutrophil cytoplasm (Figure 7). Incubation with the actin cytoskeleton inhibitor cytochalasin-B (10 $\mu\text{g}/\text{mL}$ ) prevented the uptake of the titanium nanoparticles, implicating phagocytosis in their uptake. Importantly, significantly increased and dose-dependent total and extracellular reactive oxygen species release resulted following neutrophil stimulation with the titanium implant derivatives employed at concentrations of 2000, 200, 20, and 2 ppm.<sup>330</sup>



**FIGURE 6** A, X-ray fluorescence map (250 $\mu\text{m}$  x 525 $\mu\text{m}$ ) of the associated distribution of titanium (Ti) in peri-implant skin tissues (6 $\mu\text{m}$  thickness) taken from adjacent to a commercial pure titanium bone-anchored hearing aid. Legend refers to increasing titanium fluorescence values. B, X-ray fluorescence map (50 $\mu\text{m}$  x 200 $\mu\text{m}$ ) of the associated distribution of titanium in oral epithelial tissues (6 $\mu\text{m}$  thickness) taken from adjacent to a commercial pure titanium dental implant. Legend refers to increasing titanium fluorescence values.<sup>330</sup>



**FIGURE 7** Enlarged transmission electron microscope images (JEOL 1200) acquired at original magnification of 10000 $\times$  of isolated neutrophils after 1 h stimulation with titanium oxide as anatase ( $\leq 45$  nm) dispersed in phosphate-buffered saline at a concentration of 2000 ppm. From Kalra.<sup>330</sup>

Collectively, the aforementioned data demonstrate that neutrophils are more abundant in peri-implantitis than in periodontitis. The accumulation of neutrophils in peri-implantitis tissues, together with high levels of reactive oxygen species, nitric oxide synthase, and NETosis, facilitates the disruption of the oxidant-antioxidant balance, leading to cytotoxicity, genotoxicity, and tissue damage. Titanium particles have been implicated in the elevated inflammatory response, but their role in peri-implantitis needs to be further elucidated. Although our understanding of the pathogenesis of peri-implantitis remains limited, it seems likely that neutrophils play a key role in the immune mechanisms involved in the disease.

## 14 | SUMMARY/CONCLUSIONS

We have reviewed neutrophil biology in the context of the pathogenesis of periodontitis and its association with systemic noncommunicable diseases of aging. Neutrophils play a critical role in acute inflammation, in the defense against pathogenic challenge, and in the active resolution of acute inflammation and subsequent wound healing. They are highly destructive cells when exposed to certain signals, but they are equally capable of subtle synthetic and cell-to-cell communications, collaborating with multiple host cell types, including epithelial and stromal cells and T- and B-lymphocytes, where they facilitate antigen presentation as well as serving as phagocytes for Ig-mediated microbial killing. However, as detailed in this review, when exposed to the wrong signals, neutrophils lack restraint and engage in catastrophic behaviors, including releasing extracellular cytokines, proteases, and reactive oxygen species into their surrounding environment. They also exhibit a hyperreactive and hyperactive phenotype when stressed, and excess neutrophil reactive oxygen species release induces oxidative stress and collateral host tissue damage, and is also associated with systemic oxidative stress, which has recently been shown to be a causal link with a number of systemic noncommunicable diseases of aging. Neutrophil subsets have been identified, including N1, N2, and circadian phenotypes, which vary in their physiologic repertoires. The safe removal of neutrophils from tissues can be subverted by microbial virulence factors and nonapoptotic forms

of cell death, such as NETosis, which is associated with autoimmunity. The hyperreactivity of peripheral blood neutrophils in periodontitis patients may in part be due to myeloid training following their return to bone marrow to influence myelopoiesis and generate hypersensitive progenitors, but also due to priming within the circulation. The dominant inflammatory infiltrate in peri-implantitis comprises plasma cells and neutrophils, with neutrophil counts being 25 times higher in preclinical peri-implantitis models than in periodontitis. The rapid and relentless destruction of peri-implant bone may in part relate to the predominance of neutrophils in peri-implantitis lesions. Though the primary etiologic agent is a pathogenic plaque biofilm, the accumulation of titanium breakdown products within peri-implant tissues may be of importance as a modifying factor, since such corrosion products have been demonstrated in peri-implant tissues and have been shown to enter neutrophils and trigger reactive oxygen species production. It is interesting to speculate that accumulation with time of such titanium species within tissues may, in time, exceed a threshold that triggers bone destruction in an initially plaque-initiated lesion in certain individuals. In summary, neutrophil-mediated pathology appears to be a major factor in the pathobiology of periodontitis and its association with systemic noncommunicable diseases and of peri-implantitis, and an improved understanding of neutrophil biology is necessary to develop novel preventative and therapeutic approaches to managing neutrophil-mediated conditions.

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## ORCID

Iain L. C. Chapple  <https://orcid.org/0000-0003-2697-7082>

## REFERENCES

- Liew PX, Kubes P. The neutrophil's role during health and disease. *Physiol Rev.* 2019;99(2):1223-1248.
- Borregaard N. Neutrophils, from marrow to microbes. *Immunity.* 2010;33(5):657-670.
- Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood.* 1997;89(10):3503-3521.
- Cramer E, Pryzwansky KB, Villeval JL, Testa U, Breton-Gorius J. Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood.* 1985;65(2):423-432.
- Csernok E, Lüdemann J, Gross WL, Bainton DF. Ultrastructural localization of proteinase 3, the target antigen of anti-cytoplasmic antibodies circulating in Wegener's granulomatosis. *Am J Pathol.* 1990;137(5):1113-1120.
- Ohlsson K, Olsson I. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. *Eur J Biochem.* 1974;42(2):519-527.
- Perera NC, Schilling O, Kittel H, et al. NSP4, an elastase-related protease in human neutrophils with arginine specificity. *Proc Natl Acad Sci USA.* 2012;109(16):6229-6234.
- Rice WG, Ganz T, Kinkade JM Jr, et al. Defensin-rich dense granules of human neutrophils. *Blood.* 1987;70(3):757-765.
- Weiss J, Olsson I. Cellular and subcellular localization of the bactericidal/permeability-increasing protein of neutrophils. *Blood.* 1987;69(2):652-659.
- Sengeløv H, Follin P, Kjeldsen L, et al. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol.* 1995;154(8):4157-4165.
- Kjeldsen L, Bainton DF, Sengeløv H, Borregaard N. Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. *Blood.* 1993;82(10):3183-3191.
- Sengeløv H, Nielsen MH, Borregaard N. Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. *J Biol Chem.* 1992;267(21):14912-14917.
- Borregaard N, Kjeldsen L, Rygaard K, et al. Stimulus-dependent secretion of plasma proteins from human neutrophils. *J Clin Invest.* 1992;90(1):86-96.
- Velloso FJ, Trombetta-Lima M, Anschau V, Sogayar MC, Correa RG. NOD-like receptors: major players (and targets) in the interface between innate immunity and cancer. *Biosci Rep.* 2019;39(4):BSR20181709.
- Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol.* 2008;8(5):349-361.
- Summers C, Rankin SM, Condliffe AM, et al. Neutrophil kinetics in health and disease. *Trends Immunol.* 2010;31(8):318-324.
- Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000.* 2015;69(1):7-17.
- Sanz M, Marco Del Castillo A, Jepsen S, et al. Periodontitis and cardiovascular diseases: consensus report. *J Clin Periodontol.* 2020;47(3):268-288.
- de Pablo P, Chapple IL, Buckley CD, Dietrich T. Periodontitis in systemic rheumatic diseases. *Nat Rev Rheumatol.* 2009;5(4):218-224.
- Sharma P, Fenton A, Dias IHK, et al. Oxidative stress links periodontal inflammation and renal function. *J Clin Periodontol.* 2021;48(3):357-367.
- Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. *J Leukoc Biol.* 2013;94(4):595-601.
- Pillay J, den Braber I, Vrisekoop N, et al. In vivo labeling with  $^2\text{H}_2\text{O}$  reveals a human neutrophil lifespan of 5.4 days. *Blood.* 2010;116(4):625-627.
- Jethwaney D, Islam MR, Leidal KG, et al. Proteomic analysis of plasma membrane and secretory vesicles from human neutrophils. *Proteome Sci.* 2007;5:12.
- Roberts H, White P, Dias I, et al. Characterization of neutrophil function in Papillon-Lefèvre syndrome. *J Leukoc Biol.* 2016;100(2):433-444.
- Roos D. Chronic granulomatous disease. *Methods Mol Biol.* 2019;1982:531-542.
- Klein JB, Buridi A, Coxon PY, et al. Role of extracellular signal-regulated kinase and phosphatidylinositol-3 kinase in chemoattractant and LPS delay of constitutive neutrophil apoptosis. *Cell Signal.* 2001;13(5):335-343.
- Cooper PR, Palmer LJ, Chapple IL. Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? *Periodontol 2000.* 2013;63(1):165-197.
- Chicca IJ, Milward MR, Chapple ILC, et al. Development and application of high-content biological screening for modulators of NET production. *Front Immunol.* 2018;9:337.
- White PC, Chicca IJ, Cooper PR, Milward MR, Chapple IL. Neutrophil extracellular traps in periodontitis: a web of intrigue. *J Dent Res.* 2016;95(1):26-34.
- Yousefi S, Stojkov D, Germic N, et al. Untangling "NETosis" from NETs. *Eur J Immunol.* 2019;49(2):221-227.
- Wang J, Hossain M, Thanabalasuriar A, et al. Visualizing the function and fate of neutrophils in sterile injury and repair. *Science.* 2017;358(6359):111-116.
- Allen EM, Matthews JB, O'Halloran DJ, Griffiths HR, Chapple IL. Oxidative and inflammatory status in type 2 diabetes patients with periodontitis. *J Clin Periodontol.* 2011;38(10):894-901.
- Matthews JB, Chen FM, Milward MR, et al. Effect of nicotine, cotinine and cigarette smoke extract on the neutrophil respiratory burst. *J Clin Periodontol.* 2011;38(3):208-218.
- von Vietinghoff S, Ley K. IL-17A controls IL-17F production and maintains blood neutrophil counts in mice. *J Immunol.* 2009;183(2):865-873.
- Schwarzenberger P, Huang W, Ye P, et al. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J Immunol.* 2000;164(9):4783-4789.
- Griffin GK, Newton G, Tarrío ML, et al. IL-17 and TNF- $\alpha$  sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation. *J Immunol.* 2012;188(12):6287-6299.
- Song L, Tan J, Wang Z, et al. Interleukin-17A facilitates osteoclast differentiation and bone resorption via activation of autophagy in mouse bone marrow macrophages. *Mol Med Rep.* 2019;19(6):4743-4752.
- Lee JS, Tato CM, Joyce-Shaikh B, et al. Interleukin-23-independent IL-17 production regulates intestinal epithelial permeability. *Immunity.* 2015;43(4):727-738.
- Zindel J, Kubes P. DAMPs, PAMPs, and LAMPs in immunity and sterile inflammation. *Annu Rev Pathol.* 2020;15:493-518.
- Somers WS, Tang J, Shaw GD, Camphausen RT. Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. *Cell.* 2000;103(3):467-479.
- Bunting M, Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Curr Opin Hematol.* 2002;9(1):30-35.
- Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. *Immunity.* 2014;41(5):694-707.
- Qin CC, Liu YN, Hu Y, Yang Y, Chen Z. Macrophage inflammatory protein-2 as mediator of inflammation in acute liver injury. *World J Gastroenterol.* 2017;23(17):3043-3052.



44. McPhail LC, Clayton CC, Snyderman R. The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. *J Biol Chem.* 1984;259(9):5768-5775.
45. Wang S, Voisin MB, Larbi KY, et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *J Exp Med.* 2006;203(6):1519-1532.
46. Choi EY, Chavakis E, Czabanka MA, et al. Del-1, an endogenous leukocyte-endothelial adhesion inhibitor, limits inflammatory cell recruitment. *Science.* 2008;322(5904):1101-1104.
47. Deban L, Russo RC, Sironi M, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat Immunol.* 2010;11(4):328-334.
48. Erreni M, Manfredi AA, Garlanda C, Mantovani A, Rovere-Querini P. The long pentraxin PTX3: a prototypical sensor of tissue injury and a regulator of homeostasis. *Immunol Rev.* 2017;280(1):112-125.
49. Eskan MA, Jotwani R, Abe T, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat Immunol.* 2012;13(5):465-473.
50. Hajishengallis G, Moutsopoulos NM, Hajishengallis E, Chavakis T. Immune and regulatory functions of neutrophils in inflammatory bone loss. *Semin Immunol.* 2016;28(2):146-158.
51. Kempf T, Zarbock A, Widera C, et al. GDF-15 is an inhibitor of leukocyte integrin activation required for survival after myocardial infarction in mice. *Nat Med.* 2011;17(5):581-588.
52. Wischhusen J, Melero I, Fridman WH. Growth/differentiation factor-15 (GDF-15): from biomarker to novel targetable immune checkpoint. *Front Immunol.* 2020;11:951.
53. van Staveren S, Ten Haaf T, Klöpping M, et al. Multi-dimensional flow cytometry analysis reveals increasing changes in the systemic neutrophil compartment during seven consecutive days of endurance exercise. *PLoS ONE.* 2018;13(10):e0206175.
54. Bongers SH, Chen N, van Grinsven E, et al. Kinetics of neutrophil subsets in acute, subacute, and chronic inflammation. *Front Immunol.* 2021;12:674079.
55. Carlucci PM, Purmalek MM, Dey AK, et al. Neutrophil subsets and their gene signature associate with vascular inflammation and coronary atherosclerosis in lupus. *JCI Insight.* 2018;3(8):e99276.
56. Denny MF, Yalavarthi S, Zhao W, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol.* 2010;184(6):3284-3297.
57. Watt S, Vasquez L, Walter K, et al. Genetic perturbation of PU.1 binding and chromatin looping at neutrophil enhancers associates with autoimmune disease. *Nat Commun.* 2021;12(1):2298.
58. Miralda I, Uriarte SM, McLeish KR. Multiple phenotypic changes define neutrophil priming. *Front Cell Infect Microbiol.* 2017;7:217.
59. Fossati G, Bucknall RC, Edwards SW. Insoluble and soluble immune complexes activate neutrophils by distinct activation mechanisms: changes in functional responses induced by priming with cytokines. *Ann Rheum Dis.* 2002;61(1):13-19.
60. Swain SD, Rohn TT, Quinn MT. Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxid Redox Signal.* 2002;4(1):69-83.
61. Sreejit G, Nooti SK, Jaggars RM, et al. Retention of the NLRP3 inflammasome-primed neutrophils in the bone marrow is essential for myocardial infarction-induced granulopoiesis. *Circulation.* 2022;145(1):31-44.
62. Buckley CD, Ross EA, McGettrick HM, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol.* 2006;79(2):303-311.
63. Grieshaber-Bouyer R, Radtke FA, Cunin P, et al. The neutrotime transcriptional signature defines a single continuum of neutrophils across biological compartments. *Nat Commun.* 2021;12(1):2856.
64. Regev A, Teichmann SA, Lander ES, et al. The human cell atlas. *Elife.* 2017;6:e27041.
65. O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci.* 2008;5(1):1-8.
66. Wang Y, Jönsson F. Expression, role, and regulation of neutrophil Fcγ receptors. *Front Immunol.* 2019;10:1958.
67. Weiß E, Schlatterer K, Beck C, Peschel A, Kretschmer D. Formylpeptide receptor activation enhances phagocytosis of community-acquired methicillin-resistant *Staphylococcus aureus*. *J Infect Dis.* 2019;221(4):668-678.
68. Mortaz E, Adcock IM, Ito K, et al. Cigarette smoke induces CXCL8 production by human neutrophils via activation of TLR9 receptor. *Eur Respir J.* 2010;36(5):1143-1154.
69. Adrover JM, Del Fresno C, Crainiciuc G, et al. A neutrophil timer coordinates immune defense and vascular protection. *Immunity.* 2019;50(2):390-402.e10.
70. Aroca-Crevillén A, Adrover JM, Hidalgo A. Circadian features of neutrophil biology. *Front Immunol.* 2020;11:576.
71. Ella K, Csépanyi-Kömi R, Káldi K. Circadian regulation of human peripheral neutrophils. *Brain Behav Immun.* 2016;57:209-221.
72. Wilson D, Drew W, Jasper A, et al. Frailty is associated with neutrophil dysfunction which is correctable with phosphoinositol-3-kinase inhibitors. *J Gerontol A Biol Sci Med Sci.* 2020;75(12):2320-2325.
73. Panda A, Arjona A, Sapey E, et al. Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol.* 2009;30(7):325-333.
74. Bailey KL, Smith LM, Heires AJ, et al. Aging leads to dysfunctional innate immune responses to TLR2 and TLR4 agonists. *Aging Clin Exp Res.* 2019;31(9):1185-1193.
75. Fulop T, Larbi A, Douziech N, et al. Signal transduction and functional changes in neutrophils with aging. *Aging Cell.* 2004;3(4):217-226.
76. Grieshaber-Bouyer R, Nigrovic PA. Neutrophil heterogeneity as therapeutic opportunity in immune-mediated disease. *Front Immunol.* 2019;10:346.
77. Wu J, Li Y, Schuller RM, et al. The nonconservative CD177 single-nucleotide polymorphism c.1291G>A is a genetic determinant for human neutrophil antigen-2 atypical/low expression and deficiency. *Transfusion.* 2019;59(5):1836-1842.
78. Sachs UJ, Andrei-Selmer CL, Maniar A, et al. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). *J Biol Chem.* 2007;282(32):23603-23612.
79. Bauer S, Abdgawad M, Gunnarsson L, et al. Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils. *J Leukoc Biol.* 2007;81(2):458-464.
80. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol.* 1991;147(11):3815-3822.
81. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-β: "N1" versus "N2" TAN. *Cancer Cell.* 2009;16(3):183-194.
82. Masucci MT, Minopoli M, Carriero MV. Tumor associated neutrophils. Their role in tumorigenesis, metastasis, prognosis and therapy. *Front Oncol.* 2019;9:1146.
83. Wang X, Qiu L, Li Z, Wang XY, Yi H. Understanding the multifaceted role of neutrophils in cancer and autoimmune diseases. *Front Immunol.* 2018;9:2456.
84. Tsuda Y, Takahashi H, Kobayashi M, et al. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity.* 2004;21(2):215-226.

85. Fine N, Hassanpour S, Borenstein A, et al. Distinct oral neutrophil subsets define health and periodontal disease states. *J Dent Res*. 2016;95(8):931-938.
86. Dahlstrand Rudin A, Amirbeagi F, Davidsson L, et al. The neutrophil subset defined by CD177 expression is preferentially recruited to gingival crevicular fluid in periodontitis. *J Leukoc Biol*. 2021;109(2):349-362.
87. Cianciola LJ, Genco RJ, Patters MR, McKenna J, Oss CJV. Defective polymorphonuclear leukocyte function in a human periodontal disease. *Nature*. 1977;265(5593):445-447.
88. Peters NC, Egen JG, Secundino N, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science*. 2008;321(5891):970-974.
89. Xu N, Lei X, Liu L. Tracking neutrophil intraluminal crawling, transendothelial migration and chemotaxis in tissue by intravital video microscopy. *J Vis Exp*. 2011;(55):e3296.
90. Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. *Cell Mol Immunol*. 2020;17(5):433-450.
91. Filippi M-D. Neutrophil transendothelial migration: updates and new perspectives. *Blood*. 2019;133(20):2149-2158.
92. Bouti P, Webbers SDS, Fagerholm SC, et al.  $\beta$ 2 integrin signaling cascade in neutrophils: more than a single function. *Front Immunol*. 2020;11:619925.
93. Zarbock A, Ley K. Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol*. 2008;172(1):1-7.
94. Manley HR, Keightley MC, Lieschke GJ. The neutrophil nucleus: an important influence on neutrophil migration and function. *Front Immunol*. 2018;9:2867.
95. Hind LE, Vincent WJ, Huttenlocher A. Leading from the back: the role of the uropod in neutrophil polarization and migration. *Dev Cell*. 2016;38(2):161-169.
96. Youn YJ, Shrestha S, Lee YB, et al. Neutrophil-derived trail is a proinflammatory subtype of neutrophil-derived extracellular vesicles. *Theranostics*. 2021;11(6):2770-2787.
97. Cheng Y, Felix B, Othmer HG. The roles of signaling in cytoskeletal changes, random movement, direction-sensing and polarization of eukaryotic cells. *Cells*. 2020;9(6):1437.
98. Arts JGG, Mahlandt EK, Schimmel L, et al. Endothelial focal adhesions are functional obstacles for leukocytes during basolateral crawling. *Front Immunol*. 2021;12:667213.
99. François J, Kandasamy A, Yeh YT, et al. The interplay between matrix deformation and the coordination of turning events governs directed neutrophil migration in 3D matrices. *Sci Adv*. 2021;7(29):eabf3882.
100. Yaseen R, Blodkamp S, Lüthje P, et al. Antimicrobial activity of HL-60 cells compared to primary blood-derived neutrophils against *Staphylococcus aureus*. *J Negat Results Biomed*. 2017;16(1):2.
101. Manda-Handzlik A, Bystrzycka W, Wachowska M, et al. The influence of agents differentiating HL-60 cells toward granulocyte-like cells on their ability to release neutrophil extracellular traps. *Immunol Cell Biol*. 2018;96(4):413-425.
102. Babatunde KA, Wang X, Hopke A, et al. Chemotaxis and swarming in differentiated HL-60 neutrophil-like cells. *Sci Rep*. 2021;11(1):778.
103. Koelink PJ, Overbeek SA, Braber S, et al. Collagen degradation and neutrophilic infiltration: a vicious circle in inflammatory bowel disease. *Gut*. 2014;63(4):578-587.
104. Shum DKY, Chan SCH, Ip MSM. Neutrophil-mediated degradation of lung proteoglycans. *Am J Respir Crit Care Med*. 2000;162(5):1925-1931.
105. Moseley R, Waddington RJ, Embery G. Degradation of glycosaminoglycans by reactive oxygen species derived from stimulated polymorphonuclear leukocytes. *Biochim Biophys Acta*. 1997;1362(2-3):221-231.
106. Lu J, Liu J, Li A. Roles of neutrophil reactive oxygen species (ROS) generation in organ function impairment in sepsis. *J Zhejiang Univ Sci B*. 2022;23(6):437-450.
107. Van Dyke TE, Horoszewicz HU, Cianciola LJ, Genco RJ. Neutrophil chemotaxis dysfunction in human periodontitis. *Infect Immun*. 1980;27(1):124-132.
108. Hirschfeld J, Johansson A, Claessonand R, Chapple ILC. Modulation of neutrophil chemotaxis by *Aggregatibacter actinomycetemcomitans* leukotoxin. *J Oral Microbiol*. 2017;9(Suppl 1):1325259.
109. White PC, Hirschfeld J, Milward MR, et al. Cigarette smoke modifies neutrophil chemotaxis, neutrophil extracellular trap formation and inflammatory response-related gene expression. *J Periodontol Res*. 2018;53(4):525-535.
110. Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). *Publications and Reports of the Surgeon General. How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*. Centers for Disease Control and Prevention (US); 2010.
111. Zhu Y, Huang Y, Ji Q, et al. Interplay between extracellular matrix and neutrophils in diseases. *J Immunol Res*. 2021;2021:8243378.
112. Kinane DF, Cullen CF, Johnston FA, Evans CW. Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (I). Leading front analysis in Boyden chambers. *J Clin Periodontol*. 1989;16(4):242-246.
113. Jones MM, Vanyo ST, Visser MB. The C-terminal region of the major outer sheath protein of *Treponema denticola* inhibits neutrophil chemotaxis. *Mol Oral Microbiol*. 2017;32(5):375-389.
114. Armstrong CL, Miralda I, Neff AC, et al. *Filifactor alocis* promotes neutrophil degranulation and chemotactic activity. *Infect Immun*. 2016;84(12):3423-3433.
115. Jannat RA, Robbins GP, Ricart BG, Dembo M, Hammer DA. Neutrophil adhesion and chemotaxis depend on substrate mechanics. *J Phys Condens Matter*. 2010;22(19):194117.
116. Paul CD, Hung WC, Wirtz D, Konstantopoulos K. Engineered models of confined cell migration. *Annu Rev Biomed Eng*. 2016;18:159-180.
117. Makino A, Prossnitz ER, Bünemann M, et al. G protein-coupled receptors serve as mechanosensors for fluid shear stress in neutrophils. *Am J Physiol Cell Physiol*. 2006;290(6):C1633-C1639.
118. Oakes PW, Patel DC, Morin NA, et al. Neutrophil morphology and migration are affected by substrate elasticity. *Blood*. 2009;114(7):1387-1395.
119. Lam P-y, Fischer RS, Shin WD, Waterman CM, Huttenlocher A. Spinning disk confocal imaging of neutrophil migration in zebrafish. In: Quinn MT, DeLeo FR, eds. *Neutrophil Methods and Protocols*. Humana Press; 2014:219-233.
120. Yipp BG, Petri B, Salina D, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med*. 2012;18(9):1386-1393.
121. van Kessel KPM, Bestebroer J, van Strijp JAG. Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. *Front Immunol*. 2014;5:467.
122. Simon SI, Schmid-Schönbein GW. Biophysical aspects of microsphere engulfment by human neutrophils. *Biophys J*. 1988;53(2):163-173.
123. Nordenfelt P, Tapper H. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol*. 2011;90(2):271-284.
124. Vandendriessche S, Cambier S, Proost P, Marques PE. Complement receptors and their role in leukocyte recruitment and phagocytosis. *Front Cell Dev Biol*. 2021;9:624025.
125. Foote JR, Levine AP, Behe P, Duchon MR, Segal AW. Imaging the neutrophil phagosome and cytoplasm using a ratiometric pH indicator. *J Vis Exp*. 2017;(122):e55107.

126. Hänisch GM. Host defence against bacterial biofilms: "mission impossible"? *ISRN Immunol.* 2012;2012:853123.
127. Filep JG. Targeting neutrophils for promoting the resolution of inflammation. *Front Immunol.* 2022;13:866747.
128. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol.* 2015;15(1):30-44.
129. Uribe-Querol E, Rosales C. Control of phagocytosis by microbial pathogens. *Front Immunol.* 2017;8:1368.
130. Allen LA, Beecher BR, Lynch JT, Rohner OV, Wittine LM. *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol.* 2005;174(6):3658-3667.
131. Miralda I, Uriarte SM. Periodontal Pathogens' strategies disarm neutrophils to promote dysregulated inflammation. *Mol Oral Microbiol.* 2021;36(2):103-120.
132. Bhansali RS, Yeltiwar RK, Bhat KG. Assessment of peripheral neutrophil functions in patients with localized aggressive periodontitis in the Indian population. *J Indian Soc Periodontol.* 2013;17(6):731-736.
133. Johnstone AM, Koh A, Goldberg MB, Glogauer M. A hyperactive neutrophil phenotype in patients with refractory periodontitis. *J Periodontol.* 2007;78(9):1788-1794.
134. Carvalho RPM, Mesquita JS, Bonomo A, Elsas PX, Colombo APV. Relationship of neutrophil phagocytosis and oxidative burst with the subgingival microbiota of generalized aggressive periodontitis. *Oral Microbiol Immunol.* 2009;24(2):124-132.
135. Van Dyke TE, Zinney W, Winkel K, et al. Neutrophil function in localized juvenile periodontitis: phagocytosis, superoxide production and specific granule release. *J Periodontol.* 1986;57(11):703-708.
136. Katsuragi Y, Matsuda N, Nakamura M, Murayama Y. Neutrophil functions in patients with severe periodontal disease. *Adv Dent Res.* 1988;2(2):359-363.
137. Guentsch A, Puklo M, Preshaw PM, et al. Neutrophils in chronic and aggressive periodontitis in interaction with *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. *J Periodontol Res.* 2009;44(3):368-377.
138. Dimou NL, Nikolopoulos GK, Hamodrakas SJ, Bagos PG. Fcγ receptor polymorphisms and their association with periodontal disease: a meta-analysis. *J Clin Periodontol.* 2010;37(3):255-265.
139. Maney P, Emecen P, Mills JS, Walters JD. Neutrophil formylpeptide receptor single nucleotide polymorphism 348T>C in aggressive periodontitis. *J Periodontol.* 2009;80(3):492-498.
140. Mócsai A, Walzog B, Lowell CA. Intracellular signalling during neutrophil recruitment. *Cardiovasc Res.* 2015;107(3):373-385.
141. Assinger A, Laky M, Schabbauer G, et al. Efficient phagocytosis of periodontopathogens by neutrophils requires plasma factors, platelets and TLR2. *J Thromb Haemost.* 2011;9(4):799-809.
142. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000.* 2007;43:160-232.
143. Miralda I, Vashishta A, Rogers MN, Lamont RJ, Uriarte SM. The emerging oral pathogen, *Fillifactor alocis*, extends the functional lifespan of human neutrophils. *Mol Microbiol.* 2022;117(6):1340-1351.
144. Allen DB, Maguire JJ, Mahdavian M, et al. Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg.* 1997;132(9):991-996.
145. Gabig TG, Bearman SI, Babior BM. Effects of oxygen tension and pH on the respiratory burst of human neutrophils. *Blood.* 1979;53(6):1133-1139.
146. Eggert FM, Drewell L, Bigelow JA, Speck JE, Goldner M. The pH of gingival crevices and periodontal pockets in children, teenagers and adults. *Arch Oral Biol.* 1991;36(3):233-238.
147. Mettraux GR, Gusberti FA, Graf H. Oxygen tension (pO<sub>2</sub>) in untreated human periodontal pockets. *J Periodontol.* 1984;55(9):516-521.
148. Mendes RT, Nguyen D, Stephens D, et al. Hypoxia-induced endothelial cell responses – possible roles during periodontal disease. *Clin Exp Dent Res.* 2018;4(6):241-248.
149. Graves D. Cytokines that promote periodontal tissue destruction. *J Periodontol.* 2008;79(8 Suppl):1585-1591.
150. Lodge KM, Vassallo A, Liu B, et al. Hypoxia increases the potential for neutrophil-mediated endothelial damage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2022;205(8):903-916.
151. Gustafsson A, Asman B. Increased release of free oxygen radicals from peripheral neutrophils in adult periodontitis after Fcγ-receptor stimulation. *J Clin Periodontol.* 1996;23(1):38-44.
152. Fredriksson M, Gustafsson A, Asman B, Bergström K. Hyperreactive peripheral neutrophils in adult periodontitis: generation of chemiluminescence and intracellular hydrogen peroxide after in vitro priming and FcγR-stimulation. *J Clin Periodontol.* 1998;25(5):394-398.
153. Fredriksson M, Gustafsson A, Asman B, Bergström K. Periodontitis increases chemiluminescence of the peripheral neutrophils independently of priming by the preparation method. *Oral Dis.* 1999;5(3):229-233.
154. Fredriksson MI, Gustafsson AK, Bergström KG, Asman BE. Constitutionally hyperreactive neutrophils in periodontitis. *J Periodontol.* 2003;74(2):219-224.
155. Gronert K, Kantarci A, Levy BD, et al. A molecular defect in intracellular lipid signaling in human neutrophils in localized aggressive periodontal tissue damage. *J Immunol.* 2004;172(3):1856-1861.
156. Kobayashi T, Westerdal NA, Miyazaki A, et al. Relevance of immunoglobulin G Fc receptor polymorphism to recurrence of adult periodontitis in Japanese patients. *Infect Immun.* 1997;65(9):3556-3560.
157. Matthews JB, Wright HJ, Roberts A, Cooper PR, Chapple IL. Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clin Exp Immunol.* 2007;147(2):255-264.
158. Matthews JB, Wright HJ, Roberts A, et al. Neutrophil hyperresponsiveness in periodontitis. *J Dent Res.* 2007;86(8):718-722.
159. Wright HJ, Matthews JB, Chapple IL, Ling-Mountford N, Cooper PR. Periodontitis associates with a type 1 IFN signature in peripheral blood neutrophils. *J Immunol.* 2008;181(8):5775-5784.
160. Dias IH, Matthews JB, Chapple IL, et al. Activation of the neutrophil respiratory burst by plasma from periodontitis patients is mediated by pro-inflammatory cytokines. *J Clin Periodontol.* 2011;38(1):1-7.
161. Wright HJ, Chapple IL, Matthews JB, Cooper PR. *Fusobacterium nucleatum* regulation of neutrophil transcription. *J Periodontol Res.* 2011;46(1):1-12.
162. Dias IH, Chapple IL, Milward M, et al. Sulforaphane restores cellular glutathione levels and reduces chronic periodontitis neutrophil hyperactivity in vitro. *PLoS ONE.* 2013;8(6):e66407.
163. Oyake J, Otaka M, Matsushashi T, et al. Over-expression of 70-kDa heat shock protein confers protection against monochloramine-induced gastric mucosal cell injury. *Life Sci.* 2006;79(3):300-305.
164. Ling MR, Chapple IL, Matthews JB. Neutrophil superoxide release and plasma C-reactive protein levels pre- and post-periodontal therapy. *J Clin Periodontol.* 2016;43(8):652-658.
165. Brock GR, Butterworth CJ, Matthews JB, Chapple IL. Local and systemic total antioxidant capacity in periodontitis and health. *J Clin Periodontol.* 2004;31(7):515-521.
166. Chapple IL, Brock GR, Milward MR, Ling N, Matthews JB. Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J Clin Periodontol.* 2007;34(2):103-110.

167. Chapple IL. Role of free radicals and antioxidants in the pathogenesis of the inflammatory periodontal diseases. *Clin Mol Pathol*. 1996;49(5):M247-M255.
168. Grant MM, Brock GR, Matthews JB, Chapple IL. Crevicular fluid glutathione levels in periodontitis and the effect of non-surgical therapy. *J Clin Periodontol*. 2010;37(1):17-23.
169. Matthews JB, Chen FM, Milward MR, Ling MR, Chapple IL. Neutrophil superoxide production in the presence of cigarette smoke extract, nicotine and cotinine. *J Clin Periodontol*. 2012;39(7):626-634.
170. Hatanaka E, Monteagudo PT, Marrocos MS, Campa A. Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. *Clin Exp Immunol*. 2006;146(3):443-447.
171. Ling MR, Chapple IL, Matthews JB. Peripheral blood neutrophil cytokine hyper-reactivity in chronic periodontitis. *Innate Immun*. 2015;21(7):714-725.
172. Hajshengallis G, Chavakis T. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nat Rev Immunol*. 2021;21(7):426-440.
173. Mitroulis I, Ruppova K, Wang B, et al. Modulation of myelopoiesis progenitors is an integral component of trained immunity. *Cell*. 2018;172(1-2):147-161.e12.
174. Li X, Wang H, Yu X, et al. Maladaptive innate immune training of myelopoiesis links inflammatory comorbidities. *Cell*. 2022;185(10):1709-1727.e18.
175. Kalafati L, Kourtzelis I, Schulte-Schrepping J, et al. Innate immune training of granulopoiesis promotes anti-tumor activity. *Cell*. 2020;183(3):771-785.e12.
176. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat Rev Immunol*. 2016;16(6):378-391.
177. Figueredo CM, Gustafsson A, Asman B, Bergström K. Increased release of elastase from in vitro activated peripheral neutrophils in patients with adult periodontitis. *J Clin Periodontol*. 1999;26(4):206-211.
178. Armstrong DG, Jude EB. The role of matrix metalloproteinases in wound healing. *J Am Podiatr Med Assoc*. 2002;92(1):12-18.
179. Masciantonio MG, Lee CKS, Arpino V, Mehta S, Gill SE. The balance between metalloproteinases and TIMPs: critical regulator of microvascular endothelial cell function in health and disease. *Prog Mol Biol Transl Sci*. 2017;147:101-131.
180. Gethin G. Understanding the inflammatory process in wound healing. *Br J Community Nurs*. 2012;17(Suppl 3):17-22.
181. Becirovic-Agic M, Chalise U, Daseke MJ 2nd, et al. Infarct in the heart: what's MMP-9 got to do with it? *Biomolecules*. 2021;11(4):491.
182. Bouchery T, Harris N. Neutrophil-macrophage cooperation and its impact on tissue repair. *Immunol Cell Biol*. 2019;97(3):289-298.
183. Opdenakker G, Vermeire S, Abu El-Asrar A. How to place the duality of specific MMP-9 inhibition for treatment of inflammatory bowel diseases into clinical opportunities? *Front Immunol*. 2022;13:983964.
184. Wen F, White GJ, VanEtten HD, Xiong Z, Hawes MC. Extracellular DNA is required for root tip resistance to fungal infection. *Plant Physiol*. 2009;151(2):820-829.
185. Hawes MC, Curlango-Rivera G, Wen F, et al. Extracellular DNA: the tip of root defenses? *Plant Sci*. 2011;180(6):741-745.
186. Leffler J, Martin M, Gullstrand B, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol*. 2012;188(7):3522-3531.
187. Kessenbrock K, Krumbholz M, Schönemarck U, et al. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med*. 2009;15(6):623-625.
188. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303(5663):1532-1535.
189. Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol*. 2007;5(8):577-582.
190. Hoskin TS, Crowther JM, Cheung J, et al. Oxidative cross-linking of calprotectin occurs in vivo, altering its structure and susceptibility to proteolysis. *Redox Biol*. 2019;24:101202.
191. Fuchs TA, Abed U, Goosmann C, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;176(2):231-241.
192. Palmer LJ, Damgaard C, Holmstrup P, Nielsen CH. Influence of complement on neutrophil extracellular trap release induced by bacteria. *J Periodontol Res*. 2016;51(1):70-76.
193. Hirschfeld J, Dommisch H, Skora P, et al. Neutrophil extracellular trap formation in supragingival biofilms. *Int J Med Microbiol*. 2015;305(4-5):453-463.
194. Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*. 2010;10(12):826-837.
195. Chen T, Li Y, Sun R, et al. Receptor-mediated NETosis on neutrophils. *Front Immunol*. 2021;12:775267.
196. Clark SR, Ma AC, Tavener SA, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*. 2007;13(4):463-469.
197. Vitkov L, Muñoz LE, Knopf J, et al. Connection between periodontitis-induced low-grade endotoxemia and systemic diseases: neutrophils as protagonists and targets. *Int J Mol Sci*. 2021;22(9):4647.
198. Papapanagioutou D, Nicu EA, Bizzarro S, et al. Periodontitis is associated with platelet activation. *Atherosclerosis*. 2009;202(2):605-611.
199. Leppke M, Knopf J, Naschberger E, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. *EBioMedicine*. 2020;58:102925.
200. Vitkov L, Knopf J, Krunic J, et al. Periodontitis-derived dark-NETs in severe Covid-19. *Front Immunol*. 2022;13:872695.
201. Larvin H, Wilmott S, Wu J, Kang J. The impact of periodontal disease on hospital admission and mortality during COVID-19 pandemic. *Front Med (Lausanne)*. 2020;7:604980.
202. Marouf N, Cai W, Said KN, et al. Association between periodontitis and severity of COVID-19 infection: a case-control study. *J Clin Periodontol*. 2021;48(4):483-491.
203. Aquino-Martinez R, Hernández-Vigueras S. Severe COVID-19 lung infection in older people and periodontitis. *J Clin Med*. 2021;10(2):279.
204. Lloyd-Jones G, Molayem S, Pontes C, Chapple I. The COVID-19 pathway: a proposed oral-vascular-pulmonary route of SARS-CoV-2 infection and the importance of oral healthcare measures. *J Oral Med Dent Res*. 2021;2:1-25.
205. Palmer LJ, Cooper PR, Ling MR, et al. Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin Exp Immunol*. 2012;167(2):261-268.
206. Pilszczek FH, Salina D, Poon KK, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol*. 2010;185(12):7413-7425.
207. Palmer LJ, Chapple IL, Wright HJ, Roberts A, Cooper PR. Extracellular deoxyribonuclease production by periodontal bacteria. *J Periodontol Res*. 2012;47(4):439-445.
208. Vitkov L, Klappacher M, Hannig M, Krautgartner WD. Extracellular neutrophil traps in periodontitis. *J Periodontol Res*. 2009;44(5):664-672.
209. Vitkov L, Klappacher M, Hannig M, Krautgartner WD. Neutrophil fate in gingival crevicular fluid. *Ultrastruct Pathol*. 2010;34(1):25-30.
210. Nesse W, Westra J, van der Wal JE, et al. The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation. *J Clin Periodontol*. 2012;39(7):599-607.

211. White P, Sakellari D, Roberts H, et al. Peripheral blood neutrophil extracellular trap production and degradation in chronic periodontitis. *J Clin Periodontol*. 2016;43(12):1041-1049.
212. Farrera C, Fadeel B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol*. 2013;191(5):2647-2656.
213. Dema B, Charles N. Autoantibodies in SLE: specificities, isotypes and receptors. *Antibodies (Basel)*. 2016;5(1):2.
214. Hakkim A, Fürnrohr BG, Amann K, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA*. 2010;107(21):9813-9818.
215. Fauzi AR, Kong NC, Chua MK, et al. Antibodies in systemic lupus antineutrophil cytoplasmic erythematosus: prevalence, disease activity correlations and organ system associations. *Med J Malaysia*. 2004;59(3):372-377.
216. Liu J, Gao J, Wu Z, et al. Anti-citrullinated protein antibody generation, pathogenesis, clinical application, and prospects. *Front Med (Lausanne)*. 2021;8:802934.
217. Golub LM, Payne JB, Reinhardt RA, Nieman G. Can systemic diseases co-induce (not just exacerbate) periodontitis? A hypothetical "two-hit" model. *J Dent Res*. 2006;85(2):102-105.
218. Bartold PM, Lopez-Oliva I. Periodontitis and rheumatoid arthritis: an update 2012-2017. *Periodontol 2000*. 2020;83(1):189-212.
219. de Pablo P, Dietrich T, Chapple IL, et al. The autoantibody repertoire in periodontitis: a role in the induction of autoimmunity to citrullinated proteins in rheumatoid arthritis? *Ann Rheum Dis*. 2014;73(3):580-586.
220. Spengler J, Lugonja B, Ytterberg AJ, et al. Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. *Arthritis Rheumatol*. 2015;67(12):3135-3145.
221. Ellis S, Lin EJ, Tartar D. Immunology of wound healing. *Curr Dermatol Rep*. 2018;7(4):350-358.
222. Shim HB, Deniset JF, Kubes P. Neutrophils in homeostasis and tissue repair. *Int Immunol*. 2022;34(8):399-407.
223. Flitter BA, Fang X, Matthay MA, Gronert K. The potential of lipid mediator networks as ocular surface therapeutics and biomarkers. *Ocul Surf*. 2021;19:104-114.
224. Burnet M, Metcalf DG, Milo S, et al. A host-directed approach to the detection of infection in hard-to-heal wounds. *Diagnostics (Basel)*. 2022;12(10):2408.
225. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175.
226. Fortingo N, Melnyk S, Sutton SH, Watsky MA, Bollag WB. Innate immune system activation, inflammation and corneal wound healing. *Int J Mol Sci*. 2022;23(23):14933.
227. Phillipson M, Kubes P. The healing power of neutrophils. *Trends Immunol*. 2019;40(7):635-647.
228. Wang J. Neutrophils in tissue injury and repair. *Cell Tissue Res*. 2018;371(3):531-539.
229. Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol*. 2017;17(4):248-261.
230. Chen K, Bao Z, Gong W, et al. Regulation of inflammation by members of the formyl-peptide receptor family. *J Autoimmun*. 2017;85:64-77.
231. Luo B, Gan W, Liu Z, et al. Erythropoietin signaling in macrophages promotes dying cell clearance and immune tolerance. *Immunity*. 2016;44(2):287-302.
232. Kolb JP, Martinez J. Bon EPOtit! S1P-mediated EPO signaling whets a macrophage's appetite for apoptotic cells. *Immunity*. 2016;44(2):209-211.
233. Dalli J, Serhan C. Macrophage proresolving mediators—the when and where. *Microbiol Spectr*. 2016;4(3):1-23.
234. Serhan CN, Dalli J, Karamnov S, et al. Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. *FASEB J*. 2012;26(4):1755-1765.
235. Norris PC, Libreros S, Serhan CN. Resolution metabolomes activated by hypoxic environment. *Sci Adv*. 2019;5(10):eaax4895.
236. Kajikawa T, Wang B, Li X, et al. Frontline science: activation of metabolic nuclear receptors restores periodontal tissue homeostasis in mice with leukocyte adhesion deficiency-1. *J Leukoc Biol*. 2020;108(5):1501-1514.
237. Griffiths HR, Gao D, Pararasa C. Redox regulation in metabolic programming and inflammation. *Redox Biol*. 2017;12:50-57.
238. Gauer JS, Ajjan RA, Ariens RAS. Platelet-neutrophil interaction and thromboinflammation in diabetes: considerations for novel therapeutic approaches. *J Am Heart Assoc*. 2022;11(20):e027071.
239. Karima M, Kantarci A, Ohira T, et al. Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. *J Leukoc Biol*. 2005;78(4):862-870.
240. Yang S, Hu L, Han R, Yang Y. Neuropeptides, inflammation, biofilms, and diabetic foot ulcers. *Exp Clin Endocrinol Diabetes*. 2022;130(7):439-446.
241. Stark MA, Huo Y, Burcin TL, et al. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22(3):285-294.
242. Moutsopoulos NM, Konkel J, Sarmadi M, et al. Defective neutrophil recruitment in leukocyte adhesion deficiency type I disease causes local IL-17-driven inflammatory bone loss. *Sci Transl Med*. 2014;6(229):229ra40.
243. Martinez-Navarro FJ, Martinez-Morcillo FJ, de Oliveira S, et al. Hydrogen peroxide in neutrophil inflammation: lesson from the zebrafish. *Dev Comp Immunol*. 2020;105:103583.
244. Paget C, Doz-Deblauwe E, Winter N, Briard B. Specific NLRP3 inflammasome assembling and regulation in neutrophils: relevance in inflammatory and infectious diseases. *Cells*. 2022;11(7):1188.
245. Nikoloudaki G, Brooks S, Peidl AP, Tinney D, Hamilton DW. JNK signaling as a key modulator of soft connective tissue physiology, pathology, and healing. *Int J Mol Sci*. 2020;21(3):1015.
246. Cheat B, Torrens C, Foda A, et al. NLRP3 is involved in neutrophil mobilization in experimental periodontitis. *Front Immunol*. 2022;13:839929.
247. Kryczka J, Boncela J. Leukocytes: the double-edged sword in fibrosis. *Mediators Inflamm*. 2015;2015:652035.
248. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci*. 2004;9:283-289.
249. Julier Z, Park AJ, Briquez PS, Martino MM. Promoting tissue regeneration by modulating the immune system. *Acta Biomater*. 2017;53:13-28.
250. Silva LM, Kim TS, Moutsopoulos NM. Neutrophils are gatekeepers of mucosal immunity. *Immunol Rev*. 2022;314(1):125-141.
251. Lerman YV, Kim M. Neutrophil migration under normal and sepsis conditions. *Cardiovasc Hematol Disord Drug Targets*. 2015;15(1):19-28.
252. Casey SG, Shafer WM, Spitznagel JK. Anaerobiosis increases resistance of *Neisseria gonorrhoeae* to O<sub>2</sub>-independent antimicrobial proteins from human polymorphonuclear granulocytes. *Infect Immun*. 1985;47(2):401-407.
253. Miyasaki KT, Bodeau AL. In vitro killing of oral *Capnocytophaga* by granule fractions of human neutrophils is associated with cathelicin G activity. *J Clin Invest*. 1991;87(5):1585-1593.
254. de Araújo MF, Etchebere RM, de Melo MLR, et al. Analysis of CD15, CD57 and HIF-1 $\alpha$  in biopsies of patients with peri-implantitis. *Pathol Res Pract*. 2017;213(9):1097-1101.
255. Aristotelous AC. Biofilm neutrophils interactions under hypoxia: a mathematical modeling study. *Math Biosci*. 2022;352:108893.
256. Xu Q, Zhao W, Yan M, Mei H. Neutrophil reverse migration. *J Inflamm (Lond)*. 2022;19(1):22.
257. Shofler D, Rai V, Mansager S, Cramer K, Agrawal DK. Impact of resolving mediators in the immunopathology of diabetes and wound healing. *Expert Rev Clin Immunol*. 2021;17(6):681-690.

258. Ariel A, Fredman G, Sun YP, et al. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat Immunol*. 2006;7(11):1209-1216.
259. Maekawa T, Hosur K, Abe T, et al. Antagonistic effects of IL-17 and D-resolvins on endothelial Del-1 expression through a GSK-3 $\beta$ -C/EBP $\beta$  pathway. *Nat Commun*. 2015;6:8272.
260. Herrera BS, Hasturk H, Kantarci A, et al. Impact of resolvin E1 on murine neutrophil phagocytosis in type 2 diabetes. *Infect Immun*. 2015;83(2):792-801.
261. Williams DW, Greenwell-Wild T, Brenchley L, et al. Human oral mucosa cell atlas reveals a stromal-neutrophil axis regulating tissue immunity. *Cell*. 2021;184(15):4090-4104.e15.
262. Zenobia C, Herpoldt K-L, Freire M. Is the oral microbiome a source to enhance mucosal immunity against infectious diseases? *NPJ Vaccines*. 2021;6(1):80.
263. Landzberg M, Doering H, Aboodi GM, Tenenbaum HC, Glogauer M. Quantifying oral inflammatory load: oral neutrophil counts in periodontal health and disease. *J Periodontol Res*. 2015;50(3):330-336.
264. Wellappuli NC, Fine N, Lawrence HP, et al. Oral and blood neutrophil activation states during experimental gingivitis. *JDR Clin Trans Res*. 2018;3(1):65-75.
265. Rijkschroeff P, Loos BG, Nicu EA. Impaired polymorphonuclear neutrophils in the oral cavity of edentulous individuals. *Eur J Oral Sci*. 2017;125(5):371-378.
266. Schapher M, Koch M, Weidner D, et al. Neutrophil extracellular traps promote the development and growth of human salivary stones. *Cells*. 2020;9(9):2139.
267. Lantzman E. Comparison of the morphology and viability of human salivary and blood leukocytes. *J Periodontol*. 1976;47(2):72-78.
268. Rijkschroeff P, Jansen ID, van der Weijden FA, et al. Oral polymorphonuclear neutrophil characteristics in relation to oral health: a cross-sectional, observational clinical study. *Int J Oral Sci*. 2016;8(3):191-198.
269. Moonen CGJ, Hirschfeld J, Cheng L, et al. Oral neutrophils characterized: chemotactic, phagocytic, and neutrophil extracellular trap (NET) formation properties. *Front Immunol*. 2019;10:635.
270. Wilton JM, Renggli HH, Lehner T. The role of Fc and C3b receptors in phagocytosis by inflammatory polymorphonuclear leucocytes in man. *Immunology*. 1977;32(6):955-961.
271. Miyazaki A, Kobayashi T, Suzuki T, Yoshie H, Hara K. Loss of Fc $\gamma$  receptor and impaired phagocytosis of polymorphonuclear leukocytes in gingival crevicular fluid. *J Periodontol Res*. 1997;32(5):439-446.
272. Hirschfeld J. Neutrophil subsets in periodontal health and disease: a mini review. *Front Immunol*. 2019;10:3001.
273. Groeger S, Meyle J. Oral mucosal epithelial cells. *Front Immunol*. 2019;10:208.
274. Magán-Fernández A, O'Valle F, Abadía-Molina F, et al. Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis: a pilot study. *J Periodontol Res*. 2019;54(3):218-224.
275. Mesa MA, Vasquez G. NETosis. *Autoimmune Dis*. 2013;2013:651497.
276. Lakschevitz FS, Aboodi GM, Glogauer M. Oral neutrophil transcriptome changes result in a pro-survival phenotype in periodontal diseases. *PLoS ONE*. 2013;8(7):e68983.
277. Nicu EA, Rijkschroeff P, Wartewig E, Nazmi K, Loos BG. Characterization of oral polymorphonuclear neutrophils in periodontitis patients: a case-control study. *BMC Oral Health*. 2018;18(1):149.
278. Domnich M, Riedesel J, Pylaeva E, et al. Oral neutrophils: underestimated players in oral cancer. *Front Immunol*. 2020;11:565683.
279. Klinkhamer JM, Zimmerman S. The function and reliability of the orogranulocytic migratory rate as a measure of oral health. *J Dent Res*. 1969;48(5):709-715.
280. Bender JS, Thang H, Glogauer M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J Periodontol Res*. 2006;41(3):214-220.
281. Huard B, McKee T, Bosshard C, et al. APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *J Clin Invest*. 2008;118(8):2887-2895.
282. Scapini P, Bazzoni F, Cassatella MA. Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunol Lett*. 2008;116(1):1-6.
283. Costa S, Bevilacqua D, Cassatella MA, Scapini P. Recent advances on the crosstalk between neutrophils and B or T lymphocytes. *Immunology*. 2019;156(1):23-32.
284. Kristyanto H, Blomberg NJ, Slot LM, et al. Persistently activated, proliferative memory autoreactive B cells promote inflammation in rheumatoid arthritis. *Sci Transl Med*. 2020;12(570):eaaz5327.
285. Puga I, Cols M, Barra CM, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol*. 2011;13(2):170-180.
286. Kim JH, Podstawka J, Lou Y, et al. Aged polymorphonuclear leukocytes cause fibrotic interstitial lung disease in the absence of regulation by B cells. *Nat Immunol*. 2018;19(2):192-201.
287. Lelis FJN, Jaufmann J, Singh A, et al. Myeloid-derived suppressor cells modulate B-cell responses. *Immunol Lett*. 2017;188:108-115.
288. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a crosstalk between human neutrophils and Th17 cells. *Blood*. 2010;115(2):335-343.
289. Abi Abdallah DS, Egan CE, Butcher BA, Denkers EY. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *Int Immunol*. 2011;23(5):317-326.
290. Scapini P, Marini O, Tecchio C, Cassatella MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. *Immunol Rev*. 2016;273(1):48-60.
291. Gao Y, Min K, Zhang Y, et al. Female-specific downregulation of tissue polymorphonuclear neutrophils drives impaired regulatory T cell and amplified effector T cell responses in autoimmune dry eye disease. *J Immunol*. 2015;195(7):3086-3099.
292. De Filippo K, Henderson RB, Laschinger M, Hogg N. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J Immunol*. 2008;180(6):4308-4315.
293. Barry KC, Fontana MF, Portman JL, Dugan AS, Vance RE. IL-1 $\alpha$  signaling initiates the inflammatory response to virulent *Legionella pneumophila* in vivo. *J Immunol*. 2013;190(12):6329-6339.
294. Takano T, Azuma N, Satoh M, et al. Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions. *Arch Virol*. 2009;154(5):775-781.
295. Soehnlein O, Zernecke A, Eriksson EE, et al. Neutrophil secretion products pave the way for inflammatory monocytes. *Blood*. 2008;112(4):1461-1471.
296. Allenbach C, Zufferey C, Perez C, et al. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by *Leishmania major*. *J Immunol*. 2006;176(11):6656-6664.
297. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol*. 2014;14(3):166-180.
298. Bagaitkar J, Huang J, Zeng MY, et al. NADPH oxidase activation regulates apoptotic neutrophil clearance by murine macrophages. *Blood*. 2018;131(21):2367-2378.
299. Chernykh ER, Sakhno LV, Shevela EY, et al. Phenotypic and functional changes of GM-CSF differentiated human macrophages following exposure to apoptotic neutrophils. *Cell Immunol*. 2018;331:93-99.

300. Chakravarti A, Raquil MA, Tessier P, Poubelle PE. Surface RANKL of toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood*. 2009;114(8):1633-1644.
301. Hu X, Sun Y, Xu W, Lin T, Zeng H. Expression of RANKL by peripheral neutrophils and its association with bone mineral density in COPD. *Respirology*. 2017;22(1):126-132.
302. Kim AR, Kim JH, Choi YH, et al. The presence of neutrophils causes RANKL expression in periodontal tissue, giving rise to osteoclast formation. *J Periodontol Res*. 2020;55(6):868-876.
303. Cao G, Zhang X, Song Y, et al. Local promotion of B10 function alleviates experimental periodontitis bone loss through antagonizing RANKL-expressing neutrophils. *J Periodontol*. 2021;92(6):907-920.
304. Kim AR, Lim YK, Kook JK, Bak EJ, Yoo YJ. Lipopolysaccharides of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* increase RANKL-expressing neutrophils in air pouches of mice. *Lab Anim Res*. 2021;37(1):5.
305. Poubelle PE, Chakravarti A, Fernandes MJ, Doiron K, Marceau AA. Differential expression of RANK, RANK-L, and osteoprotegerin by synovial fluid neutrophils from patients with rheumatoid arthritis and by healthy human blood neutrophils. *Arthritis Res Ther*. 2007;9(2):R25.
306. Hajishengallis G, Liang S, Payne MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe*. 2011;10(5):497-506.
307. Heyman O, Horev Y, Koren N, et al. Niche specific microbiota-dependent and independent bone loss around dental implants and teeth. *J Dent Res*. 2020;99(9):1092-1101.
308. Heyman O, Horev Y, Mizraji G, et al. Excessive inflammatory response to infection in experimental peri-implantitis: resolution by resolvin D2. *J Clin Periodontol*. 2022;49(11):1217-1228.
309. Berglundh T, Armitage G, Araujo MG, et al. Peri-implant diseases and conditions: consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol*. 2018;89(Suppl 1):S313-S318.
310. Belibasakis GN. Microbiological and immuno-pathological aspects of peri-implant diseases. *Arch Oral Biol*. 2014;59(1):66-72.
311. Berglundh T, Gislason O, Lekholm U, Sennerby L, Lindhe J. Histopathological observations of human periimplantitis lesions. *J Clin Periodontol*. 2004;31(5):341-347.
312. Carcuac O, Abrahamsson I, Albouy JP, et al. Experimental periodontitis and peri-implantitis in dogs. *Clin Oral Implants Res*. 2013;24(4):363-371.
313. Carcuac O, Berglundh T. Composition of human peri-implantitis and periodontitis lesions. *J Dent Res*. 2014;93(11):1083-1088.
314. Lindhe J, Berglundh T, Ericsson I, Liljenberg B, Marinello C. Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog. *Clin Oral Implants Res*. 1992;3(1):9-16.
315. Rakic M, Radunovic M, Petkovic-Curcin A, et al. Study on the immunopathological effect of titanium particles in peri-implantitis granulation tissue: a case-control study. *Clin Oral Implants Res*. 2022;33(6):656-666.
316. El-Benna J, Hurtado-Nedelec M, Marzaioli V, et al. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol Rev*. 2016;273(1):180-193.
317. Rohrbach AS, Slade DJ, Thompson PR, Mowen KA. Activation of PAD4 in NET formation. *Front Immunol*. 2012;3:360.
318. Saini R, Singh S. Inducible nitric oxide synthase: an asset to neutrophils. *J Leukoc Biol*. 2019;105(1):49-61.
319. Dionigi C, Larsson L, Carcuac O, Berglundh T. Cellular expression of DNA damage/repair and reactive oxygen/nitrogen species in human periodontitis and peri-implantitis lesions. *J Clin Periodontol*. 2020;47(12):1466-1475.
320. Bressan E, Ferroni L, Gardin C, et al. Metal nanoparticles released from dental implant surfaces: potential contribution to chronic inflammation and peri-implant bone loss. *Materials (Basel)*. 2019;12(12):2036.
321. Mijiritsky E, Ferroni L, Gardin C, et al. Presence of ROS in inflammatory environment of peri-implantitis tissue: in vitro and in vivo human evidence. *J Clin Med*. 2019;9(1):38.
322. Zhao Y, Zheng Z, Zhang M, et al. Design, synthesis, and evaluation of mono-carbonyl analogues of curcumin (MCACs) as potential antioxidants against periodontitis. *J Periodontol Res*. 2021;56(4):656-666.
323. Fretwurst T, Buzanich G, Nahles S, et al. Metal elements in tissue with dental peri-implantitis: a pilot study. *Clin Oral Implants Res*. 2016;27(9):1178-1186.
324. Grande F, Tucci P. Titanium dioxide nanoparticles: a risk for human health? *Mini Rev Med Chem*. 2016;16(9):762-769.
325. Matusiewicz H. Potential release of in vivo trace metals from metallic medical implants in the human body: from ions to nanoparticles—a systematic analytical review. *Acta Biomater*. 2014;10(6):2379-2403.
326. Wennerberg A, Ide-Ektessabi A, Hatkamata S, et al. Titanium release from implants prepared with different surface roughness. *Clin Oral Implants Res*. 2004;15(5):505-512.
327. Noronha Oliveira M, Schunemann WVH, Mathew MT, et al. Can degradation products released from dental implants affect peri-implant tissues? *J Periodontol Res*. 2018;53(1):1-11.
328. Kumazawa R, Watari F, Takashi N, et al. Effects of Ti ions and particles on neutrophil function and morphology. *Biomaterials*. 2002;23(17):3757-3764.
329. Heyman O, Koren N, Mizraji G, et al. Impaired differentiation of langerhans cells in the murine oral epithelium adjacent to titanium dental implants. *Front Immunol*. 2018;9:1712.
330. Kalra S. *The Distribution and Pro-Inflammatory Impact of Titanium Debris Accumulation in the EPRI-Implant Environment*. [PhD thesis]. The University of Birmingham, UK 2013 <https://etheses.bham.ac.uk/id/eprint/4759/5/Kalra14PhD.pdf>
331. Asman B, Engström PE, Olsson T, Bergström K. Increased luminol enhanced chemiluminescence from peripheral granulocytes in juvenile periodontitis. *Scand J Dent Res*. 1984;92(3):218-223.
332. Ellegaard B, Borregaard N, Ellegaard J. Neutrophil chemotaxis and phagocytosis in juvenile periodontitis. *J Periodontol Res*. 1984;19(3):261-268.
333. Henry CA, Winford TE, Laohapund P, Yotnuengnit P. Neutrophil chemi-luminescence and opsonic activities of young people with periodontitis in Thailand. *Arch Oral Biol*. 1984;29(8):623-627.
334. Asman B, Bergström K, Wijkander P, Lockowandt B. Influence of plasma components on luminol-enhanced chemiluminescence from peripheral granulocytes in juvenile periodontitis. *J Clin Periodontol*. 1986;13(9):850-855.
335. Åsman B. Peripheral PMN cells in juvenile periodontitis. *J Clin Periodontol*. 1988;15(6):360-364.
336. Asman B, Bergström K, Wijkander P, Lockowandt B. Peripheral PMN cell activity in relation to treatment of juvenile periodontitis. *Scand J Dent Res*. 1988;96(5):418-420.
337. Whyte GJ, Seymour GJ, Cheung K, Robinson MF. Chemiluminescence of peripheral polymorphonuclear leukocytes from adult periodontitis patients. *J Clin Periodontol*. 1989;16(2):69-74.
338. Mattout CMJ, Capo C, Bongrand P, Fourel J. Impairment of neutrophil functions: study of a family with a case of juvenile periodontitis. *J Parodontol*. 1990;9:297-308.
339. Guarnieri C, Zucchelli G, Bernardi F, et al. Enhanced superoxide production with no change of the antioxidant activity in gingival fluid of patients with chronic adult periodontitis. *Free Radic Res Commun*. 1991;15(1):11-16.
340. Shapira L, Borinski R, Sela MN, Soskolne A. Superoxide formation and chemiluminescence of peripheral polymorphonuclear leukocytes in rapidly progressive periodontitis patients. *J Clin Periodontol*. 1991;18(1):44-48.

341. Zafiropoulos GG, Flores-de-Jacoby L, Plate VM, Eckle I, Kolb G. Polymorphonuclear neutrophil chemiluminescence in periodontal disease. *J Clin Periodontol*. 1991;18(8):634-639.
342. Asman B, Bergström K. Expression of Fc-γ-RIII and fibronectin in peripheral polymorphonuclear neutrophils with increased response to Fc stimulation in patients with juvenile periodontitis. *Arch Oral Biol*. 1992;37(12):991-995.
343. Kimura S, Yonemura T, Kaya H. Increased oxidative product formation by peripheral blood polymorphonuclear leukocytes in human periodontal diseases. *J Periodontol Res*. 1993;28(3):197-203.
344. Gomez RS, da Costa JE, Lorentz TM, de Almeida Garrocho A, Nogueira-Machado JA. Chemoluminescence generation and MTT dye reduction by polymorphonuclear leukocytes from periodontal disease patients. *J Periodontol Res*. 1994;29(2):109-112.
345. Leino L, Hurttia HM, Sorvajärvi K, Sewon LA. Increased respiratory burst activity is associated with normal expression of IgG-Fc-receptors and complement receptors in peripheral neutrophils from patients with juvenile periodontitis. *J Periodontol Res*. 1994;29(3):179-184.
346. Mouynet P, Delamaire M, Legoff MC, et al. Ex vivo studies of polymorphonuclear neutrophils from patients with early-onset periodontitis (II). Chemiluminescence response analysis. *J Clin Periodontol*. 1994;21(8):533-539.
347. Gustafsson A, Asman B, Bergström K. Priming response to inflammatory mediators in hyperreactive peripheral neutrophils from adult periodontitis. *Oral Dis*. 1997;3(3):167-171.
348. Hurttia H, Saarinen K, Leino L. Increased adhesion of peripheral blood neutrophils from patients with localized juvenile periodontitis. *J Periodontol Res*. 1998;33(5):292-297.
349. Biasi D, Bambara LM, Carletto A, et al. Neutrophil migration, oxidative metabolism and adhesion in early onset periodontitis. *J Clin Periodontol*. 1999;26(9):563-568.
350. Fredriksson MI, Figueredo CM, Gustafsson A, Bergström KG, Asman BE. Effect of periodontitis and smoking on blood leukocytes and acute-phase proteins. *J Periodontol*. 1999;70(11):1355-1360.
351. Gainet JDP, Chollet-Martin S, Brion M, et al. Neutrophil dysfunctions, IL-8, and soluble L-selectin plasma levels in rapidly progressive versus adult and localized juvenile periodontitis: variations according to disease severity and microbial flora. *J Immunol*. 1999;163:5013-5019.
352. Gustafsson A, Asman B, Bergström K. Cigarette smoking as an aggravating factor in inflammatory tissue-destructive diseases. Increase in tumor necrosis factor-alpha priming of peripheral neutrophils measured as generation of oxygen radicals. *Int J Clin Lab Res*. 2000;30(4):187-190.
353. Zekonis G, Zekonis J. Effect of bacterial stimulants on release of reactive oxygen metabolites from peripheral blood neutrophils in periodontitis. *Medicina (Kaunas)*. 2004;40(3):260-264.
354. Sadzeviciene R, Zekonis J, Zekonis G. Generation of superoxide anion by peripheral blood leukocytes in periodontitis patients with type 1 diabetes mellitus. *Medicina (Kaunas)*. 2005;41(6):477-481.

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