Morphological Characterization of Para and Pro-Inflammatory Neutrophil Phenotypes Using Transmission Electron Microscopy

by

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Faculty of Dentistry
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Abstract

Neutrophils play a crucial role in the immune response, leaving the circulation to participate in an inflammatory response to perceived pathogens. Yet in the oral cavity, they are constantly recruited into the gingival sulcus even in health, producing no apparent clinical inflammation. It has been demonstrated that in this homeostatic state the neutrophils present a “partially active” phenotype, termed para-inflammation. To further investigate this phenotype, we studied the morphology of neutrophils obtained in vivo under transmission electron microscopy (TEM), in health and Chronic Periodontitis (CP), in the blood and the oral cavity. To study and compare the changes induced by the commensal oral bacteria in vitro, blood neutrophils were also incubated separately with *S. oralis* cultures. Our hypothesis was that the neutrophils in the healthy oral cavity will present a morphology consistent with para-inflammation, in contrast to the naïve phenotype of blood neutrophils, and the fully active, pro-inflammatory oral neutrophils in CP.

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Abbreviations

BOP – Bleeding on Probing

CAL – Clinical Attachment Level

CD – Cluster of Differentiation

CGD – Chronic Granulomatous Disease

CP – Chronic Periodontitis

E/A – Epon Araldite

LAD – Leukocyte Adhesion Deficiency Syndrome

MMP – Matrix metalloproteinases

NE – Neutrophil Elastase

NETs – Neutrophil Extracellular Traps

PAD4 – Peptidyl Arginine Deiminase 4

PAMP – Pathogen Associated Molecule-Patterns

PBS – Phosphate Buffered Saline

PD – Probing Depth

PKC – Protein Kinase C

PMA – Phorbol-12-Myristate 13-Acetate

PMN – Polymorphonuclear Neutrophils

PO – Propylene Oxide

ROS – Reactive Oxygen Species

TEM – Transmission Electron Microscopy

TLR – Toll Like Receptors

VAMP – Vesicle Associated Membrane Protein

Chapter 1:

Introduction

Polymorphonuclear neutrophils (PMNs) are an integral component of the host response in periodontal disease¹. They exit the gingival plexus of blood vessels and enter the gingival crevice, where they represent the overwhelming majority of leukocytes recruited in response to the tooth associated biofilm². While they play an important protective role by mediating the destruction of invading pathogens, neutrophils can also cause substantial collateral damage to host tissue, particularly when inflammation levels are overly elevated or in-effect, dysregulated³. Chronic periodontitis (CP), a highly prevalent inflammatory condition of the oral cavity, is a prime example of the tissue destruction mediated by the pro-inflammatory PMN response. CP is characterized by increased PMN recruitment, gingival inflammation and progressive, usually irreversible loss of periodontal attachment^{4,5}. However, even in periodontal health, PMNs are constantly being recruited to the oral cavity, without inducing clinically evident inflammation or tissue destruction^{6,7}. This is likely related to the fact that even in the absence of so-called invading pathogens or an over-abundance of biofilm related microbes, there is always still a ‘health-associated’ level of oral microbes that would illicit the need for protection by the innate immune system in general and PMN cells in particular. Thus, it seems that while the blood PMNs are naïve, becoming primed or activated only during certain disease states, oral PMNs in health, which have undergone extravasation and exposure to the oral biofilm (even a health-associated or commensal biofilm as noted above) are *partially* activated, and do not cause any overt clinical signs of inflammation. This intermediary immune state has been termed “para-inflammation” and the PMNs associated with this can be referred to as being in a parainflammatory state^{8,9}. Consequently, events and conditions that induce PMNs to switch from a para-inflammatory to a pro-inflammatory state are likely to be central in the pathogenesis

of CP as well as other chronic inflammatory diseases associated with biofilm-bearing tissues. Mechanisms that restrain PMN function by supporting a para-inflammatory state for PMNs, including tolerance of the commensal microbiota without host tissue damage, are essential for maintenance of oral health. In order to begin to understand the roles and differences between parainflammatory and inflammatory PMNs, it is proposed that an initial understanding of their ultrastructural/morphological and functional differences between para- and pro-inflammatory PMNs must be developed.

Transmission electron microscopy (TEM) has been used extensively in the past to study the ultrastructural morphological characteristics of PMNs allowing for inference regarding their functional properties¹⁰⁻¹². This said, the majority of studies done in this area have focused principally on PMNs from the peripheral circulation. Few studies have examined the morphology of oral PMNs, and most TEM studies of periodontal inflammation and disease focused on the composition of the tissue as a whole, rather than the morphological features of individual cells⁶. Studies examining individual PMNs in periodontal disease have focused on impaired functionality of these cells, with no assessment of their morphological changes in health and disease¹³⁻¹⁶. In order to investigate morphological and functional differences between para- and pro-inflammatory PMNs we used high resolution TEM to analyze blood and oral PMNs of healthy volunteers and patients with CP. Furthermore, whole blood was incubated with oral commensal bacteria to assess the morphological changes observed in naïve blood PMNs by TEM. Our hypothesis was that PMNs in the healthy oral cavity will present with a distinctive morphology consistent with para-inflammation characterized by low granulation, light cytoplasm and high amount of euchromatin, while the fully active, pro-inflammatory phenotype of oral PMNs in CP will be characterized by a higher number of phagosomes, lower granularity, lighter cytoplasm, and larger amount of euchromatin. We present data on the morphological diversity

between the three groups, as well as the response induced in naïve cells after incubation with bacteria.

Chapter 2: Literature Review

2.1 PMN Homeostasis and the Concept of Para-inflammation

2.1.1 PMNs in Health

As alluded to above, bacterial challenge is unceasing in the oral cavity. Biofilm associated with teeth starts to form in the mouth within minutes after mechanical removal of the biofilm/plaque following oral hygiene practices, and if left unchecked the biofilm will continue to grow thereby presenting an increasingly large microbial load in the mouth that can lead to gingival inflammation and disease^{17, 18}. PMNs, the primary first responders of the innate immune system, are recruited into the oral cavity continuously as part of the host response to this challenge even in health and the presence of a commensal oral biofilm. Even though PMNs are recruited into the mouth under overt conditions of health, this action does not lead to the development of any clinical signs of inflammation or disease⁶. Hence, this low level of PMN migration into the mouth under overt conditions of health should be interpreted as being physiological in nature, part of the process of maintaining homeostasis between the host and commensal oral bacteria. Indeed, the disruption of this homeostatic relationship might be the root cause of or trigger for initiation of the pathophysiological processes that lead to the development of periodontal disease^{19, 20}.

2.1.2 Paradoxical Impact of Changes in the Innate Immune System on Periodontal Health

Although it is established that PMNs and over-activity in the innate immune system likely plays a major and probably causal role in the initiation and progression of periodontal disease, it should be recognized that reduced numbers of PMNs or PMNs with impaired function can also have a negative effect on periodontal health. For instance, severe deficiency in PMN numbers,

migration or antimicrobial activity is linked strongly with severe and rapid breakdown of the periodontal tissue. Agranulocytosis, and most forms of neutropenia, whether chronic, cyclic, idiopathic or familial - are all associated with severe periodontal breakdown²¹⁻²⁵. Furthermore, treatment of these patients and elevation of PMN levels using colony stimulating factors have been shown to significantly improve their periodontal state^{26, 27}. Hereditary conditions which do not affect the number of circulating PMNs but cause severe deficiency in their functionality are also associated with aggressive forms of periodontal destruction. Leukocyte adhesion deficiency syndrome (LAD I-III) is a term for a collection of disorders of the leukocyte recruitment cascade. They are associated with severe bacterial infections, rapid alveolar bone loss, and premature loss of dentition²⁸. On the other hand, in otherwise healthy individuals, the evidence that connects PMN dysfunction with periodontitis is limited at best. Most of the work on this effect examined aggressive periodontitis, and indeed some studies have shown reduced PMN chemotaxis and impaired phagocytosis. However, other studies contradicted these findings, demonstrating normal PMN activity, no correlation between PMN impairment and disease severity, and even elevated PMN functionality²⁹⁻³¹. In actuality, major damage to the periodontal tissue in periodontitis is caused by the activity of the PMNs themselves, whether directly from factors such as matrix metalloproteinases (MMPs) and reactive oxygen species(ROS), or indirectly through secretion of pro-inflammatory cytokines and activators of bone resorption²⁰. This is the case both for the aggressive form of the disease, but also for CP¹⁹. Therefore, although the tissue changes observed under conditions such as neutropenia and LAD are often referred to as chronic (albeit severe) periodontitis, we suggest that in fact these conditions are more representative of severe infections and microbially-mediated damage (as well as some degree of host mediated damage). The mechanisms of tissue destruction are different in that they could not be mediated by the antibacterial activity of PMNs given the paucity of PMN numbers or activity. This

concept is demonstrated in Papillion-Lefevre syndrome, which is among other things characterized by an inherited defect in the gene for Cathepsin C, an important activator of PMN serine protease. The defect causes some antimicrobial impairment in the PMN cells in patients with this condition, but the same PMNs also express marked elevations in the synthesis of ROS leading to high levels of oxidative stress³². Accordingly, these patients are also extremely prone to destruction of periodontal tissue which in this case could be considered as extremely severe CP as compared to conditions where PMN numbers or functionality are reduced. Interestingly, compared to healthy individuals these patients show no increased susceptibility to other bacterial infections, possibly because their PMNs are, if anything, highly active and protective (at least extraorally) while their increased tendency for oxidative burst in the oral cavity leads ultimately to the tissue destruction discussed previously^{33, 34}.

CP is characterized by increased PMN recruitment, gingival inflammation and progressive, irreversible loss of periodontal attachment. It is a highly prevalent inflammatory condition of the oral cavity and a model of tissue destruction mediated by the pro-inflammatory PMN response^{4,5}. Hyperresponsiveness of the PMNs, or their persistence in the tissues due to “frustrating” inability to resolve the bacterial infection can be a major contributor to the progressive tissue damage seen in periodontitis^{35, 36}. Therefore, if PMNs were to be continuously fully activated in the oral cavity, clinical inflammation and progressive tissue damage would occur. However, remaining inactivated after extravasation will lead to uncontrolled bacterial oral infection and severe disease, as seen in chronic or acute PMN deficiencies. Thus, it seems that in health the oral PMNs are primed to an intermediate state of partial activation, which lies between inactivity and pro-inflammation. This state has been termed “para-inflammation”³⁷. Fine et al. (2016) studied the differences between oral and circulatory PMNs in humans, in health and CP, characterizing the surface cluster of differentiation (CD) marker expression on these cells using flow cytometry.

They demonstrated that in the healthy periodontium, the oral PMNs express a para inflammatory phenotype, distinct from the pro inflammatory phenotype seen in CP, or the naïve phenotype seen in circulatory PMNs. Significant changes were observed mainly in the expression of CD markers associate with degranulation, cell activation, and adhesion⁸. Our current work was designed, in part, to expand the investigation of these phenotypes via direct observation.

2.2 Electron Microscopy

TEM has been used extensively in the past to study the morphology and function of PMNs. It is a viable method of assessing the cells structure, organelles and interaction with the environment¹⁰⁻¹². Although a vast amount of PMNs studies under TEM exists, they mainly focus on PMNs in the circulation. Relatively few studies examined the morphology of oral PMNs, and an even smaller number examined oral PMNs in periodontal disease. In 1976 Page and Schroeder published their seminal work on the pathophysiology of periodontal disease, which relied in part on extensive TEM study of gingival tissues⁶. However, similar to other studies done at this time, the main focus was on the composition of the tissue as a whole, rather than the ultrastructural characteristics of individual cells. The different cell types and their proportion, as a function of disease progression was described, while the morphological or functional changes in the actual cells (in this case PMNs) were not. Furthermore, the studies which did examine individual PMNs in periodontal disease mostly focused on its aggressive form, attempting to find impaired functionality of these cells. To the best of our knowledge, no TEM study of the morphological structure of oral PMNs in CP has been done. Using TEM, Carranza et al. (1983) examined the surrounding tissue of a tooth extracted from a 15-year-old patient suffering from localized juvenile periodontitis in order to characterize the invading microorganisms in the tissue. They also observed an infiltrate of PMNs in several layers of the epithelium. However,

only few of the PMNs engulfed the nearby bacteria, or showed signs of degranulation¹³.

Newman et al. (1982) obtained PMNs from patients with periodontosis (i.e. localized juvenile periodontitis) and controls, and used *Candida guilliermondiae* yeast to examine their phagocytic ability. None of the PMNs from these patients were capable of phagocytosing the yeast organisms, and TEM images showed most of the PMNs were in what appeared to be a degenerative state. The control oral PMNs had some phagocytic activity, but much less than anticipated in comparison to blood PMNs. TEM images revealed that most of the phagocytosed organisms were not broken down or lysed¹⁴. However, a study by Barnett et al. (1983) obtained PMNs using a crevicular washing method from patients with gingivitis, juvenile periodontitis and juvenile diabetes. In this particular investigation normal phagocytic activity of PMNs was demonstrated. In gingivitis, the authors found the cell membranes to be intact, and the cells had irregular surface shape, with numerous cell processes, several vacuoles and a decreased number of granules. In the juvenile periodontitis patients, there was less phagocytic activity and little evidence of degranulation. In contrast, a large number of cells from diabetic patients showed phagocytosis in progress, with many cells showing phagocytic vacuoles and few remaining granules¹⁵. In light of these studies, it seems useful to assess PMN activity in CP under TEM which should permit a much more detailed and hopefully more reliable assessment of their ultrastructural characteristics. From this, some aspects of their functionality can be inferred while also assessing their phagocytic capabilities more directly.

2.3 The Function and Morphological Features of PMNs

2.3.1 Granules

2.3.1.1 Granule variability

One of the main characteristics of PMNs is their intra-cellular granularity. When PMNs were first described, structures of interests were first termed “specific granules”, to differentiate them from other granule-looking artifacts³⁸. Some of these granules were then recognized as a different and separate subset, based on their affinity to basic blue dye, and therefore were named “azurophilic granules”. The development of fixation and dying techniques for electron microscopy have revealed that the major difference between these two types is that the azurophilic granules contain myeloperoxidase (MPO), whereas the specific granules do not. It was also noted that the larger azurophilic granules form first, during the promyelocyte stage, and in addition to MPO they contain other lysosomal enzymes. Therefore, they were considered a specialized form of lysosome. In contrast, the smaller specific granules are formed during the myelocyte stage, and were perceived as secretory vesicles contributing to the regulation of the inflammatory response^{11, 39, 40}. Further research and the sophistication of histochemical analysis methods have revealed a more complex picture, and has allowed these granules to be classified according to their content as opposed to merely their appearance. It is generally accepted that granules in the mature PMN can be divided into four main subtypes: primary (azurophilic), secondary (specific), tertiary (gelatinase), and secretory vesicles. Each type is characterized by the dominant protein it contains; MPO for the primary granules, lactoferrin in the secondary, and gelatinase in the tertiary. The secretory vesicles are unique in that they contain only plasma proteins, and therefore their marker is albumin⁴¹.

2.3.1.2 Primary and Secondary Granules

The creation of ROS is one of the main components of the antibacterial activity of the PMN. The contents of the PMN's granules can be thought of as the components of a highly reactive product, which are kept separately in different containers until they are mixed to create the active compound. Thus, the components of the PMN's bactericidal response are kept separately in the primary and secondary granules until needed. For example, the MPO in the primary granules turns H_2O_2 into hypochlorous acid, but H_2O_2 is created by NADPH. Some of the essential components of NADPH, such as cytochrome b_{558} , are stored in the secondary granules⁴². Furthermore, protease and elastase from the primary granules can proteolytically turn preform gelatinase and collagenases in other granules into active proteases⁴³. Additionally, the primary and secondary granules seem to contain the main cache of the PMN's antibacterial molecules such as proteinase 3, cathepsin G, α -defensins, elastase and lactoferrin.

2.3.1.3 Tertiary Granules

The tertiary granules are high in gelatinase, which can degrade type IV collagen, the major component of the basement membrane⁴⁴. Hence, it is likely that these granules participate in extravasation by promoting MMP mediated degradation of the basement membrane. This will enable the PMN to travel through the tissue without releasing the harmful serine proteases in the primary and secondary granules^{43, 45}. The tertiary and secondary granules' membranes are also the main source of MAC-1 (CD11b/CD18) in the cell, and therefore assist in phagocytosis^{46, 47}.

2.3.1.4 Secretory Vesicles

The significance of the secretory vesicles is in their membranes, are rich in various signaling proteins such as CXC chemotactic receptors and toll-like receptors (TLRs)^{48, 49}. Interaction of ligands with selectins on the surface of the PMN on activated endothelium promotes the fusion

of the secretory vesicles with the cell membrane, causing upregulation of β_2 -integrins and chemotactic receptors⁵⁰. Thus, the secretory vesicles are instrumental in making the PMN environment-sensitive, and in activating it for firm adhesion with the endothelium and for chemotactic migration, all essential steps in the inflammatory cascade. Furthermore, as this process of secretory vesicles fusion with the plasma membrane does not result in the extracellular release of any antimicrobial or proteolytic proteins (because they do not contain any)⁵¹, it highlights the notion that the granules' membrane are as important as are their contents. Hence, the PMN's granules are more than simple storage vacuoles, and they play an important part in the regulation of surface protein expression and in the cell's function and activation.

2.3.1.5 Granule Release Regulation

Another interesting aspect of the granules' membrane is the presence of VAMP-2 (vesicle-associated membrane protein 2), which enables it to fuse with the cell's membrane. Higher expression of VAMP-2 on the granule's surface will render it more exocytosis ready, and it is therefore assumed that the different proportion of VAMP-2 in different granule type plays an important part in the hierarchy and time control of their release⁵². For example, of all the granules, the secretory vesicles display the highest amount of VAMP-2, and thus are released the most readily. This is appropriate for their role in initially activating the PMN for adhesion and migration. The tertiary granules have the second highest amount of VAMP-2 on their surface, and therefore will be released next, promoting extravasation as described above. The secondary granules have even less VAMP-2, whereas on the primary granules it is mostly undetectable⁵¹. Thus, the control of granule release is quantity derived. Also, it is important to note that granule release, or degranulation, starts with the initiation of the inflammatory response, long before the PMN starts its actual bacteria killing activity.

2.3.2 Cytoplasm

2.3.2.1 Functionality and Structure of the Cytoplasm

The PMN's cytoplasm houses the nucleus, granules and all other organelles of the cell. However, it is more than just a filler liquid in which free particles float. Considering the high protein concentration in the cytoplasm and its bulk biophysical properties, the cytoplasm should be considered as a highly congested protein solution, rather than a simple undiluted and unbound aqueous one⁵³. Moreover, it has been suggested that the cytoplasm should be looked at as a dynamic structural continuum, in which the organelles, cytoskeletal filaments, ribosomes, and "soluble" enzymes are suspended⁵⁴. Advances in electron microscopy in the 1970s have enabled ultrastructural assessment of cells in minute detail in various regions; for example, cytoplasm and a variety of organelles. Consequently, the existence of a fine, anastomosing network of filaments dubbed "the microtubular lattice" was identified and is consistent with a model suggesting that cytoplasmic structure is dynamic in nature^{54, 55}. However, this has been seriously criticized by other investigators as being an artefact of the sample preparation process, and today it is generally accepted that the microtubular lattice does not exist^{56, 57} (it should be noted here that despite the similar nomenclature, the microtubular lattice is not related to the cytoskeleton and its microtubules, which are described below). Still, the true nature of the cytoplasm is still debated today, with the two main models suggesting that it is either a well-mixed, concentrated solution containing macromolecules and inorganic ions, or a more structured entity, with most macromolecules and metabolites being structure-associated or structure-forming within the cell⁵⁸. There is evidence showing altered function of proteins induced by concentration changes in other proteins alone, supporting the classic solution model^{59, 60}. This also suggests that "crowding" of the proteins in certain areas of the cytoplasm is not random, but part of the regulatory process. The real structure of the cytoplasm is probably an intermediate between the

solution and structured models, but it accounts for the different electron-density observed in the cytoplasm as it relates to function^{58, 61}.

2.3.2.2 The Cytoskeleton

The cytoplasm is filled with an interconnecting network of filaments and tubules known as the cytoskeleton. Its three main components are actin microfilaments, intermediate filaments, and microtubules. Apart from providing a physical scaffold to the cell, the various components of the cytoskeleton participate in different functions. In the PMN, the cytoskeleton appears to have a complex role in regulating the release of granules. As described above, once a target has been phagocytosed, granules are merged with the phagosome to incorporate their antimicrobial content. It was shown that the cytoskeleton's microtubules have a major role in directing the primary granules to the phagosome. However, the microtubules have much less control over the peroxidase negative granules, and this suggests an added mechanism in the degranulation hierarchy, in addition to the VAMP-2 mechanism described previously. Interestingly, both are controlled through the Ca^{2+} gradient in the cell⁶². With regard to the actin microfilaments, early studies with electron microscopy have demonstrated the formation of microfilaments during phagocytosis at sites of the stretching lamellipodia and the consequently engulfed phagosome. This is followed by rapid disassociation of the microfilaments, and fusion of the granules through these "barren" regions, suggesting that the actin microfilaments serve as a barrier which additionally regulates granule release⁶³. It was also shown that localized actin polymerization, mediated by Rac-1, participates in the control of the degranulation of primary granules⁶⁴. Therefore, polymerization of the actin network may be involved in active transport of granules, with its disassociation permitting degranulation⁶⁵. Another interesting observation are the data which have demonstrated various associations of actin with granules that were isolated from human PMNs. The highest amount of actin was observed with secretory vesicles, followed by

the tertiary granules, secondary granules, and the primary granules with the least amount of actin. This is again similar to the different VAMP-2 amounts found on different granule subsets, and suggests an actin binding membrane protein on the granules, which participates in regulating the degranulation hierarchy⁶⁶. In the context of microscopy, this association and disassociation of actin/f-actin has been shown to affect the cytoplasm compactness as it is visualized under TEM^{61, 67, 68}.

2.3.3 The Nucleus

2.3.3.1 Nuclear morphology

The segmented, multi-lobed nucleus of the PMN is one of its main morphological characteristics. It is generally thought that this arrangement allows for easier deformation of the nucleus, and therefore enables the PMN to pass through the tissue more easily after leaving the circulation⁶⁹. Chromosomes are dispersed randomly between the lobes, and their arrangement is based on gene density, with gene-poor chromatin located towards the periphery⁷⁰. When viewed under TEM, there is a clear distinction between the dark, electron-dense hetero-chromatin, and the lighter, more lightly packed euchromatin, which is therefore readier for gene transcription. The tightly packed heterochromatin, together with silenced genes, are usually localized at the nuclear periphery, which supports the theory that gene translation might be suppressed in this region, while the nuclear interior is a site of transcription activity⁷¹. Interestingly, this partitioning of the nucleus into active/silenced regions requires actual movement of genes inside the nucleus during cell maturation and activation. Indeed, it has been shown that “tethering” active genes to the nuclear lamina represses their transcription, or at least alters their expression^{72, 73}. It has also been shown that chromatin organization can change upon stimulation of the cell with LPS⁷⁴. In addition, it has been suggested that the placement of heterochromatin at the nuclear lamina contributes to the physical stability of the nucleus⁷⁵. Therefore, more than just a “holding sack” for chromatin, the

nucleus should be considered as an active organelle with morphological features that might explain different cell functions.

2.3.3.2 PMN Extracellular Traps

One of the PMN's most interesting anti-bacterial mechanisms is the release of Neutrophil Extracellular Traps (NETs). NETs are chromatin fibers bound to histones and various granular proteins such as elastase, cathepsin G, MPO, MMP-9, lactoferrin and bactericidal permeability inducing protein⁷⁶. As their name suggests, NETs are released outside of the PMN in order to trap and kill pathogens, and they are also able to destroy bacterial virulence factors⁷⁷. This is usually a part of specialized cell death dubbed "NETosis", although situations in which cells retained their vitality after NET release have been reported⁷⁸. NET release can be induced by numerous factors. These include bacterial⁷⁹, fungal⁸⁰ or viral⁸¹ infections, hydrogen peroxide⁸², antibody and antibody-antigen complex⁸³, bacterial LPS⁸⁴, and activated platelets⁸⁵.

An important aspect of NET release is that it requires the stimulation of the PMN's integrin receptors, and therefore their attachment to a substrate. This probably prevents excessive NET formation in the circulation⁸⁶. After this activation, the cell flattens and attaches firmly to the substrate. The NET signaling cascade starts, and it includes Protein Kinase C (PKC), the RAF-MEK-ERK kinase pathway, and Rac2^{84, 87}. These lead to the activation of the NADPH oxidase enzyme complex, which enables the creation of ROS by MPO. MPO and neutrophil elastase (NE) are released from primary granules into the nucleus, where they degrade the linker histones H1 and process core histones⁸⁸. Another prominent feature is the citrullination of core histones by peptidyl arginine deaminase 4 (PAD4), which is probably essential for the decondensation of the chromatin^{89, 90}. Morphologically, at this point the eu- and heterochromatin lose their segregation, and the nucleoplasm starts to appear homogenous. Eventually, the cells round up

and contract until the cytoplasmic membrane is ruptured and the NETs are expelled extracellularly⁸².

In the context of periodontal disease, NETs have been shown to be abundant in the gingival crevice, where phagocytosis by PMNs is relatively low, and are therefore thought to be the main mechanism in which PMNs manage the subgingival plaque⁹¹. Some bacteria can evade NETs by producing DNase. It has been demonstrated that some orange and red complex bacteria can produce DNase, and therefore it has been suggested that this can explain, at least in part, their increased virulence with respect to the roles they play in the initiation and progression of periodontitis^{92, 93}. Furthermore, while NETs are effective at trapping and killing pathogens, they also have the potential to cause tissue damage. Thus, exaggerated NETosis induced by LPS or genetic predisposition will cause continuous “bystander damage”, and may contribute to the progressive attachment loss seen in periodontitis⁹⁴. Additionally, citrullinated histones in NETs can induce an auto-immune antibody response, as was demonstrated in rheumatoid arthritis⁹⁵.

2.3.4 Phagocytosis

2.3.4.1 Engulfment of Bacteria

Phagocytosis is the main method used by PMNs to kill bacteria. The engulfment of pathogens or cell debris relies mainly on direct interaction via pathogen-associated-molecule-patterns (PAMPs), or indirectly via opsonization - the process in which pathogens are marked for digestion by specific antibodies or complement factors⁹⁶. The PMN makes close contact with the target, its membrane flowing around the surface of the target until it is completely enclosed. The process results in the internalization of the offending factor into a vacuole which is called the phagosome. With bacteria, very little extracellular fluid enters the phagosome and so it is a tight space initially, a discrete environment which is excluded from the surrounding medium, in this

case the cytoplasm⁹⁷. The phagosome itself is harmless to the bacteria, and a maturation process is required for it to become lethal. This initiates with the fusion of PMN granules with the phagosome's membrane, a process which dramatically raises luminal protein concentration, and eventually causes volume expansion by osmotic influx⁹⁸. In contrast to what is seen in macrophages, although the PMN granules themselves are acidic and contain V-ATPases which are incorporated to the phagosome membrane, the initial phagosomal pH is alkaline. This is achieved mainly through the activity of NADPH oxidase, as described below, but after several minutes the luminal pH balances and remains neutral⁹⁹.

2.3.4.2 Mechanisms of Intracellular Bacterial Killing

As described above, the granules contain an array of antimicrobial molecules which are delivered into the phagosome, thus creating an extremely inhospitable environment for the entrapped microbe(s). These added molecules include but are not limited to azurocidin, cathelicidin, serine protease and α -defensins, which disrupt the bacterial cell membrane, lysozyme which destroys peptidoglycan, iron sequestering lactoferrin and lipocalin, and elastase which degrades bacterial virulence factors^{100, 101}. The initial acidity of the phagosome is considered essential for the activity of the serine proteases elastase and Cathepsin G¹⁰². Some periodontal pathogens such as *P. gingivalis*, *T. denticola* and *A. actinomycetemcomitans* show resistance to cathelicidin and phagocytosis, and these features might explain partially why these microbial cells appear to be more virulent as compared to other bacteria insofar as periodontitis is concerned¹⁰³.

The processes described above comprise the so-called oxygen-independent killing mechanism of the PMN, but the cell also possesses an oxygen-dependent mechanism, which is extremely important to its efficient antimicrobial activity. This mechanism creates ROS, in what is referred to as the oxidative burst. The process starts with the assembly of NADPH oxidase, a large

complex of at least 7 different proteins, on the phagosome membrane. Some of the components of NADPH oxidase come from the cytosol, but one essential protein, cytochrome *b558*, originates in the secondary granules¹⁰⁴. Once NADPH oxidase is assembled, it starts the cascade by reducing molecular oxygen into superoxide ions, which are not strong antioxidant themselves but are the starting point of various potential reactions that will create ROS. One of the main reactions is thought to be the dismutation of the superoxide ions into hydrogen peroxide, which is then used by granule-born MPO to create hydroxide radicals and hypochlorous acid, which are very volatile - they efficiently disrupt the bacterial membrane, fragment bacterial proteins and induce DNA damage^{98, 105, 106}.

2.3.4.3 Impaired ROS production

The critical importance of ROS has been well described in patients who harbor mutations in the genes encoding for the NADPH oxidase subunits, collectively known as chronic granulomatous diseases (CGD). These patients do not produce adequate amount of superoxide, and therefore their microbe killing ability is severely impaired, rendering them highly susceptible to infections¹⁰⁷. An interesting aspect of the disease is that catalase-negative bacteria can still be controlled, as they produce their own hydrogen peroxide which can be used by the host as a substrate in the ROS cascade¹⁰⁸. In contrast, individuals with MPO deficiency usually do not display significant clinical effects, and appear to have normal immunity. This implies that MPO is not essential for antimicrobial activity, and that other ROS such as superoxide, hydrogen peroxide, hydroperoxyl radical and peroxynitrite can fill the gap¹⁰⁹. Alternatively, these observations can be explained by the lack of distinction between partial and complete MPO deficiency. Indeed, partially MPO deficient individuals show little to no symptoms¹¹⁰. This is similar to what is seen in CGD, where even 1% NADPH oxidase activity dramatically improves prognosis¹¹¹.

Objectives

1. To study the different morphology of blood and oral neutrophils in healthy and chronic periodontitis patients using transmission electron microscopy.
2. To study the function of blood and oral neutrophils in healthy and chronic periodontitis patients using transmission electron microscopy.
3. To use these characteristics to define the pro and para-inflammatory phenotypes of blood and oral neutrophils in health and in periodontal disease.

Hypotheses

1. Oral and blood neutrophils, in health and in Chronic Periodontitis, will show significant morphological differences in terms of total cell size, and nuclear size.
2. Oral and blood neutrophils, in health and in Chronic Periodontitis, will show significant functional differences as seen by changes in granularity, Euchromatin fraction in the nucleus, and number and type of phagosomes.
3. The differences will be most significant in Chronic Periodontitis patients, creating 3 different phenotypes – blood, oral health, and oral Chronic Periodontitis.

Chapter 3: Materials and Methods

3.1 Participants and Clinical Assessments

Participants were recruited from Toronto General Hospital's Nephrology Center and University of Toronto's Graduate Periodontology Clinic. The study was approved by the University of Toronto's Research Ethics Board (REB #30044 & 29410) as well as the University Health Network Research Ethics Board (no. 13-6896-AE). Prior to inclusion, a signed informed consent was obtained from all participants. Patients were excluded based on the following exclusion criteria: 1) severe systemic conditions, 2) pregnancy, 3) antibiotic or surgical periodontal therapy within the last 6 months, 4) patients with dental implants, 4) complete edentulism and 5) other active oral disease including caries, endodontic lesions and mucocutaneous diseases. Following a complete dental examination, a total of six patients with generalized severe CP¹¹² were included in the study. Five patients with a healthy periodontium were also recruited as controls. Clinical parameters including bleeding on probing (BOP), probing depth (PD), recession, and clinical attachment level (CAL) were measured at six sites on each tooth. The degree of inflammation was determined by percentage of sites that displayed bleeding upon probing. A site was determined to have active disease upon the concurrent finding of bleeding on probing as well as having a probing depth greater than or equal to 5mm. Lastly, a site was said to have had a history of disease if clinical attachment levels were measured to be greater than or equal to 5mm.

3.2 Electron Microscopy

Blood and oral samples were obtained as previously described^{113, 114}. Briefly, participants were asked to rinse with 5mL 0.9% isotonic sodium chloride solution (Baxter, Toronto, ON) for 30 seconds before any instrumentation to avoid initial bleeding that might interfere with the results.

Participants were then asked to expectorate the rinse sample into a sterile 50-mL falcon tube. Blood samples were drawn into a vacutainer containing 0.1 volumes of sodium citrate as anticoagulant. Erythrocytes were eliminated by isotonic lysis. Oral samples were pelleted at 690 g and resuspended in 10 mL of cold phosphate-buffered saline, and epithelial cells were removed by filtration as previously described.¹¹⁴ Cell sorting based on CD18 expression was performed to confirm >98% purity of oral PMN populations. The cells were then pelleted and processed following a modified technique by Gelderblom¹¹⁵. In brief, the cells were fixed with Karnovsky's style fixative (3.2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1M Sorensen's phosphate buffer, pH 6.8 - 7.0) for 1 hour. Fresh fixative was replaced and samples were left over night at 4C. After removal of the fixative the pellets were washed with Sorensen's phosphate buffer (a mixture of 0.1 M monobasic and 0.1 M dibasic sodium phosphate salts in a ratio to give the required pH) and then post-fixed with 1% osmium tetroxide for 1 hour. Following another wash the pellets were dehydrated using a graded series of ethanol/distilled water at 30%, 50%, 70%, 95% and 100% ethanol. The pellets were then washed with the transitional solvent propylene oxide. Infiltration was performed using Epon Araldite resin, prepared as described previously by Coulter¹¹⁶, using a graded series of Epon Araldite (E/A) and propylene oxide (PO) at 1:1 E/A and PO for half an hour, 3:1 E/A and PO for 2 hours, and 100% E/A overnight. Another infiltration using 100% E/A was performed the next day for another 2 hours. The samples were then placed in polyethylene BEEM capsules with fresh E/A, and placed in an oven for polymerization for 48 hours at 60° C. After complete polymerization, the solid resin blocks containing the samples were sectioned on a Reichert Ultracut E microtome at 60 to 90 nm thickness and collected on 200 mesh copper grids. The sections were counter stained using saturated uranyl acetate for 15-20 minutes, rinsed in distilled water, followed by Reynold's lead citrate for 15-20 minutes and rinsed again in distilled water. The sections were examined

and photographed in a Hitachi H7000 transmission electron microscope at an accelerating voltage of 75Kv. At least 113 images were taken for each of the four groups: blood PMN from controls (bCTL), blood PMNs from CP patients (bCP), oral PMNs from controls (oCTL), and oral PMNs from CP patients (oCP). Image analysis was performed using ImageJ software. Total area, nucleus and cytoplasm area were measured for individual cells. The absolute number of granules in each cell was counted, and granularity was defined as the ratio of number of granules/cytoplasm area. Euchromatin fraction was calculated as the ratio of euchromatin area/total nuclear size. Grey values for cytoplasm grey value were measured from a sample clear cytoplasm area in each cell. The grey value was assigned by the imaging software, ranging from 0-255 where “0” indicates a completely black area, and “255” completely white. Therefore, a higher grey value indicates a lighter shade of the cytoplasm. For oral PMNs, the number of phagosomes in the cytoplasm was counted, while also distinguishing between early and late phagosomes. A phagosome was labeled “early” if it contained at least one intact bacteria. Phagosomes which contained only bacterial fragments or debris were labeled “late”. In addition, whole tissue samples were obtained from 3 patients with CP who had undergone periodontal surgery as part of their treatment plan thus providing so-called ‘gingival discards’ from open flap surgery. All treated sites had PDs $\geq$ 6mm with BOP. The sections were kept in Karnovsky’s fixative, cut to pellet size using #15 scalpels, and prepared for TEM viewing as described above.

3.3 Bacterial stimulation

In order to examine the effect of bacterial stimulation on PMNs, blood samples from healthy individuals were incubated with *Streptococcus oralis* (sk39) and examined under TEM. Bacteria were grown overnight in THYE broth, and diluted to 5×10^7 cells in 200 μ l phosphate buffered saline (PBS). This volume was added to 100 μ l containing 5×10^6 isolated PMNs, and incubated in

37°C for 20 minutes. The samples were then prepared for TEM viewing in the same manner described above.

3.4 Statistical analysis

One-way analysis of variance was performed with a post hoc Bonferroni's test for pairwise comparisons. $P \leq 0.05$ was considered statistically significant. Statistical analysis was performed with GraphPad Prism software.

Chapter 4: Results

4.1 Clinical description of periodontal disease in the patient cohort

On average, patients with CP had 3.3 fewer teeth than healthy controls (24.7 ± 4.7 vs 28 ± 1.7 , $p = 0.05$). Bleeding on probing occurred at 65 percent of sites in CP patients compared to 8.6 percent of sites in healthy controls ($p < 0.001$). In patients with disease, 23 percent of all sites were deemed to be undergoing “active inflammation-mediated” periodontal tissue breakdown, while active disease was virtually absent (0.1 %) in healthy controls ($p < 0.001$). Patients with CP had an increased oral inflammatory load as demonstrated by a five-fold increase in oral PMN counts compared to healthy controls.

4.2 General Cell Morphology

Dramatic morphological changes were observed between the blood and oral PMNs, regardless of the oral health state of the subject. Similarly, these changes were observed in the blood PMNs that were incubated with bacteria. Significant changes were also observed between the oral control and oral CP groups, albeit to a lesser extent. There were no significant differences in the blood PMNs between health and disease.

We found that blood PMN cytoplasm is more electrodense, with a higher average grey value compared to the oral PMN and bacteria stimulated groups. Furthermore, CP oral PMNs had a significantly lighter cytoplasm compared to control oral PMNs, which was similar to the cytoplasm of the bacteria stimulated PMNs (Fig. 1, bCTL 111.6 ± 1.90 , bCP 116.7 ± 2.07 , oCTL 136.2 ± 2.12 , oCP 148.0 ± 1.63 , bBCT 157.5 ± 2.42 , $p < 0.001$). Euchromatin fraction of the nucleus was similar in blood PMNs, whereas it was higher in the oral control PMNs and highest in the oral CP PMNs. Bacteria stimulated PMNs had a euchromatin fraction similar to the oral CP

PMNs. (fig. 2, bCTL 24.8%±1.1, bCP 27.12%±0.8, oCTL 33.9%±1.3, oCP 38.7%±1.3, bBCT 37.9%±1.5, $p<0.0001$). The number of granules per area of cytoplasm was significantly lower in oral PMNs compared to blood PMNs, with the number of granules in oral PMNs from CP patients being significantly lower compared to control oral PMNs. In comparison, bacteria stimulated PMNs were less granular than the blood PMNs, but had more granules than the oral cells (fig. 3, bCTL 2.43±0.12 granules/ μm^2 , bCP 2.44±0.04 granules/ μm^2 oCTL 1.415±0.10 granules/ μm^2 oCP 1.094±0.06 granules/ μm^2 bBCT 2.07±0.07 granules/ μm^2 , $p<0.0001$). CP PMNs had significantly more phagosomes compared to control PMNs. Bacteria stimulated PMNs had more phagosomes than control PMNs and less than the CP cells, but no significant difference could be detected (fig. 4, oCTL 1.00±0.17, oCP 1.975±0.23, bBCT 1.56±0.28, $p<0.0001$). Comparing early phagosomes within the 3 groups showed a similar relationship (fig. 4 oCTL 0.24±0.07, oCP 1.00±0.16, bBCT 0.72±0.16, $p<0.0001$). No significant difference in average cell area or nuclear area was found between any of the groups (average cell area bCTL 39.76±1.028 μm^2 , bCP 40.14±38.66 μm^2 , oCTL 42.69±1.37 μm^2 , oCP 39.81±1.21 μm^2 , bBCT 44.61±1.48 μm^2 , $p=0.06$, nuclear area data not shown).

4.3 Morphological Characteristics of Cells within Tissue

PMNs in the gingival blood vessels (fig. 5 A and B) had morphological appearances similar to PMNs from blood (fig. 1 A). They maintained their round and smooth shape, as well as the dark and dense cytoplasm, which is filled with granules. Euchromatin fraction in the nucleus was still low compared to the extravasated PMNs. PMNs that migrated into the connective tissue had a lighter cytoplasmic density, with fewer granules and higher euchromatin fraction compared to PMNs in the circulation (Fig. 5C). Finally, in the epithelium, the cells demonstrated features similar to those isolated from saliva, including low density cytoplasm with relatively few

granules. A unique nuclear manifestation was observed in gingival tissue PMNs characterized by an increased euchromatin/heterochromatin ratio, with heterochromatin that was localized in a highly focused manner at the periphery of the nucleus, in conjunction with the nuclear envelope (fig 5 D,E).

Chapter 5: Discussion

We have performed a survey of tissue and blood PMN phenotypes in health compared to CP using high resolution TEM. Three distinct PMN phenotypes were observed based on analysis of granularity, cytoplasmic density, euchromatin/heterochromatin ratio and phagocytic activity, corresponding to naïve (bCTL, bCP), para-inflammatory (oCTL) and pro-inflammatory (oCP) states (fig. 6A, 6B, 6C). Incubating naïve blood cells with *S. oralis* caused morphological changes similar to those seen in the activated oral PMNs (fig. 6D).

5.1 Blood Vs Oral PMNs

The most dramatic morphological changes were observed between the oral and blood PMN groups, regardless of health status. It appears that in circulation, PMNs maintain a naïve phenotype. Their recruitment usually starts by changes induced in stimulated endothelium, which causes upregulation of adhesion molecules on their surface. Compatible upregulation of appropriate ligands in free circulating PMNs then causes them to tether to the vessel surface¹¹⁷. The PMNs then roll and adhere to the endothelium, and finally extravasates into the tissue, starting migration induced by chemotactic attractants¹¹⁸. This journey from the circulation into the oral cavity is complex, highly regulated, and involves the expression of numerous genes and proteins¹¹⁹. Additionally, migration between the epithelial and connective tissue cells require physical adjustment of the cell and nucleus¹⁰. Finally, the recruited oral PMN needs to manage the constant bacterial presence in the oral cavity, using its antibacterial arsenal¹²⁰. Therefore, even in “health”, the oral PMN has very different function and activity from the blood PMN. Thus, significant morphological changes are expected in granularity, the nucleus, and the cytoplasm, all of which participate in PMN activation as described below.

Very similar and robust morphological changes were observed in blood PMNs which were incubated with bacteria. Therefore, it appears that in vitro, bacterial factors are sufficient to induce the changes observed in extravasated cells.

Interestingly, no differences were observed between the blood PMNs in the health and CP groups. The host response is recognized as an important factor in CP, and within it is the function of PMNs. Different individuals show different susceptibility to CP, and impaired or altered PMN response has been suggested as a partial explanation to this variability. However, the evidence for this is not definitive for CP¹²¹. Alternatively, exacerbation of systemic disease by CP has been established in the context of diabetes mellitus¹²², and to a much lesser extent in cardiovascular disease¹²³ and adverse pregnancy outcomes¹²⁴, suggesting a pathway that might include altered PMN activity. However, when we examined systemically healthy individuals and did not observe any differences in the blood PMNs between the control and study groups, these findings would not seem to support this concept.

5.2 Oral Health vs Oral CP

In the oral PMN groups, significant changes in morphological features were observed when comparing oral PMNs from the control and CP groups. This is consistent with previous studies that have shown that oral health and CP PMNs present two different phenotypes, presenting different CD marker profile and gene expression^{8, 114}. Our findings suggest that even without clinical symptoms of inflammation and once extravasated para-inflammatory PMNs appear to be in a state of nearly-full activation.

5.3 Cytoplasm Density

One consistent observation pertained to the significant changes in the electron-density in the cytoplasm that was observed in the different groups. Compared to the dark, dense cytoplasm of the naïve PMNs, the oral PMNs have a much lighter, electron-lucent cytoplasm. This is accentuated further in the pro-inflammatory cells. It could be seen that similar cytoplasmic changes could be induced by incubating naïve blood cells with *S. oralis*. *S. oralis* is a gram positive, non-motile facultative anaerobe, and a commensal early colonizer of the oral cavity. As such, it is generally associated with periodontally healthy sites, and therefore it is interesting to see that exposing this species to naïve PMNs reproduced a similar response to that observed in para-inflammation. Here we see that even bacteria that is considered commensal can trigger the innate immunal response, which can explain why plaque that is left undisturbed leads to an inflammatory process – gingivitis – even in the absence of true “pathogens”. Whether this response will progress to periodontitis depends on other factors, ranging from microbial changes to host response. That the observed response was of lesser extent can be attributable to the fact that this incubation was done *in vitro*. In the oral cavity, the dental biofilm is a complex bacterial community, rather than one specific species.

It has been previously demonstrated that PMNs stimulated by phorbol 12-Myristate 13-acetate (PMA) produce a change in cytoplasm density as part of their activation, and similar observations have been noted in human lymphocytes^{125, 126}. This may be due to the rapid degranulation observed in the para- and pro-inflammatory cells. Furthermore, stimulated PMNs have been shown to have altered cytoplasm density due to enhanced polymerization of cytoplasmic proteins, such as monomeric actin converting to F-actin, or the translocation of cytosolic components such as protein kinase C to the cell membrane¹²⁷⁻¹²⁹. This is relevant to the

PMNs ability to migrate through the connective tissue and epithelium which necessitate cytoskeleton adaptation, as we observed in the PMNs in whole tissue sections. All of these point to structural and functional changes that are consistent with cell activation.

5.4 Euchromatin Fraction in the Nucleus

Euchromatin is the decondensed form of DNA. In contrast to the condensed heterochromatin, euchromatic DNA is more exposed and ready for gene transcription. Therefore, a more active cell with increased gene expression and protein synthesis can be characterized by the presence of a higher euchromatin/heterochromatin ratio. It has been demonstrated that in CP, there are alterations in gene-expression within oral PMNs¹¹³. This is consistent with the increased fraction of euchromatin that was observed in the para- and pro-inflammatory cells, compared to naïve blood PMNs. The increased euchromatin content in pro-inflammatory relative to para-inflammatory PMNs is also consistent with higher activation levels of the pro-inflammatory cells.

5.5 Granularity

Secretion of proteolytic enzymes associated with PMN granules is an important antimicrobial mechanism, but it also has cytotoxic capabilities that may affect the host tissues⁴¹. Severe deficiency in degranulation activity has been associated with aggressive forms of periodontitis¹³⁰. However, beyond their direct antimicrobial activity, granule contents also play a major role in the activation of the PMN, as seen by the upregulation of integrins and chemotactic receptors through fusion of the secretory vesicles with the cell membrane, and the release of collagen degrading gelatinase through the release of tertiary granules. The degranulation starts with the activation of the PMN, long before contact with its targets and the actual microbial killing.

As expected, pro-inflammatory PMNs that we observed in the oral cavity of CP patients, displayed decreased granularity. This is consistent with elevated inflammatory activity of these PMNs, which is a hallmark of periodontal disease^{113, 114, 131}. Interestingly, para-inflammatory PMNs, that we observed in the healthy oral cavity, also showed reduced granularity compared to the naïve cells, but to a lesser extent than the pro-inflammatory PMNs. Indeed, PMNs in the healthy oral cavity carry out an important housekeeping function that prevents the development of gingival disease¹⁷, therefore some level of PMN activation is expected. Incubating naïve blood PMNs with *S. oralis* induced degranulation as well, albeit to a lesser extent than the degranulation of the para-inflammatory cells *in vivo*. One of the limitations of quantifying granules using TEM is that it is virtually impossible to differentiate between the 4 different granule subtypes without specific biomarkers – it would be interesting to see if the para- and pro-inflammatory phenotypes differ not only in quantity, but also in the quality of the granule content being released to the oral cavity¹¹.

5.6 Phagocytosis

Comparison of the total number of phagocytes shows a significant increase among the pro-inflammatory cells, as well as the bacteria stimulated cells. Some genetic disorders with impaired phagocytosis are strongly associated with severe aggressive periodontal disease. In the past, this led to the theory that this PMN dysfunction is one of the main causes of aggressive periodontitis, or periodontal disease in general. However, more recent theories suggest that *hyper* activity of PMNs could be responsible for periodontal tissue damage, especially in CP^{29, 41}. This model aligns with our observation of increased phagocytosis in the oral PMNs of CP patients compared to healthy controls. Furthermore, more early phagosomes were observed in the pro-inflammatory PMNs. These phagosomes show engulfed bacteria that has not yet been digested. It was

previously demonstrated that periodontal pathogens like *P. gingivalis* can inhibit phagocytosis by using microbial factors such as gingipains,¹³² therefore it is interesting that the health associated *S. oralis*, in contrast, induced phagocytosis in the naïve cells.

5.7 Summary

We have characterized the morphology of the para-inflammatory phenotype displayed by oral PMNs in health, and compared it to the naïve morphology of the blood PMN and the pro-inflammatory expression in oral PMNs in CP. This morphology can be partially induced by incubation of PMNs *in vitro* with bacteria. As bacterial challenge is constant in the oral cavity, para-inflammation is a normal physiologic process carried out by PMNs in order to contain the commensal biofilm without exposing the host to the harmful effect of a fully active PMN response. However, this is a delicate balance, and dysbiosis of microbial populations in the oral cavity can cause a shift towards a pro-inflammatory environment which includes full activation of PMNs⁸, which promotes progression to periodontal tissue loss and periodontal disease. Furthermore, inherent hyperactivity of the oral PMNs could predispose to periodontal disease, and could partially explain the different susceptibility to it between different individuals and populations. Directly visualizing cells and their components via TEM enhances our understanding of PMN activation state differences in oral health and disease obtained from molecular studies.

Figure Legend

Figure 1 - Oral PMNs have lighter cytoplasm density, a difference which is more pronounced in CP, and reproduced with bacterial stimulation. A – Blood PMN from a CP patient. B – Oral PMN during phagocytosis from the same patient. Notice lighter density of cytoplasm. C – Blood PMN after incubation with *S. oralis*. Notice similarity in cytoplasm density to (B). D – Cytoplasm density of the cells as represented by mean grey value. A higher value represents lighter cytoplasm. *, # represent a significant statistical difference ($p < 0.001$). The statistical test used was one-way ANOVA with post hoc Bonferroni's test. Control Blood $n=113$, CP blood $n=117$, Control Oral $n=125$, CP oral $n=153$, Bacteria $n=62$. All images are magX8000 and 75Kv.

Figure 2 – CP oral PMNs have a higher fraction of euchromatin than all other groups. A – CP blood PMN. B – oral PMN from the same patient. Notice high amount of euchromatin in the nucleus. C - Blood PMN after incubation with *S. oralis*. D – Euchromatin percentage of the nucleus. *, # represent a significant statistical difference ($p < 0.0001$). The statistical test used was one-way ANOVA with post hoc Bonferroni's test. Control Blood $n=113$, CP blood $n=117$, Control Oral $n=125$, CP oral $n=153$, Bacteria $n=62$. All images are magX8000 and 75Kv.

Figure 3 - The number of granules in oral and bacteria stimulated PMNs is lower than in blood, and even lower in oral PMNs from CP patients. A – Blood PMN from CP patient. B – Oral PMN from same patient. C - Blood PMN after incubation with *S. oralis*. Notice lower number of granules in (C), and lowest in (B). D – the ratio of granules and cytoplasm area. *, #, & represent a significant statistical difference ($p < 0.0001$). The statistical test used was one-way ANOVA with post hoc Bonferroni's test. Control Blood $n=113$, CP blood $n=117$, Control Oral $n=125$, CP oral $n=153$, Bacteria $n=62$. All images are magX8000 and 75Kv.

Figure 4 – CP Oral and bacteria stimulated PMNs have more phagosomes, and more late phagosomes in the cytoplasm. A – Oral PMN from CP patient during phagocytosis.

MagX9000, 75kV. B – Magnification of marked area at B mag X30000, 75kV. On the right – early phagosome, containing morphologically intact bacteria. On the left – late phagosome containing only debris, presumably bacterial fragments. C – number of phagosomes in cytoplasm. D – number of early phagosomes in cytoplasm. * represent a significant statistical difference ($p < 0.0001$). The statistical test used was one-way ANOVA with post hoc Bonferroni's test. Control Oral $n=125$, CP oral $n=153$, Bacteria $n=62$.

Figure 5 – PMNs traveling from gingival blood vessel of a CP patient into the epithelium. A

– PMN (center of square) surrounded by different leukocytes (black arrows) and erythrocytes (white arrows). MagX2500 75kV B – Magnification of squared area at A. MagX8000 75kV. Compare with figure 1a. C– PMN moving through gingival connective tissue. MagX7000, 75kV. D– PMNs moving from gingival connective tissue (CT) into the epithelium (E). MagX2500. E – PMNs moving inside the epithelium. MagX5000, 75kV. F – PMNs moving inside the epithelium. MagX6000, 75kV.

Figure 6 – morphological differences between the four PMN groups. A - naïve control

blood PMN. B - Oral PMN from a healthy control. C - Oral PMN from a CP patient. Note decreasing granularity, decreasing cytoplasm density, increasing euchromatin fraction in the nucleus, and increasing number of phagosomes. D - naïve blood PMNs after incubation with S. oralis. a, b, c, d – Graphical summary of size, cytoplasm mean grey value, granularity, euchromatin fraction and number of phagosomes for each group. Control Blood $n=113$, CP blood $n=117$, Control Oral $n=125$, CP oral $n=153$, Bacteria $n=62$. All images are magX8000 and 75Kv.

Figures

Figure 1. Oral PMNs have lighter cytoplasm density, a difference which is more pronounced in CP, and reproduced with bacterial stimulation.

Figure 2. CP oral PMNs have a higher fraction of euchromatin than all other groups.

Figure 3. The number of granules in oral and bacteria stimulated PMNs is lower than in blood, and even lower in oral PMNs from CP patients.

Figure 4. CP Oral and bacteria stimulated PMNs have more phagosomes, and more late phagosomes in the cytoplasm.

Figure 5. PMNs traveling from gingival blood vessel of a CP patient into the epithelium.

Figure 6. morphological differences between the four PMN groups.

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