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Polymorphonuclear Leukocyte and Dendritic Cell Interactions in the Presence of Infection by Periodontal Pathogen *Porphyromonas gingivalis*

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Abstract of the Dissertation

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Polymorphonuclear leukocytes (PMNs) act as first-responders to microbial infections in the human gingiva, primarily responsible for bacterial clearance. Dendritic cells (DCs) serve as the sentinels of the immune system and bridge the innate and adaptive immune responses by processing and presenting antigens to guide the T cell response. Recent research has shown that physical associations between these two cell types may result in mutually beneficial outcomes for both PMNs and DCs. Because the periodontal red complex pathogen *Porphyromonas gingivalis* through its minor fimbriae interacts with the C-type lectin receptor DC-SIGN on the surface of DCs, the same receptor that ligates to the Mac-1 integrin and CEACAM molecules on the surface of PMNs, it was hypothesized that this pathogen would interfere in DC-PMN conjugation. Infection of DCs with *P. gingivalis* did in fact thwart DC-PMN conjugation, however initial infection of PMNs with *P. gingivalis* caused an upregulation in surface integrins that leads to increased DC-PMN association. DC-PMN interaction in the face of *P. gingivalis* also lead to partially matured, highly

migratory DCs that were capable of reversing apoptosis of infected PMNs and receiving bacteria by transfer from PMNs. Furthermore, the ability of PMNs to kill *P. gingivalis* while DCs appear to harbor viable bacteria suggests a complicated pathogen-host response interaction that may underlie the chronic nature of periodontal disease. Understanding the nature of the interactions between DCs and PMNs and their effects on *P. gingivalis* infection as well as the consequences for the innate immune response in the face of infection with this pathogen may lead to the detailed insight required for more effective, lasting means for treating chronic periodontitis.

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List of Abbreviations

DC- Dendritic cell

mDC- myeloid dendritic cell

pDC- plasmacytoid dendritic cell

MoDC- monocyte-derived dendritic cell

PMN- Polymorphonuclear leukocyte

DC-SIGN, also DCS- dendritic cell specific ICAM-3 non-grabbing integrin

PRR- pattern recognition receptor

PAMP- pathogen-associated molecular pattern

CP- Chronic periodontitis

CFSE- Carboxyfluorescein succinimidyl ester

Mfa-1- *P. gingivalis* 67-kDa minor fimbriae

FimA- *P. gingivalis* 41-kDa major fimbriae

Porphyromonas gingivalis isogenic mutant strains:

WT- Mfa-1^{+}FimA^{+}

DPG-3- Mfa-1^{+}FimA^{-}

MFI- Mfa-1^{-}FimA^{+}

MFB- Mfa-1^{-}FimA

LPS- lipopolysaccharide

TNFα- tumor necrosis factor alpha

CD- cluster of differentiation

CEACAM- Carcinoembryonic antigen-related cell adhesion molecule

CCR- chemokine receptor

CXCR- C-X-C chemokine receptor

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Chapter 1
Introduction

I. An Overview:

Chronic periodontitis (CP) is a destructive disease characterized by inflammation of the dentition supporting soft tissues of the oral cavity, often resulting in tooth loss and absorption of the underlying alveolar bone [1, 2]. In the United States alone, some form of periodontitis affects as much as 47% percent of the adult population [3] and has been linked to systemic diseases such as diabetes and atherosclerosis (ATH) [4, 5]. In a 2012 statement by the American Heart Association, periodontal disease was named as an independent risk factor for the development of atherosclerotic vascular disease [6]. Many etiologic factors contribute to the pathogenesis of this disease, including formation of a complex, subgingival microbial biofilm, genetic predisposition, and host behaviors [7]. While the disease is initiated by biofilm formation and presence of bacterial products such as endotoxin, progression and recurrence of CP seems to be a product of the host's unharnessed inflammatory immune response [8].

The first line of defense in the inflammatory response involves polymorphonuclear leukocytes (PMNs), the primary cell responsible for the killing and clearing of pathogens [9]. Recently, PMNs have been found to have complex interactions with other immune cell types, especially dendritic cells [10-12]. Elucidating these interactions may lead to a better understanding of how the innate immune system contributes to chronic inflammatory processes. Furthermore, it is unknown how bacterial infections are cleared by these cell complexes or how pathogens may interfere with the formation of these PMN-DC conjugates [17]. Recently, investigators have began to determine how PMN-DC interactions affect the immune response to certain pathogens [10, 11] and how each cell type affects the other [11]. Conversely, it's possible that pathogens are evolving to escape

immune cell collaboration [12] and in some cases, commandeering one or more types of immune cells to improve their dissemination [13, 14]. The following data and discussion will show a case in which a bacterial pathogen eludes an immune response in part by inhibiting interactions between neutrophils and dendritic cells. It proposed herein that P. gingivalis, a causative agent of chronic periodontitis, persists by interfering with DC-PMN conjugate formation, perhaps allowing this pathogen to become disseminated to other tissues, contributing to other systemic diseases such as CAD [15, 16].

II. Dendritic cells:

A. General characteristics and function

Dendritic cells (DCs), found in some form in every body tissue, are often considered the sentinels of the immune system, as, upon uptake of antigen, these cells acquire maturation and migration markers that allow them to home to lymph nodes and antigen present to T lymphocytes [17-19]. In this way, DCs are the most potent antigen-presenting immune cells, driving the host response to infection and bridging the innate and adaptive arms of the immune system [20]. While the immunological niche of the dendritic cells in various tissue types is well-defined, the specific function and mechanisms of action of blood dendritic cells are less understood [21]. Because DCs so heavily influence the antibody and memory response of the immune system, elucidating the mechanisms of these populations of circulating dendritic cells are thought to be the key to developing important vaccines that induce T-cell immunity against both viral and bacterial antigens as well as cancer cells [22, 23]. The last 15 years have profoundly progressed the study of DCs in terms of their maturation

expression profile, migratory mechanisms and patterns, antigen processing ability and immunometabolism [24].

B. Types of DCs

Fully differentiated immature conventional or classic DCs (cDCs), as they are called in the most recent literature, have been observed in the circulatory networks of the body, including the blood and afferent lymphatic vessels, and in lymphoid organs. Classic DCs display a high degree of plasticity within organs and lymphoid tissues and their effector functions are often regulated by tissue microenvironment [25]. However, two broad groups of cDCs can be defined on the basis of their location: DCs located in non lymphoid tissues, such as the skin, mucosa and internal organs (tissue-resident cDCs), and DCs found in lymphoid organs, blood and afferent lymphatic vessels (lymphoid tissue-resident cDCs, or blood cDCs) [26, 27]. Even in health and under homeostatic conditions, cDCs continually scan the environment for the presence of incoming pathogens [27].

Classically, both lymphoid and tissue cDCs are further classified into four types based on their lineage, expression profile, and they way in which they are differentiated from a common myeoloid progenitor cell [21]. Conventional or myeloid DCs (cDCs or mDCs) are found in both lymphoid and non lymphoid tissue as well as circulating in human blood [28]. A subset of cDCs in mice distinguished as being $CD8^+$ and $CD103^+$, and the human equivalent $CD141^+$ mDC population has been well characterized both phenotypically and genotypically [29]. This population is best known for its potent class I MHC presentation of exogenous antigens to

stimulate cytotoxic $CD8^+$ cells and the production of immunoregulatory cytokines IL-10 and IL-17 in the spleen, lymph nodes, lungs and skin in both mice and humans [30-32]. A method of tolerizing these cells in the context of donor-recipient interactions is thought to be a promising avenue to prevent rejection of transplanted tissues [33].

A second subtype of classical or mDCs are $CD1c^+$ mDCs, characterized by their expression of surface CD1c, CD11b and SIRP-α, make up the major component of circulating DCs in the human blood stream [34]. While their murine counterpart $CD11b^+$ cDCs have been studied more indepth, $CD1c^+$ mDCs have been investigated in terms of their ability to prime $CD4^+$ T helper cells and to secrete immunoregulatory cytokines such as IL-17 in the lung in response to fungal infection [35], and IL-8, -10, -23 in skin [36]. Very little is known about the unique purpose and mechanism of function of this particular DC subset except how it differs from its murine counterpart [28].

$CD14^+$ DCs are unique to humans and are found in both lymphoid and non-lymphoid tissues. While sharing traits of both circulating monocytes and macrophages, they have proven more potent antigen presenters with higher levels of expression of MHC-II and CD11c [37]. Uniquely, they appear not to express the end stage migratory marker CCR7, making them ill equipped for homing to lymph nodes [38, 39]. Their expression of TLRs 1-9 however and release of interleukins -1B, IL-6, IL-8, and IL-10 as well as the observation that they can prime T regulatory cells suggests that are immunoregulate their immediate environment [40]. In addition, they also induce helper T-cells as well as antibody secreting B-cells [41].

Plasmacytoid DCs (pDCs) make up approximately 0.2-0.8% of all circulating peripheral blood mononuclear cells (PBMCs) in both human blood and lymphoid tissue and are the most secretory of all DC types [42]. Because they were originally found to be derived from a common lymphoid progenitor under experimental conditions [43], pDCs were first considered a distinct lineage from myeloid type DCs [44]. However, due to the control of common transcription factors and their capacity, albeit restricted, to prime T cells, pDCs were re-categorized as DCs [45]. Their most unique attribute is, despite their small population, they have a potent functional capability to secrete copious amounts of type I interferons when infected by viruses [46].

Langerhans cells (LCs) are type of DC that express high levels of surface CD1a and low levels of CD11c, and are found primarily in epithelium [47]. LCs seem to promote a regulatory T cell response in an MHC-II context [48], as well as a potent T_{reg} response when challenged by certain fungal pathogens [48]. Therefore, the pathogen type and strength of the stimulus dictate the type of immune response, as is the case with mDCs.

Monocyte derived DCs (moDCs) sometimes referred to as inflammatory DCs (iDCs) are a less defined population of DCs found in vivo in all body tissues depending on the phenotypic markers used and the nature of the condition being examined [49]. As their name suggests, they originate as monocytes that are encouraged to further differentiate to DCs, expressing high levels of MHC-II, CD11c, CD11b, CD1a, and CD206. While CD14 and CD1c expression is usually downregulated compared to monocyte predecessors, moDCs may continue to express these markers at low to

moderate levels. Functionally, they prime T_{reg} and Th_1 immunity [50].

It is possible that this population in vivo is simply comprised of activated tissue-resident $CD1c^+$ DCs that have become migratory and circulate [51]. However these cells are often produced in vitro as a proxy for mDCs in order to ensure a controlled immature phenotype and higher yields than if pan DCs were isolated from human blood or tissue [49]. Immature moDCs are produced in vitro with the addition of growth factors granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 to human peripheral blood mononuclear cells (PBMCs), and with the further addition of transforming grown factor-β (TGF-β), can be engineered to take on a LC-type expression profile [52].

Other cell types have been observed promoting monocytes to differentiate into moDCs/iDCs, such as natural killer cells [53, 54], while some cell types may prohibit differentiation such as human gingival fibroblasts [55]. Similarly, pathogens may have a direct impact on the ability of monocytes to differentiate to moDCs. Hepatitis C has recently been shown to prevent the autophagic process of monocytes thereby prohibiting their differentiation to a dendritic cell phenotype [56]. Epstein Barr virus causes the differentiation of semi-mature dendritic cells from cord blood monocytes which may then have a regulatory, immunosuppressive effect [57]. Some pathogens change the nature of differentiating DCs such as the parasite *Leshmania major*, which seems to create immature DCs with low levels of marker CD1a and mature DCs that create low levels of the cytokine IL-12p70, a profile that seems to increase DC survival [58]. The oral pathogen *Porphyromonas gingivalis*, a Gram negative bacteria has also been shown to cause monocyte differentiation to DCs

possibly by means of its surface fimbriae interacting with monocyte/DC surface receptor DC-SIGN [59]. Because DCs define the nature of an immune response, pathogens that have evolved to control the process of their differentiation may be able to evade various facets of the immune system.

C. Phagocytic capacity and Antigen Processing

DCs are a diverse group of related, haematopoietic cell types that, through various receptor mechanisms, are capable of recognizing pathogens or damaged and dying host cells [60]. On their surfaces, they express various pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [61, 62], nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [63], retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors, which are able to bind molecular motifs that are characteristic of particular pathogens or that are associated with cellular damage [64]. Such receptors allow for the pathogen recognition, uptake, degradation and antigen processing for presentation of both endogenous and exogenous antigens to T lymphocytes by way of both major histocompatibility complex I (MHC I) and MHC II, respectively [65]. DCs also respond, become activated and subsequently propelled toward maturation by encountering such exogenous stimuli such as LPS [66, 67] and bacterial DNA [68], and endogenous stimuli including but not limited to T cell-derived ligands such as CD40L [69] and pro-inflammatory signals such as cytokines [70] released from other immune cells, cells undergoing apoptosis [71] or healthy cells such as endothelial cells, fibroblasts, and epithelial cells [72].

Most DCs present in circulation are resting or in an immature state and are equipped for antigen capture. One such PRR present during the phagocytic stage of the DC life cycle is the lectin receptor DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin). DC-SIGN is adept at recognizing pathogen associated molecular patterns (PAMPs) as well as self damage associated molecular patterns (DAMPs) in dying host cells, especially glycan-bound proteins with carbohydrate chains containing repeating mannose moieties [73, 74]. DC-SIGN is specifically up-regulated on the surface of DCs in their circulating immature state and acts as a DC invasin for many viral and bacterial pathogens including gp-120 on the surface of HIV virons [75], hepatitis C virons [76], glycosylation motifs on tumor cells [77], and surface antigens on Mycobacterium tuberculosis [78]. In some cases, as in hepatitis C, DC-SIGN and other lectin receptors are manipulated by pathogens to evade lysosomal degradation in DCs and persist intracellularly [79]. Ligation of DC-SIGN in general has also been shown to promote a unique pattern of differentiation of monocytes into moDCs, when blockage of the lectin receptor with two different antibodies under traditional IL-4 exposure created DCs that when treated with maturation signals, resulted in low CD80 and CD83 expression and maintained high levels of monocyte marker CD14 [80]. The effect of DC-SIGN ligation on DC maturation is still debatable in the literature, with some studies showing that bacterial pathogens interact with the receptor to promote maturation [81] and other showing that antibody ligation of DC-SIGN prohibits maturation [82]. The role of DC-SIGN and its intracellular signaling pathways remain ill-defined, but it is anticipated

that depending on the stimulus, various pathways may be triggered, determining a distinct immune response as effected by this receptor [80].

The exact mode of trafficking and processing of antigen within DCs from endosome to cytosol and back toward the cell surface for presentation is still under investigation [83]. In contrast to macrophages, which are similar cells with more efficient pathogen killing mechanisms, cDCs process pathogens much less efficiently and appear to have mechanisms to control lysosomal degradation in order to preserve peptide antigen for easier recognition by T cells [84]. The NADPH oxidase NOX2 is recruited to the DC's early phagosomes and mediates the sustained production of low levels of reactive oxygen species, and maintaining a less degradative, less cytotoxic alkalinization of the phagosomal lumen [85].

D. DC Maturation

Once antigen has been encountered and engulfed, cDCs begin the process of maturation that generally results in the downregulation of PRRs and upregulation of antigen presenting and co-stimulatory molecules on the cell surface [86]. Maturation results from the stimulation of transcription factor NF-Kβ [87]. While other transcription factors are also important, the maturation profile is largely determined by posttranscriptional and posttranslational modifications making final expression of surface markers paramount in understanding the overall function of the mature DC [88].

In classic DC maturation, there is loss of endocytic/phagocytic receptors such as DC-SIGN and an increase in surface expression of co-stimulatory molecules CD40,

CD80 and CD86, respective ligation and co-stimulation of these molecules being required for DCs to perform their antigen presenting function [89]. When T-cells engage CD80, CD86 and CD40 along with antigen presented in an MHC class I or II context, they are encouraged to further differentiate into $CD4^+$ helper cells [90]. With maturation, there is also an accompanying change in morphology to reflect a rounder shape with loss of the podosomes for which the immature phase gives DCs their name [91]. CD83, a transmemebrane molecule believed to play an immunoregulatory function, is upregulated [92, 93]. Recently, CD83 on mature DCs was shown to play a specific role in increase T lymphocyte proliferation by increase intracellular calcium release in T cells [94]. Membrane bound CD83, which along with MHC class I molecules, delivers a significant signal specifically supporting the expansion of newly DC-primed naïve $CD8^+$ T cells [95]. Class II MHC group human leukocyte antigens (HLA), specifically HLA-DR, the predominant subset in humans, is responsible for the presentation of oligopeptide ligands of exogenous antigens, and is also up-regulated as part of the DC maturation surface profile [96].

In addition to changes in the surface expression profile, maturation of DCs also results in a unique cytokine profile, both contributing to drive the type of resulting immune response [97, 98]. DCs, depending on the nature of the antigen they have acquired, the PRRs activated by the pathogen, and signals from other immune cells such as natural killer cells, can trigger one of three $CD4^+$ T-helper (Th) cell reaction types: type 1 (Th_1), type 2 (Th_2), or regulatory T cell response (Th_{reg}) [99]. Type 1 signals result from DC uptake of intracellular pathogens such as viruses and most bacteria [62]. This subset primes a pro-inflammatory pathway by the release of

interleukins IL-12, IL-23, IL-27, IL-18, IFN-a and B, and ICAM-1 [100].

In contrast, type 2 responses are induced by DCs in response to infection with large extracellular parasites, such as helminths and also mediate allergic inflammatory diseases such as asthma, allergic rhinitis and atopic dermatitis [101]. While once thought to be only DC driven, it is now known that the type 2 response results from crosstalk between DCs and other innate immune cell types such as eosinophils, basophils and mast cells [102]. The third type of helper T-cell response encouraged by DCs are Th17 cells which secrete the cytokines IL-17A, IL-17F, IL-22, GM-CSF, and TNF-α [55]. Th17 cells are key inflammatory T cells that play a pathogenic role in many autoimmune diseases [103]. T_{reg} cells also play a major protective role in immunity to pathogens, in particular against fungi and bacteria, through the recruitment and activation of neutrophils to the site of infection [104].

Recently, DCs deemed to be semi-mature have become of interest as an intermediate phenotype between the immature and mature states has been discovered [105]. While steady-state immature DCs constantly sample the host environment and promote tolerance by presenting "self" antigens with the purpose of quelling immune responses [106, 107], mature dendritic cells have been challenged by a "non-self "entity that causes a potent DC-initiated immune response [108]. Then, DCs coordinate their proteolytic processes in the cytosol such as proteasomes, lysosomes by regulation of lysosomal hydrolases, and the endoplasmic reticulum to breakdown "non-self" proteins and present resulting antigens via MHC class I and II molecules to T and B cells [105]. Roughly defined by either low expression of costimulatory molecules CD80, CD83 and CD86 or by low or lacking cytokine

secretion (ie. IL-12, IL-10, IL-6, TNF,IL-1B), the semi-mature phenotype of DCs and the ability to manipulate it by altering the cells' environment may be the link between the immune system's promotion of self-tolerance and pathogen or cancer cell destruction/clearance [109, 110]. For instance, it has been shown that priming monocytes with IFN-α instead of the classically used growth factor IL-4 creates semi-mature DCs that is more migratory, pro-inflammatory and cytotoxic to tumor cells [111]. Also, cancer cells have been shown to be successful in maintaining the semi-mature state in which DCs display aberrant motility, reduced antigen-presenting abilities, and decreased endocytosis, all characteristics that would benefit the persistence of tumors [112]. Similarly, pathogens such as Epstein Barr virus can cause the differentiation of semi-mature DCs from monocytes, weakening the DC response against the virus by allowing for high expression of costimulatory markers and decreased secretion of cytokines [57]. Harnessing the power of this intermediate type of DC and understanding its immuno-regulatory functions may contribute to the success of priming DCs for vaccination [22, 107]. Additionally, knowing how pathogens and other cell types contribute to determine the exact phenotype of these semi-mature DCs, may enable an explanation for the persistence of chronic diseases and infections as well as cancer [108].

E. DC Migration Capabilities

Both immature and mature DCs are capable of migrating, although they respond to different sets of chemokines and express different migration markers [113]. Immature DCs are highly motile as they seek out pathogens, and respond to

chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-3α and RANTES/CCL5. CCR2, a receptor primarily expressed on monocytes, but retained on immature moDCs, responds to CXCL8 to initiate chemotaxis of immature moDCs and monocytes to sites of inflammation [114]. CCR6, a Gi protein coupled receptor responds to MIP-3α (CCL20), triggering a signaling cascade that induces actin assembly and chemotaxis [115]. CCR6 is usually upregulated in the presence of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS), and after stimulation with LPS, TNF-a or CD40 ligand, MIP-3α (CCL20) becomes the main chemoattractant for DCs [116]. However, with complete maturation, IL-4 and TNF- α mediate the down regulation of CCR6 [117] [118]. High levels of inflammatory chemokines like MIP-1α are produced short-term after DC stimulation with LPS, TNF-α or CD40L, while CCL5 is first downregulated and then upregulated [119]. CXCR4, a receptor upregulated on mature DCs responds to ligand CXCL-12 in a process that controls both chemotaxis and promotes DC survival [120].

CCR7 is a Gi protein coupled receptor which triggers integrin-mediated adhesion and induction of PKB and MAPK activation [121]. It is a marker for late DC maturation and plays a role in the migration of T cells and DCs to secondary lymphoid tissue by responding to its ligands CCL19 and CCL21 [122, 123]. In summary, when DCs mature, CCR1, CCR2 and CCR5 [124] as well as CCR6 are downregulated by endogenous cytokines as well as exogenous signals such as LPS, while CCR7 and CXCR4 expression levels are drastically upregulated [125, 126].

Once DCs have encountered and engulfed pathogens, their maturation process

also involves an alteration in surface molecule expression that makes them able to migrate through the lymphatic system to lymph nodes for antigen presentation to T cells [127]. Various chemokines and other ligands synergize to promote a repertoire that differs between and uniquely dictates the migration of CD14+ monocytes, immature and mature DCs [128]. Immature dendritic cells are drawn to inflammatory cytokines, matrix metalloproteinases (MMPs), as well as exogenous pathogen products such as LPS [129]. With maturation, inflammatory chemokine receptors are downregulated and CCR7 and CXCR4 chemokine receptors are upregulated [130]. However, even in conditions of health and homeostasis, DC migration takes place to lymph nodes in a process that might be responsible for promoting peripheral tolerance [131]. In mice, deletion of CCR7 receptor lead to autoimmune type reactions in multiple organ systems [132].

F. Role in Pathogen Dissemination

It has been shown that DCs can respond with common and predictable pathogen clearance and antigen presentation or in ways that are specific to a particular pathogen. In an evolutionary arms race, it seems that some viral and bacterial pathogens have been able to influence the DC response and even hijack the cells themselves to benefit their own persistence and infectivity [133]. This protection and mobilization of microbes by DCs may be a mechanism by which hosts build immune tolerance to both commensal and harmful organisms [134].

Blood myeloid DCs are not efficient killers of microrganisms [135], and thus some pathogens have been shown to reside in DC phagosomes such as mycobacteria,

which just as it resides latent in macrophages, does the same after being taken up in a DC-SIGN-mediated manner [136, 137]. This allows the bacteria to travel throughout the host until a time when conditions improve and the infection can be reactivated, as well as influencing the DC response by inhibiting complete maturation and inducing DC secretion of the anti-inflammatory cytokine IL-10 [132].

Similarly, *Chlamydophila pneumoniae* has also been shown to be taken up by moDCs, in which they survive well enough to be viably recovered from the cells after lysis and culturing of the moDC lysate [138]. Hepatitis C virus evades degradation in DC lysosomes [79], and is known to alter the ability of DCs to reach traditional maturation especially in individuals with chronic infection, suggesting that the virus persists by using moDCs as a extra-hepatic reservoir and is able to re-emerge to cause acute inflammation intermittently [139, 140]. Perhaps the most thoroughly studied pathogen to employ DC dissemination is HIV, which also gains access to DCs by the DC-SIGN receptor via its glycosylated envelope protein gp120 and replicates within DCs prolonging infection and providing a mechanism for access of the virus to T cells [141, 142].

Recently, the oral bacterial pathogen *P. gingivalis* was also shown to be viable and recoverable after uptake by DCs also in a DC-SIGN mediated manner [15, 251]. This, as well as the fact that patients with periodontitis exhibit increased populations of blood mDCs [15], and that *P. gingivalis* is able to promote differentiation of monocytes to moDCs [14], points to circulating DCs as a niche for this causative agent of the disease leading to the survival and dissemintation of *P. gingivalis.* and offers an

explanation as to why *P. gingivalis* is found at distant sites of inflammation such as atheromatous plaques in patients with coronary artery disease (CAD) [15, 131].

II. Polymorphonuclear Leukocytes:

A. Function and Antimicrobial Mechanisms

Polymorphonuclear leukocytes (PMNs), or neutrophils, are the most prevalent type of innate immune cell, and are characterized as the short-lived first-responders at the onset of localized infection and/or inflammation [143]. Until recently, PMNs were thought to be fully differentiated cells that indiscriminately phagocytized and metabolized pathogens with intra- and extra-cellular enzymatic excretions such as NADPH [144]. However, they are increasingly being looked at for their dynamic phenotype, one that has been shown to differ depending on the pathogen challenge and to even reflect a DC-like receptor pattern [143, 144]. Recent studies have shown that PMNs can restrain their pro-inflammatory capacity and become equipped for antigen presentation, while still maintaining classic neutrophil traits such as their migration patterns and ability to degrade antigens [145, 146]. This newly uncovered expansion in function allows them to be looked at as both the beginning and ending of the innate arm of the immune response [144].

PMNs must be constantly controlled to strike a balance between their protective and potentially destructive effects, including their production, trafficking and clearance [147]. The granulocyte colony-stimulating factor (G-CSF) regulates both granulopoiesis and PMN release from bone marrow as well as playing a role in their survival [148]. Extravasation of PMNs occurs by a series of low- and high-

affinity adhesions and integrins between the cells, such as CD11a/CD18 LFA-1 and CD11b/CD18 Mac-1 integrins and the vascular endothelium [149]. The site for PMN extravasation is determined by tissue-derived cytokines which can change the morphology of cell surface integrins to promote migration toward and through the endothelium [150]. Once transmigration through the endothelium has transpired, PMNs are attracted to sites of inflamed or infected tissue by cytokines or chemoattractants derived from infecting bacteria such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) [151]. Upon encountering pathogen, PMNs are highly phagocytic and very efficient killers of bacteria, capable of emulsifying microbes [143].

B. Apoptosis and NETosis in PMNs

PMNs constantly undergo cell death both to maintain homeostasis as in aseptic regions of inflammation [152] and in direct response to pathogen contact [153]. Apoptosis, a complex and controlled form of cell death, is even automatically defaulted to once these cells mature and enter into the blood stream unless a survival signal is detected. Although the exact pro-apoptotic signal that initiates this process is unclear, it is know that encountering certain molecules such as TNF-α, pathogens such as viruses and bacteria, and even detection of the cells own ROS as produced by NADPH oxidase enzymes can accelerate apoptosis in neutrophils.

Bacterial species shown to elicit apoptosis in neutrophils include *Escherichia coli, Burkholderia cepacia,* and *Mycobacterium tuberculosis* [154]. When neutrophils engulf microbes they undergo phagocytosis-induced apoptosis, beginning with the

activation of inducer caspases 3 and 8 [154, 155]. Similar to the mechanism of TNF-α induced apoptosis, after phagocytosis, ROS are produced by enzyme NADPH oxidase. Inhibition of these products has been shown to prohibit apoptosis, suggesting they play a direct role, upstream of caspases, in the induction of this form of cell death [154]. ROS levels and therefore the survival or death of PMNs are directly determined by the amount of bacterial challenge compared to the number of cells [154]. ROS also reinforces its direct effects by indirectly potentiating apoptosis through the release of cathepsin B from lysosomes, an enzyme that also mediates caspase activation [156]. The importance of ROS in phagocytosis-driven apoptosis is further patients with CDG, who due to their low levels of NADPH oxidase produce too little ROS, resulting in decreased levels of apoptosis in the face of bacterial challenges [157].

PMNs have recently been reported to possess an antigen trapping mechanism such as neutrophil extracellular traps or NETs, a fibrous network of DNA and granular antimicrobial proteins [158]. The result of a novel type of cell death with traits of both apoptosis and necrosis and yet distinctly different in that it resembles a control necrosis, termed NETosis [159, 160], NETs play a role in the immune defense against pathogens such as *Helicobacter pylori* [161] and *Escherichia coli*, [162] contribute to the inflammatory aspect of the formation of blood clots as in deep vein thrombosis [163], and even have implications in the host defense against cancer cells [164]. Through charged interactions between NETs and the surface components of pathogens, neutrophils are able to contain and eliminate even large pathogens that cannot be phagocytized, and makes these cells potent killers even after their deaths

[165, 166]. The downside of this process is that it is a violent one, resulting in the extracellular release of proteases and oxidative neutrophil constituents that may contribute to inflammatory injury of surrounding otherwise healthy tissues [167, 168], and clearing a path for the onslaught of other inflammatory cells [169]. The newest classification of NETosis keeps this process as an outlier as it remains to be seen if it is its own unique mechanism of cell death or a subset or resultant process of another type of cell death, and it is possible for NETs to form without the cell undergoing NETosis [170].

C. Mac-1 Integrin

Integrins are expressed as αβ heterodimeric transmembrane glycoproteins present in cell membranes that function to mediate adhesion between cells, cells and extracellular matrix and cells and pathogens. In vertebrates, there are identified 18 α chains and 8 β chains that can be combined into 24 pairs. The β2 (CD18) integrin is expressed on several cell types around in the body, leukocytes amongst them and when combined with a unique a chain, form the most important integrins on the surface of leukocytes [171]. Mac-1 when activated is comprised of CD11b/CD18, is primarily expressed on myeloid lineage cells such as neutrophils, monocytes and macrophages, and serves in neutrophils primarily by binding to endothelial ICAM-1 and mediating adhesion and transendothelial migration [172]. Activated Mac-1 is described as the conformational change that occurs between the two separate components CD11b and CD18 when neutrophils become activated, resulting in a higher affinity and avidity for its ligands [173]. The β2 integrin also plays an essential

role in the phagocytosis of complement opsonized particles by neutrophils and other phagocytic leukocytes, and ligation of this integrin has been shown to play a role in neutrophil apoptosis [157]. In patients with leukocyte adhesion deficiency type I (LAD1) a heritable defect of the CD18 gene, neutrophil recruitment to infection sites is impaired as is the ability of neutrophils to clear pathogens, resulting in recurrent bacterial infections and in some cases chronic disease [174, 175].

D. CEACAM Family

Members of the carcinoembryonic antigen family includes the CEACAM subgroup, widely expressed on the surface of many cell types including epithelial, endothelial, and hematopoietic cells including neutrophils, T-cells and natural killer cells [176]. They initiate signaling cascades which have many effects depending on the tissue including tumor promotion or suppression [177], angiogenesis, [178] and regulation of the cell cycle and adhesion [179, 180]. In neutrophils, members of this family that are also part of the immunoglobulin gene superfamily cause activation of these cells. CD66 monoclonal antibodies react with members of the CEACAM family, specifically CD66a corresponds to CEACAM-1 and CD66b corresponds to CEACAM-8, both of which contribute the ability of neutrophils to adhere to the endothelium in an interdependent manner, a step that precedes extravasation of neutrophils to sites of infection [181].

CD66 mAbs have been reported to activate neutrophils via their corresponding CEACAM receptor as determined by the cells increased ability to adhere to human umbilical vein endothelial cells (HUVECs) [182]. CD66 mAb binding

to the neutrophil surface triggers a fleeting activation signal that relies on extracellular calcium that in turn regulates the adhesive activity of Mac-1 (CD11/CD18) [182, 183]. It has also been reported that CEACAMs are capable of regulating the exact function of CD11/CD18 as well as inducing an increase in intracytoplasmic calcium and an oxidative burst in neutrophils, [184, 185, 186] therefore initiating adhesion to promote the start of the inflammatory reaction and the release of neutrophil contents to promote the continuation of inflammation [187]. CEACAM-1 has also been shown to regulate neutrophil apoptosis, specifically delaying both spontaneous and fas ligand-induced apoptosis, and therefore may play a role in the resolution of inflammation as well [188].

Members of the CEACAM families may also bind certain types of bacteria, including *Neisseria meningiditis* and *Neisseria gonorrhea* in humans, resulting in neutrophil activation, and overall modulation of neutrophil activity. [189, 190] Specifically, bacterial invasion of some cell types through the CEACAM receptors occurs through bacterial adhesins or fimbriae as is the case with *Escherichia coli* invading epithelial cells of the intestinal lining and *N. meningitides* penetrating mucosal lining [191-193]. CEACAMs have also been observed to transmit signals regulating proliferation of epithelial cells and on the surface of DCs, stimulating the proliferation of T lymphocytes [179, 194]. Most recently, one study has shown that soluble CEACAM-8 released in response to a bacterial stimuli will bind to CEACAM-1 on the surface of granulocytes causing a reduction of the pro-inflammatory immune responses in neutrophil-driven bacterial infection of the respiratory tract [195].

IV. Interactions of Dendritic Cells and PMNs:

A. Overview

Recently, DCs have been found to conjugate directly with PMNs by the C-type lectin surface receptor DC-SIGN on dendritic cells and the integrin Mac-1 (CD18/CD11b) and CEACAM-1 (CD66a) on PMNs [59, 196-200]. DC-PMN interaction was first assumed to be mutually beneficial for both cell types, extending the life and microbicidal functions of PMNs and enhancing the activation and maturation of DCs [59, 196, 197]. However, recent evidence has shown that depletion of DCs may encourage the bacterial killing capacity of PMNs by corresponding increases in reactive oxygen species production [11]. The immune consequences of these interactions for each cell type are therefore controversial. Moreover, there is very little current understanding of how these DC-PMN interactions and consequent effects are thwarted or manipulated by opportunistic pathogens.

The last twenty years have revealed multiple ways in which DCs and PMNs interact, but most of them can be broken down into one of three types of communication: 1) neutrophil recruitment of dendritic cells to a site of acute or chronic infection by means of soluble secreted chemical mediators, 2) direct physical contact via the surface receptor-integrin interface on each cell type, 3) a phased interaction in which effete PMNs that have cleared infection are subsequently cleared by DCs.

B. DC recruitment and maturation by PMN-generated factors

Because PMNs are typically the first immune cell type to arrive to sites of acute infection, and remain in constant flux during chronic infections, it stands to reason that APCs such as DCs and macrophages are recruited by activated and effete PMN-derived soluble attractants [201]. Defensins, small antimicrobial peptides characterized by three or four disulfide bonds have been identified as molecules secreted by PMNs that result in recruitment of certain subsets of immature DCs and naïve T cells [202]. Defensins in general are classified into α and β groups, the α-defensins, specifically defensins 1,2,3, and 4 are expressed by neutrophils and are thus termed human neutrophil peptides (HNP) [203, 204]. Alpha defensins released locally by degranulating neutrophils, recruit iDCs to inflammatory sites where upon contact with antigen, become antigen presenting, express high levels of CCR7 and are thus able to migrate toward secondary lymphoid tissues [125, 205].

Alpha defensins have also been shown to have an effect on that maturation of human moDCs, causing the upregulation of CD83, CD86 and HLA-DR expression at low doses, while high doses of defensins of immature moDCs cause down-regulation of CD86 and HLA-DR when exposed to maturation factors [206, 207]. Differentiation of monocytes to moDCs is also affected in the presence of α-defensins 1-3, modulating levels of CD86, HLA-DR, CD14, and CD11c in the last 2-3 days of maturation as compared to a maturation cocktail of growth factors [206, 208]. This suggests that soluble factors from PMNs are able to influence the migration, quality and quantity of DC populations at a site of inflammation.

In addition to defensins, PMNs and other innate immune cells secrete cathelicidins, a family of mammalian antimicrobial proteins of which more than 40

have been identified [209]. PMNs release cathelicidins in response to PAMPs or inflammation due to tissue injury [210]. One such cathelicidin, LL-37 activates neutrophils and inhibits their apoptosis, as well as blocking the activation of human DCs, decreasing the expression of CCR7, CD80, CD83, CD86 and HLA-DR when challenged with TLR antagonists [211]. It has also been demonstrated that soluble factors known as serine proteases generated by PMNs decreased the allostimulatory effects of immature DCs, but not mature DCs, resulting in slowed DC-induced proliferation of T cells compared to untreated DCs [212].

A less studied paradigm is the attraction of PMNs as influenced by soluble factors that are products of DCs. It has been shown that supernatants collected from TNF-α treated moDCs increased surface expression of CD11b and CD18, subparts of of the Mac-1 integrin, on the surface of PMNs which would theoretically increase their ability to migrate across endothelium [198, 213]. Evidence shows however that only DCs subjected to inflammatory conditions successfully recruit PMNs, mostly likely by the secretion of cytokine CXCL8 [213].

C. DC-PMN Conjugation

Recent studies have shown that during infection, these cell types accumulate at the site of inflammation, physically interacting to allow mutual scanning of one another's surfaces for receptor-ligand interactions [59, 197, 214]. This association was proposed by van Gisbergen et al. [195-197] to occur by ligation of DC-SIGN on DCs and Mac-1 and CD66a (CEACAM1) on PMNs. The same study also showed evidence that it was specifically the carbohydrate moieties on the PMN integrins that bound DC-SIGN, specifically the nonsialylated Lewis-x glycosylation pattern

expressed by PMNs [200]. Typically, DC-SIGN ligates with high mannose moieties [215]. Van Gisbergen et al. demonstrated that moDC-PMN conjugation led to increased levels of CD83, a marker for DC maturation and activation, when moDCs were co-cultured only with PMNs activated by FMLP, TNF-α, or LPS [195-197]. However this study is unclear how CD83 expression was determined to only be upregulated on moDCs, as more recent publications have since shown proof of PMNs upregulating CD83 as well as acquiring some antigen presenting capabilities similar to DCs [216, 217].

Since the claims by van Gisbergen et al., various other studies have attempted to elucidate the nature and effects of the DC-PMN association especially in the face of infection with various pathogens. The role of DC-SIGN has been further implicated in the recognition of PMNs undergoing apoptosis as a result of infection with *Mycobacterium tuberculosis,* suggesting that the scanning ability of DCs is refined enough to determine which PMNs are dying and tailor their response accordingly [218]. In mice, PMNs exposed in vitro to *Aspergillus* germ tubes cause activation of cDCs isolated from lung tissue also by a contact dependent mechanism involving DC-SIGN ligation and resulting in increased DC expression of CD86 and CD40 in a dose dependent manner [219]. This study also revealed, unlike the findings of van Gisbergen et al., that some DC maturation was induced even by non stimulated PMNs [219]. Neutrophils stimulated with LPS and IL-2 or IL-15 and IL-18 in the presence of a particular subset of human DCs were shown, not by DC-SIGN-Mac-1 interactions but rather by CD18 on PMNs and ICAM-1 on DCs, to better stimulate a NK cell response than stimulated PMNs alone. The DC-PMN co-culture potentiated the

release of IFN-γ by NK cells propagating the response to the original PMN-encountered stimulus, and creating a feedback loop by which the IFN-γ release then causes IL-12p70 release by DCs [220]. Contact between PMNs and DCs has been observed in vivo as well in the intestinal mucosa of patients with Crohn's disease, suggesting that association of these cell types may be a common occurance in inflammatory processes [195]. Recently, one study showed the PMNs stimulated to produce neutrophil extraceullar traps (NETs) caused enhanced DC maturation, but no upregulation of terminal migration markers CXCR4 and CCR7, suggesting another mechanism by which PMNs are able to induce an exact DC phenotype [221].

Because of the complexity of living systems, some studies have shown intriguing ways in which each of these cells types may affect the other in vivo, but have been unable to define whether these effects were mediated by contact dependent mechanisms. Recent evidence has shown that the presence of DCs may regulate PMN homeostasis, in part by the release of cytokines that recruit and reduce apoptosis in PMN populations [222]. Furthermore, lack of DCs as shown in transgenic mice with CD11c diphtheria toxin receptor transgenic mice causes a release of PMNs from bone marrow, increasing over all bacterial clearance [223]. Therefore presence of DCs may inhibit the ability of PMNs to clear infection, perhaps acting as reservoirs for intracellular survival of pathogens and allowing low grade chronic infections to persist [15, 224, 225]. The exact mechanism by which DCs influence the PMN response has yet to be determined and may or may not be contact-dependent versus being mediated by soluble factors [226].

V. Chronic Periodontitis

A. Overview:

Adult chronic periodontitis (CP) mixed infection in which the host response to bacterial biofilms causes the creation of pro-inflammatory mediators that in some individuals lead to the degradation of connective tissue and bone resulting in irreversible loss of attachment apparatus [227, 228]. As the number one cause of tooth loss, it has a very high prevalence, the moderate form seen in more than 50% of the adult population, and the progressive form seen in 5-15% [229]. The disease results from a combination of microbial infection [230] and an unharnessed host response, but patient genetics as well as concurrent diseases such as atherosclerosis, diabetes, and COPD have been implicated as risk factors [231].

While over 500 bacterial species have been identified as inhabiting the oral cavity, there is only a handful which are considered contributors to the pathogenic process, however there is no minimum amount or specific bacterial species that is necessary for the onset the periodontal disease [230]. Genetic susceptibility such as various polymorphisms in IL-1 potentiates the disease and the severity of the host immune response is determined largely by the variability in the host response [232]. Inflammatory products such as matrix metalloproteinases (MMPs), receptor activator of nuclear factor-kappaB ligand (RANKL), their respective tissue inhibitors of metalloproteinases (TIMPs) and the loss or inundation of their natural inhibitors leads to tissue breakdown [233]. However, red complex pathogen *Porphyromonas gingivalis*, a key organism in not only CP, but has been implicated as causing inflammation in distant sites from the gingiva, and is a proteolytic bacteria capable of

causing tissue damage in its own right [234, 235]. Data to be presented here and in various other studies points to immunomodulation due to *P. gingivalis* that may lead to an unharnessed, aberrant host response [236-238].

B. Pathogenic and immune evasion mechanisms of *Porphyromonas gingivalis*

Porphyromonas gingivalis is an anaerobic, Gram negative bacterium which plays a causative role in adult chronic periodontitis (CP) [239-241]. Various markers and structures on the surface of *P. gingivalis* [242], as well as its ability to proliferate within epithelial cells in vitro [243] help this bacterium to benefit from interactions with its host's cells and increase its pathogenicity. Its LPS alone is a stimulator of proinflammatory responses, and in animal models has been shown to cause bone resorption [244]. It is however a weaker cytokine stimulator when compared with the LPS of enteropathogenic species [245]. While the lipid A component of LPS is usually a potent activator of TLR4 responses, that of *P. gingivalis* activates TLR2 primarily, sometimes acting to antagonize TLR4 to reduce the immune response [246]. In this way and others, *P. gingivalis* is adept at remaining just below the radar of the immune system, causing enough immunoactivation to induce inflammation, but being manipulative enough to evade killing [247].

The fimbriae of *P. gingivalis*, small filament-like surface protrusions, offer the pathogen a means for attachment to extracellular matrix, eukaryotic cells of the host, and each other. They allow *P. gingivalis* to become a component of biofilms, and assist in attaching and invading endothelial cells and leukocytes [248]. Both the 41kDa FimA-encoded major [249] and 67kDa mfa1-enocded minor fimbriae [250, 251] of *P. gingivalis* have been shown to interact with receptors on the surface of various host cells. Generally

speaking, the major fimbriae (FimA) functions to cause invasion of cells, and the minor (mfa-1) incite an inflammatory response [248, 252].

Our lab has shown that the minor fimbriae are capable of targeting DC-SIGN for direct uptake by DCs, where *P. gingivalis* accumulates, essentially intact in DC-SIGN-rich intracellular compartments [253]. This ligation of DC-SIGN provides a mechanism by which *P. gingivalis* suppresses the host immune response and capitalizes on the motility of DCs, using DCs for systemic dissemination [224]. DC-SIGN ligation with the minor fimbriae may also lead to differentiation of monocytes to immature DCs, perhaps explaining the increased numbers of myeloid DCs in circulation in patient's with CP [15].

In addition to the invasin DC-SIGN, *P. gingivalis* has also been shown to enter immune cells, namely macrophages, via the β2 integrin portion of the heterodimer Mac-1 in a fimbriae-dependent manner [254-256]. Mac-1 usually offers a safe point of entry for the intracellular persistence of *P. gingivalis* [254, 256]. While it has been shown that *P. gingivalis* interaction with Mac-1 occurs when the integrin is expressed on PMNs, and that this integrin is upregulated in response to the pathogen [257], the exact way this interaction tailors the immune response is only now being uncovered. While components of *P. gingivalis* have been shown to impair PMN function as well, suggesting another cell type evaded by this pathogen [258-260], PMNs have still been shown in vivo to be the premier clearers of periodontal pathogens [261, 262]. The advent of NETs has caused PMN-*P. gingivalis* interactions to be reexamined, with a recent publication citing certain strains as primarily causing a NET response rather than phagocytosis of the pathogen [263]. The story of PMNs interaction with *P. gingivalis* and their contribution to the host response that perpetuates the disease is therefore constantly evolving.

VI. Summary

The present study demonstrates that PMNs, while increasing expression of surface Mac-1 and other integrins upon activation by fimbriated strains of *P. gingivalis*, seem to be effective killers of this bacteria. Conversely, we show that monocyte-derived dendritic cells (moDCs) harbor intracellular *P. gingivalis* which can be recovered with antibiotic protection up to 24 hours post-infection. The following data will also demonstrate the ability of *P. gingivalis* to modulate DC-PMN ligation either by blocking cell interactions by competitively inhibiting DC-SIGN via its fimbriae, or enhancing DC-PMN interactions by influencing the integrin profile and eliciting fimbriae-mediated, caspase-induced apoptosis in PMNs. Similar to other studies of antigen transfer from PMNs to DCs [214], we will also demonstrate the capacity of *P. gingivalis* to invade uninfected DCs after previous uptake by PMNs.

Increased contact mediated interactions with PMNs and antigen transfer from PMNs can alter DC phenotype, and therefore DC antigen presentation and migration capabilities [218, 219, 264]. The level of PMN activation was found to be relevant only in terms of certain DC maturation and migration markers. However, activation and elicitation of apoptosis by fimbriated strains of *P. gingivalis* are required for PMN driven attraction of primary isolated blood DCs. In addition, we demonstrate that PMNs are able to cause differentiation of monocytes to a mature DC phenotype at a much faster rate than IL-4 and GM-CSF, growth factors traditionally used in vitro to drive differentiation. The following data imply the important role of physical cell interactions between PMN and DCs for modulating innate, and possibly adaptive, immunity. This is an example of a unique pathogenic strategy to interfere with the formation of DC-PMN conjugates and the subsequent host response.

Chapter 2
Aims of Research

1. **To determine the fate of *Porphymonas gingivalis* after uptake by DCs and PMNs**

We hypothesized that because DCs are poor microbicidal cells, due to their proclivity to preserve antigens for presentation, that *P. gingivalis* would remain viable and be recoverable after uptake by moDCs with antibiotic protection. *P. gingivalis* would be killed however in infection of PMNs, due to the vital role PMN have been show to have in the clearance of periodontal pathogens and due to the cells' aggressive means of pathogen destruction.

2. **To characterize the necessity of DC-SIGN in DC-PMN conjugation and what effect *Porphymonas gingivalis* will have on the formation of these conjugates**

We hypothesized that DC-SIGN as evidenced by cocultures with Raji DC-SIGN+/- cells and PMNs as well as co-cultures of moDCs and PMN, will be an important factor in the physical association formed between these two cell types. We also anticipate that because the minor fimbriae of *P. gingivalis* has been shown to engage DC-SIGN, this pathogen will prohibit DC-PMN association in vitro by occupying DC-SIGN and encouraging the uptake of this receptor from the cell surface, decreasing its availability.

3. **To observe the effects of contact-dependent interaction on both DCs and PMNs in both the presence and absence of *Porphyromonas gingivalis* infection**

We hypothesized that PMNs, especially LPS/*P. gingivalis* activated PMNs, will promote maturation of moDCs by physical contact of these cell types, resulting in a surface expression profile of increased CD86, CD80, cD83 HLA-DR, CCR7, and CXCR4, and decreased CCR2, DC-SIGN, CCR5, and CCR6. In addition, stimulated/*P. gingivalis* infected PMNs co-cultured with moDCs will exhibit lower levels of apoptosis than PMNs alone. This mutually beneficial association should exist to benefit the host, however if hypothesized effects of Aim 2 hold true, *P. gingivalis* may compromise these positive effects.

Chapter 3
Materials and Experimental Methods

1. Isolation of Human Peripheral Blood PMNs and PBMCs

The Committee on Research Involving Human Subjects Research at Stony Brook University approved all protocols involving human subjects. Informed consent was obtained from all healthy volunteers before commencement of the study. Peripheral human blood was collected from healthy volunteers by venipuncture into spray coated sodium heparin collection tubes (BD). Separation of granulocyte component of whole blood was accomplished as previously described [265]. Briefly, 5mL of whole blood was layered onto Mono-poly Resolving Medium Ficol Hypaque (MP Biomedicals, 091698049) and a layer of Histopaque 1077 (Sigma-Aldrich Labs, 10771). Samples were centrifuged at room temperature for 30 minutes at 500 RCF. Cells from the granulocytic and monocytic bands were washed multiple times and resuspended in HBSS without Ca+2 and Mg+2. Residual red blood cells were lysed for 10 minutes of resuspension of chilled red blood cell lysing buffer by Hybri-Max (Sigmal Aldrich, R7757) on ice. Purity was estimated to be >95%, and viability >95% as assessed by flow cytometry counts gated for forward and side scatter characteristics of granulocytes (fsc-h 3000-5000 and ssc-h 3000-6000) and monocytes (fsc-h 2000-4000 and ssc-h 2000-4000) and by flow cytometry detection of propridium iodide uptake, respectively.

2. Generation of Monocyte-derived Dendritic Cells

MoDCs were generated as described previously. Monocytes were isolated from mononuclear fractions of the peripheral blood of healthy donors and seeded in the presence of growth factors GM-CSF (100mg/ml, PeproTech Inc. Cat # 300-03) and IL-4

(25ng/ml, R&D Systems Cat# 204-IL-010) at a concentration of 1-2 x 105 cells/ml in RPMI 1640 cell media (Cellgro) containing 10% HI-FBS (Lonza) and antibiotic/antimycotic (HyClone) for 5 days with frequent media changes. Flow cytometry was employed to confirm immature DC phenotype ($CD14^{lo}CD83^{-}CD1c^{+}DC\text{-}SIGN^{+}$). The following antibodies were used in four-color immunofluorescence staining for cell surface detection of these markers: mAb CD83 (eBiosciences 11-0839-42), mAb CD14 (eBiosciences 17-0149-42), mAb CD1c (MACs 130-090-507), mAb CD209 (eBiosciences 45-20990-42).

3. Bacterial growth and culture conditions, bacterial labeling, and infection of PMNs

Wild-type (WT) Pg381, expressing both the minor and major fimbriae (Pg maj+/min+), isogenic major fimbriae-deficient mutant DPG3 (Pg min^{+}/maj^{-}), isogenic minor fimbriae-deficient mutant MFI (Pg $min^{-}.maj^{+}$), and the double fimbriae mutant MFB (Pg maj^{-}/min^{-}) were grown and maintained under anaerobic conditions (10% H2, 10% CO_2, and 80% N_2) in a vinyl anaeorbic glove box (Coy Laboratories) at 37°C in Wilkins-Chalgren anaerobe broth (Acumedia) [266]. Erythromycin (5ug/ml) and tetracycline (2ug/ml) were added to strains according to selection criteria of each isogenic mutant[267]. Bacterial suspension were prepare for experiments by washing three times with PBS and resuspending at an OD of 0.11 at 660nm, previously determined to reflect a concentration of 5 x 107 CFU [268]. When appropriate, Pg were stained with CFSE for visualization and detection by flow cytometry, added at a final concentration of 5 uM for 30 minutes at 37°C and subsequently protected from light.

PMNs were infected with strains of Pg at multiplicity of infection (MOI) of either 1 or 10 for 15, 30, or 45 minutes to reflect a realistic bacterial challenge the cells may face in

Pg infected blood or tissue prior to co-cultures with moDCs.

4. Separation of Human Peripheral Blood panDCs

PanDCs were isolated from total PBMCs using Robosep automated negative selection by removal of all cells targeted by tetrameric antibody complexes recognizing CD3, CD9, CD14, CD16, CD19, CD34, CD56, CD66b, glycophorin A and dextran-coated magnetic particles (Stemcell Technologies 19251). Resulting cells were phenotype tested as $CD141^{high}$, $CD123^{high}$, $CD1c^{high}$, $CD14^{lo}$ by four-color flow cytometry analysis.

5. Intracellular Bacterial Survival Assay

Wild type Pg381 was used to infect MoDCs, or PMNs at a multiplicity of infection of 100. Uptake of the bacteria by human cells was confirmed by observing complete internalization of CFSE-stained *P. gingivalis* via epifluorescence microscopy as soon as 60 minutes after inoculation. Cells were then washed twice in PBS and re-suspended in culture medium for continued incubation. At each time point, cells were re-suspended in sterile water on ice for 20 minutes to initiate cell lysis. Remaining bacteria released from within the cells was re-suspended in PBS, and streaked on anaerobic 5% blood agar plates in triplicate under anaerobic conditions (10% H2, 5% CO_2 in nitrogen) at a one in ten dilution. Plates were incubated in anaerobic conditions at 35°C for 14 days after which colonies were numerated and surviving cell forming units (CFU) per mL were determined.

6. Tissue collection, sectioning and immunofluorescence

Oral mucosal tissues from the human gingiva were collected from untreated CP patients using a biopsy technique previously reported [269]. Immediately after collection,

the tissues were rinsed with sterile saline to remove traces of blood and embedded in Tissue Tek OCT compound (Sakura) and snap frozen in liquid nitrogen, and sectioned into 7 μM thick sections using a cryostat (Leica CM1850). For immunofluorescent staining, sections were fixed in acetone for 5 min at −20°C, rehydrated in PBS lacking Ca^{+2} and Mg^{+2} (PBS-), blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS- along with anti-human FcR blocking reagent (Miltenyi Biotec) for 1h, and washed. Sections were incubated for 30 minutes at RT with conjugated primary antibodies diluted in PBS-, and washed before mounting. All sections were mounted with VectaShield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc.). To identify DC-SIGN within gingival tissue sections, RPE-conjugated mouse anti-human CD209 (AbD Serotec) were used.

7. Transmission Electron Microscopy

After fixation of moDC/PMNs, preparation of samples for microscopy were carried out at the Electron Microscopy and Histology Core, Department of Cellular Biology and Anatomy, Georgia Regents University. The cells were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate (NaCac) buffer, pH 7.4, postfixed in 2% osmium tetroxide in 0.1M NaCac, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series and embedded in Epon-Araldite resin. Thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Cells were observed in transmission electron microscope (JEM 1230—JEOL USA Inc.) at 110 kV and imaged with a CCD camera and first light digital camera controller (Gatan Inc.).

8. Apoptosis and Caspase Activation Assays and PMN phenotyping

Freshly isolated PMNs were resuspended at a concentration of 4 x 10^6 cells/ml and infected with WT and mutant strains of *P. gingivalis* at an MOI of 10 for 3 hours [270]. Positive controls were PMNs exposed to TNF-a at a concentration of 10ng/mL of TNF-a for 3 hours, conditions previously reported to cause 75% of cells to undergo apoptosis [271]. Annexin V-FITC and propidium iodide uptake were determined by flow cytometry using cell death detection kits (eBioscience BMS500Fl/300). Annexin V staining was performed according to manufacturer's instructions. Cells were collected after infection, washed in PBS to remove excess bacteria and then washed in annexin-V binding buffer. Next, the FITC-annexin V conjugate was incubated with the cells for 30 minutes after which unbound antibody was washed away. Cells were then analyzed immediately by flow cytometry.

Similarly, isolated PMNs were infected with WT and mutant strains of *P. gingivalis* at an MOI of 10 for 3 hours at 37°C and analyzed for caspase activity using Vybrant FAM FLICA Polycaspase kits (Immunochemistry 9122) according to manufacturer instructions. Briefly, PMNs were washed and resuspended in caspase binding buffer. FLICA probes for caspase-1, -3, -7 and -8 were attached to each respective caspase displaying binding sequences exposed upon activation. Flow cytometry and immunofluorescence microscopy was used to determine the mean fluorescence activity for each experimental setup.

In co-culture assays, fixed immature 6-day old moDCs were added at PMN:moDC ratios of 1:1 to optimize cell-to-cell contact for 3 hours at 37°C and 5% CO_2 and gentle agitation. MoDCs were fixed in order to specifically see the contact dependent effect created by moDCs on dying PMNs rather than the response to secretion of soluble factors and cytokines. PMNs were then analyzed by flow cytometry for markers of apoptosis as explained above.

9. PMN-Raji Cell Co-Cultures

PMNs were challenged with WT and isogenic mutant strains of P. gingivalis at an MOI of 10 for 3 hours of 37°C before they were fixed in chilled 1% glutaraldehyde in 0.1M PBS solution for 20 minutes on ice to achieve crosslinking of membranes and preservation of surface protein expression patterns. Cells were thoroughly washed three times in PBS. PMNs were resuspended in RPMI cell media containing 10% FBS and pulsed with Raji cells.

Raji cell lines were obtained courtesy of D. R. Littman (Skirball Institute of Biomolecular Medicine, New York University, New York, NY) maintained in 10% heat-inactivated FBS (Invitrogen), RPMI 1640 with L-glutamine and NaHCO3 (Sigma-Aldrich) at 37°C in 5% CO_2. Raji cells were previously sampled and stained to verify surface receptor expression with the following antibodies: mouse mAb anti-human CD14-FITC (eBiosciences 17-0149-42), mouse mAb anti-human PE-CD19 (BD Biosciences, 612130), mouse anti-human PerCP-CD209 (BD Biosciences 558263), and anti-CD29-PE (BD Biosciences catalog no. 555443). Raji cells were either DC-SIGN$^-$ or stably transfected to express DC-SIGN. Each type of Raji cell was combined with uninfected/infected and fixed PMNs in a 1:1 ratio for 30 minutes with gentle agitation. Samples were kept on ice during co-culture due to the rapid cycling of DC-SIGN on Raji cells maintained at 37°C. Ratios were set at 1:1 and total cell concentration relatively low (5×10^5 cells/mL) in order to encourage doublet formation and discourage cluster of cells that would disrupt laminar flow. Cells were fixed in 1% glutaraldehyde in 0.1M PBS solution on ice for 20 minutes. Then cells were prepared for analysis by flow cytometry as previously described [272]. In brief, cells were was 3 times in PBS, and resuspended in 5% BSA containing humnan FcR

blocker (MACs 130-059-901) for 30 minutes. Antibodies used to stain cell types were mouse anti-human PE-CD19 (BD Biosciences, 612130) for Raji-DC-sign -/+ cells and mouse anti-human FITC-CD66a/b/d/e (BD Biosciences, 551479). Antibody binding was allowed to continue for 30 minutes at 4°C. Flow cytometry was done with an Acuri C6 Flow Cytometer. Gates for doublets were determined by overlapping doublet peaks on histograms of side scatter characteristics and double positives for both FITC- and PE-conjugated antibodies after color compensation to account for cross-channel bleeding of fluorescence, signifying that both cell types were present together [273] [274].

As with Raji-PMN co-cultures, uninfected/infected fixed and washed PMNs were resuspended in RPMI cell media containing 10% FBS, and pulsed with 6-day old immature moDCs. For conjugation experiments via flow cytometry, co-cultures were at a ratio of 1:1 PMNs:moDCs to discourage clustering which would interfere with laminar flow during flow cytometry data collection and register outside the selected gates. Cells were co-cultured with gentle agitation for 30 minutes at 37°C and 5% CO_2. Antibodies used to stain cell types were mouse anti-human PE-CD1c (BD Biosciences 564900) and mouse anti-human FITC-CD66a/b/d/e (BD Biosciences, 551479). Flow cytometry was performed as described for PMN-Raji cell co-cultures. Again, Gates for doublets were determined by overlapping doublet peaks on histograms of side scatter characteristics and double positives for both FITC- and PE-conjugated antibodies after color compensation to account for cross-channel bleeding of fluorescence, signifying that both cell types were present together (ie. Double positives in the upper right corner of scattergram FITC vs. PE and gated for doublets) [273] [274].

A second set of experiments were conducted with either Raji-DC-SIGN+ cells or moDCs infected first with all strains of Pg at an MOI of 10 for 30 minutes. These cells

were then washed twice, fixed and exposed to isolated PMNs at a ratio of 1:1 PMNs:moDCs/Raji cells with gentle agitation for 30 minutes. Cells were stained as described above and flow cytometry ran and interpreted in the same way.

10. Phenotyping moDCs Exposed to PMNs

PMNs were left untreat, or infected with WT and mutant strains of Pg, or exposed to isolated Pg LPS (10ng/mL) or leukotoxin isolated from *Actinobacillus actinomycetemcomitans* (Aa, LtxA 20ng/mL), for 3 hours at 37°C and in 5% CO_2. Aa leukotoxin was a kind gift from Dr. Scott Kachlany (Rutgers, School of Dental Medicine), and was used as a control for a known substance that causes PMN degranulation [275]. PMNs were fixed in a 1% glutaraldehyde fixative solution on ice for 20 minutes. PMNs washed three times and co-cultured with 6-day old immature moDCs for 24 hours at 37°C and 5% CO_2 and gentle agitation at a PMN:moDC ratio of 5:1 to best emulate comparative en vivo cell concentrations. Cells were collected an moDCs were then fixed with 1% glutaraldehyde solution on ice for 20 minutes. PMN moDCs co-cultures were then resuspended in 5% BSA containing human FcR blocker (MACs 130-059-901) for 30 minutes. Cells were stained with the following antibodies for 30 minutes at 4°C (all antibodies are monoclonal mouse anti-human unless otherwise noted and were added at concentrations recommended by respective manufacturers): PE-DC-SIGN (BD Biosciences, 551265), FITC-CD80 (BD Biosciences, 555683) PerCP-HLA-DR (BD Biosciences, 347364), FITC-CD83 (BD Biosciences, 556910) , APC-CD86 (BD Biosciences 560956), APC-CCR2 (R&D Systems, FAB151C), FITC-CCR5 (BD Biosciences, 555992), PE-CXCR4 (eBiosciences, 12-9999-42), PE-CCR6 (eBiosciences, 12-1969-42) and rat anti-human APC-CCR7 (eBiosciences, 17-1979-42).

11. Immunofluorescence Microscopy

For cellular immunofluorescence, PMNs were infected with PMN-Raji-DC-SIGN -/+ and PMN-moDC co-cultures at a PMN-Raji/moDC ratio of 5:1 were stained with mouse anti-human TxRed DC-SIGN (Miltenyi Biotec, clone DCN47.5) and were pelleted and resuspended in Shandon Cytospin fluid (Thermo 6768315). Cells were then adhered to microscope slides using Shandon EZ Cytofunnels (Thermo A78710004) and a ThermoScientific Cytospin 4. Vectashield mounting media containing DAPI for nucleic acid staining was used to prepare the slides. All images were taken on a Zeiss LSM 510 META NLO Two-photon Laser Scanning Confocal microscope system at 63x magnification for tissues and 100x magnification for suspension cells and captured and analyzed using Nikon NES elements software.

12. Migration Assay

Transendothelial migration assays were performed using a 48-well microchemotaxis chamber (NeuroProbe P48) with nitrocellulose filters (Neuroprobe SCB8) with 5uM pore size were used. The bottom (chemoattractant) chamber of these plates containing 1 x 10^6 live PMNs suspended in 600uL of 0.05% BSA. The negative control was uninfected PMNs. Experimental wells contained PMNs infected with either WT Pg381 or Pg MFB at MOI of 10 for 30 minutes at 37°C and then washed twice and resuspended. PanDCs purified from whole blood at a concentration of 1 x 10^5 cells/50uL suspended in 100uL were loaded into upper chambers and migration was permitted to occur for 3 hours at 37°C and 5% CO_2. Nitrocellulose filters 100 microns thick were stained using Harris modified hematoxylin (Fisher SH30-500D) and leading edge migration depths were determined

under a 40x objective lens [276]. Cells at the farthest focal distance achievable were enumerated systematically. All samples were loaded and counted in triplicate and averages for each experimental condition were determined.

13. Cell-to-cell transfer Assay

CFSE stained bacteria were used to infect PMNs at an MOI of 10 for 30 minutes and then were washed three times to remove extracellular bacteria. PMNs were co-cultured with 6-day old immature moDCs at a ratio of 5:1 PMNs:moDCs post-infection for 3 hours incubation at 37°C and 5% CO2. In some experimental setups, either PMNs or moDCs or both were fixed with 1% glutaraldehyde in 0.1M PBS (pH=7.8) prior to co-culture. Co-cultured cells were then all fixed after 3 hours, on ice for 30 minutes with 1% gulteraldeyde in 0.1M PBS. Samples were resuspended in blocking solution for 30 minutes and stained with TexasRed-conjugated anti-DC-SIGN mAB (Miltenyi Biotec, clone DCN47.5) at 4°C for another 30 minutes. Cells were prepared for immunomicroscopy as previously described as well as for flow cytometry. For flow cytometry, only cells with SSC and FSC characteristics of moDCs were included, and positives were considered cells that generated a signal in both the red channel (DC-SIGN) and the green channel (CFSE stained Pg) signifying DC-SIGN positive moDCs that have acquired Pg from infected PMNs.

14. Statistical Analysis

For each experiment, each setup was compared to either negative and or positive controls or to each other by independent Student's T-tests. Means compared

were calculated from 4-6 independently conducted experiments. Data entry, statistical testing and graphs were done with GraphPad Prism 6 software. Significance was determined according to the Holm-Sidak method of setting significant alpha values at those less than 0.05. Significance results are denoted in figures by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$). For flow cytometry data, difference between peaks were determined significant by Student's T-tests by averaging mean fluorescence intensity (MFI), considering number of events (n) and calculating standard deviation from coefficient of variation (CV). Standard deviations were calculated as a function of the pooled variances from each experiment, feasible because each run counted the same number (n) of gated events.

Chapter 4

Results

PMNs successfully kill P. gingivalis, DCs harbor and promote the survival of P. gingivalis

In order to determine the fate of *P. gingivalis* after encountering either DCs or PMNs, a series of survival assays with antibiotic protection were conducted. As evidenced by TEM, apparently intact *P. gingivalis* is taken up by both moDCs (fig1a, i) and primary blood myeloid DCs (fig1a, ii) after 4 hours. Live *P. gingivalis* was recovered from lysed moDCs on blood agar plates after up to 24 hours of anaerobic incubation (fig1b). Before cell lysis, extracellular bacteria was eliminated with washes and treatment with gentamycin and metronidazole. In an identical experiment, PMNs successfully killed *P. gingivalis* as none was recovered after only 6 hours of anaerobic incubation, both with and without antibiotic protection (fig1c). TEM images of PMNs cultured with *P. gingivalis* for 3 hours revealed PMNs with morphologic signs of apoptosis [277] (fig1d). In addition, uptaken *P. gingivalis* appears to be being degraded within PMN vesicles, supporting the notion that PMNs are efficient killers of *P. gingivalis*.

PMNs conjugate with moDCs and DC-SIGN and CD11b/CD18 are shown to co-localize

Next, we sought to determine the nature of the interaction between PMNs and DCs and if this cell-to-cell association occurred in gingival tissues of patients with CP. MoDCs and PMNs show tight associations of their membranes after only 30 minutes of co-culture by TEM, suggesting that cell-to-cell contact is purposeful and not due only to proximity (fig2a). Co-cultures of moDCs and PMNs were immunostained with monoclonal

antibodies for DC-SIGN (red channel) and either CD11b/CD18 (fig2b i) or a nonspecific antibody for various subtypes of CEACAM molecules (green channel) (fig2b ii). These receptors on their respective cell types are co-localized as PMNs cluster around moDCs (fig2b). Similar co-localization of DCs (DC-SIGN, red channel) and PMNs (CEACAMs, green channel) was visible in the excised gingival tissue of 3 individuals with CP (fig3). These cells appear deep to the basement membrane in an area of the tissue where blood vessels are plentiful and where priming of T-cells and therefore the initiation of adaptive immunity occurs [278].

PMNs conjugation with Raji cells expressing DC-SIGN or moDCs, an interaction blocked by Pg381 in a fimbriae-dependent manner

By flow cytometry, we confirmed that DC-SIGN is required for optimum cell conjugation with PMNs (fig4). Raji cells expressing DC-SIGN and stained with B cell maker CD19 co-localized with PMNs stained with anti-CD66 for a double positive signal approximately three times greater than that formed by PMNs and Raji cells not expressing DC-SIGN (fig4a). In addition, Raji-DC-SIGN$^+$ cells treated with WT Pg381 were less able to form conjugates with PMNs. Raji cells not expressing DC-SIGN remained unable to associate with PMNs in the presence of wild-type Pg381. Non-fimbriated *P. gingivalis* mutant MFB did not inhibit Raji DC-SIGN$^+$ conjugation with PMNs (fig4a). Of all tested strains of Pg, MFI, the major fimbriated strain, most efficiently blocked conjugation even compared to the WT ($p<0.05$), however, DPG and WT strains also yielded conjugation rates significantly below those of non-infected cells and MFB infected cells ($p<0.0007$) (fig4b). The major fimbriae may be interfering however with other Raji B cell receptors that interact with PMNs [279]. Confocal fluorescence microscopy evidence (fig4c)

demonstrates PMN clustering around DC-SIGN$^+$ Raji cells (i), and no clustering in co-cultures of PMNs and DC-SIGN$^-$ Raji cells (ii). In Raji DC-SIGN$^+$ cells alone treated with WT Pg381 at an MOI of 10, bacteria adheres to the cell surface of most cells, preventing staining with anti-DC-SIGN on two of the three cells shown (iii). Similarly, Raji DC-SIGN$^+$ cells treated with a low MOI of Pg381 (MOI 1) exhibit clustering with PMNs only in sites unoccupied by Pg381. No such adherence of bacteria to cells is seen in co-cultures with Raji DC-SIGN$^-$ cells and wild type Pg381 (iv).

Similar results are seen for PMN-moDC conjugation when moDCs are infected with Pg before co-culture. MoDCs treated with WT Pg381 undergo conjugation at a rate of approximately 20% that of uninfected controls (fig5a). DPG Pg, expressing the minor fimbriae, yielded similar outcomes to the WT, while MFB and major FimA expressing mutant MFI showed significantly higher rates of conjugation allowed (fig5b). WT Pg colocalized with DC-SIGN on the surface of moDCs, and in these areas of occupied/hindered DC-SIGN, no PMNs cluster (fig6c).

PMNs treated with fimbriated strains of* P. gingivalis *demonstrate increased conjugation with DC-SIGN+ Raji cells and moDCs

While treating DC-SIGN+ Raji cells with WT Pg381 causes inhibition of conjugation with PMNs, treating PMNs with fimbriated strains of *P. gingivalis* before co-culture with Raji DC-SIGN+ cells or moDCs increases the amount of conjugation (fig6 and 7). PMNs treated with wild-type Pg381, or the mutants DPG, which expresses only the minor fimbriae (mfa), and MFI, which expresses only the major fimbriae (fimA) were more likely to cluster with both DC-SIGN+ Raji cells, as evidenced by a greater percentage of double positives in the upper right corner of FACS-generated scattergrams

(fig6a). PMNs treated similarly with non-fimbriated strain MFB showed colocalization with Raji DC-SIGN+ cells to similar degree as untreated controls, while WT and fimbriated mutant strains showed 3-4x levels of conjugation compared to uninfected cells (fig6b). Confocal microscopy evidence supports this flow cytometry data (fig5c). PMNs treated with wild type Pg381 readily uptake the bacteria but do not conjugate with Raji cells not expressing DC-SIGN (i). In the presence of DC-SIGN- Raji cells, PMNs treated with non-fimbriated mutant MFB do not exhibit any conjugation (ii). PMNs also seem unable to recognize the bacteria in the absence of fimbriae, and fail to phagocytose the MFB strain. However, PMNs treated with wild-type Pg381 cluster readily with DC-SIGN+ Raji cells (iii), as opposed to PMNs treated with MFB, which cluster only to the same degree as DC-SIGN+, untreated controls.

A similar pattern was seen with Pg381-treated PMNs co-cultured with moDCs (fig7). Wild-type Pg381, as well as minor fimbriae-expressing mutant strain DPG caused higher rates of co-localization of PMNs and moDCs as shown by an elevated number of events in the upper right quadrant, while MFB-treated PMNs showed levels of conjugation similar to untreated controls (fig7a). This suggests that PMNs are altered by fimbriated *P. gingivalis* strains in a way that improved their ability to conjugate with DCs. All strains of Pg demonstrate significant increases in moDC-PMN conjugation except MFB. Conjugation with MFB infected PMNs is significantly less than the WT, while the major fimbriated strain MFI causes conjugation rates significantly higher than the WT (fig7b). All strains also elicit significantly higher rates of conjugation when PMNs are infected first versus as compared to moDCs infected first (fig7c). Microscopic evidence demonstrates the ability of WT Pg-filled PMNs (CFSE stained Pg, green channel) to readily cluster around immature DC-SIGN expressing moDCs (red channel) (fig7d).

Fimbriated strains of Pg381 elicit Apoptosis and Phenotypic Changes in Surface Markers in PMNs

We then sought to determine what changes occurred in PMNs exposed to fimbriated strains of *P. gingivalis* that may have increased their propensity to associate with DCs. Changes in integrin and other surface marker expression in the presence of Pg381 and its mutants were determined by flow cytometric analysis, and are represented as a shift in fluorescence intensity (fig8a). Infection by fimbriated strains Pg381 WT, DPG, and MFI caused a dramatic increase in CEACAM1 (CD66a), a molecule implicated in PMN-DC conjugation, as well as CEACAM8 (CD66b). Fimbriated strains also increase expression of the activated from of Mac-1 integrin (CD18/CD11b) as well as an overall increase in the B2 integrin portion of Mac-1, its inactivated form CD11b (fig8a). The non-fimbriated strain MFB did not show apparent PMN activation by the phenotypic markers measured by flow cytometry data (fig8a). However, calculation of significance between averages based on multiple experiments by Student's T tests yielded evidence that MFB does cause significant upregulation of CD11b ($p<0.0001$) and CD66b ($p<0.05$) as compared to uninfected controls. However, activated Mac-1 and CD66a, the markers indicated in PMN-DC interactions [59] do not show a significant upregulation with MFB infection (fig8b). The largest shift in all surface markers was elicited by the major fimbriae expressing strain, MFI (fig8a and b).

Aside from phenotypic changes, fimbriated *P. gingivalis* strains initiated programmed cell-death in PMNs by apoptosis. Cell death was seen 3 hours post infection at an MOI of 10. WT Pg381, as well as MFI and DPG caused increases in early apoptosis marker, exterior membrane phosphatidylserine as observed via staining with annexin V.

Annexin V staining was increased with infection with WT Pg and DPG, and most of all MFI as compared to non-fimbriated MFB and purified Pg LPS (fig9a). Annexin V staining intensity was increased to a greater extent with exposure of PMNs to WT Pg381 than with exposure the positive control substance, the cytokine TNFa, known to a be an inducer of apoptosis [280] (fig9b). In addition, cells also experienced increased loss of membrane integrity, showing increased positivity for propridium iodide in all strains, most of all MFI, and least of all MFB, and suggesting fimbriated Pg induced eventually aponecrosis (fig9b). Staining for intracellular polycaspase activation followed a similar pattern, showing evidence of activation in PMNs infected with WT, but less so for MFB (fig9c). The involvement of the caspase pathway also confirms that the mode of programmed death is apoptosis rather than pyroptosis or necrosis [281]. The mutant strain expressing only the major fimbriae (MFI) seems to cause apoptosis to a greater extent compared to the strains only expressing the minor fimbriae (DPG) or no fimbriae (MFB). Furthermore *P. gingivalis* LPS is not sufficient to cause apoptosis. Poly caspase activation levels are also indicative of apoptosis being induced by the major fimbriae, but not MFB, DPG or LPS (Fig9c). With extended infection times, PMNs exposed to fimbriated strains eventually progress to secondary necrosis, as shown by measuring fluorescence spillover in the upper right quadrant signaling positivity for propridium iodide (PI) uptake. All fimbriated strains cause heightened annexin V staining and PI positivity after a period of 3 hours compared to MFB. Fig9d shows PMNs stained with dihydroerythrin and positive FITC-annexin V staining in those cells exposed to WT Pg381 and DPG. PMNs treated with these strains also show less retention of dihydroerythrin signifying a loss in membrane integrity. MFB shows similar levels of annexin V staining as the control, and demonstrates greater brightness of cytoplasmic stain. All images were taken 3 hours post

infection.

DC-PMN conjugation Rescues PMNs from* P. gingivalis-*induced apoptosis

It has been postulated that the life spans of PMNs may be lengthened by their interaction with DCs [198], and that DCs may reverse apoptosis in PMNs. To determine if DCs were capable of rescuing PMNs from *P. gingivalis* or otherwise induced cell death, we treated PMNs with wild type Pg381, non-fimbriated mutant MFB, minor fimbriae-expressing mutant DPG, and TNFa, a well-documented inducer of apoptosis in PMNs. Annexin V staining assessed by flow cytometry was used to determine onset of apoptosis. After multiple washes, moDCs were added to the PMNs for 60 minutes, and cells PMNs were reassessed for annexin V positivity. Fig10a shows induction of apoptosis compared to untreated PMN controls. As shown previously, fimbriated strains and TNFa induced apoptosis strongly, while MFB did not. In the presence of moDCs, apoptosis was fully reversed in the TNFa-treated PMNs, as evidenced by a leftward shift in fluorescence intensity that matches untreated PMN (fig10a ii). MoDCs added to PMN cultures decreased annexin V staining in PMNs infected with fimbriated wild-type and DPG *P. gingivalis*, but to a lesser degree than moDCs reverse apoptosis in TNF-a (fig10a ii and iii and fig10c). Poly-caspase staining is also decreased in moDCs in a similar pattern, the most drastic reversal is seen with TNF-a treatment (fig10b). While moDCs are able to rescue PMNs from cell death, degree of reversal is based on the apoptosis inciting stimulus.

PMNs cause a unique maturation profile in moDCs and rapid differentation of monocytes to immature moDCs

In order to fully understand how PMNs, whether activated or inactivated, influence the maturation of DCs, moDCs cultured with differentially treated PMNs underwent phenotypic analysis by flow cytometry. PMNs left untreated, or treated with either TNFalpha, wild-type Pg381, or mutant strains MFB or DPG, were co-cultured with immature day 6 moDCs for 24 hours. PMNs were thoroughly washed and fixed after treatment and before co-culture with moDCs to ensure that any change in DC phenotype was due strictly to cell contact, rather than being mediated by a soluble factor. Normal maturation of DCs is characterized by down-regulation of DC-SIGN and up-regulation of lymphocyte co-stimulator molecules and those involved in antigen presentation such as CD80, CD83, CD86, and HLA-DR [93]. Overall these maturation markers were upregulated with exposure to PMNs, treated or untreated, while DC-SIGN, characteristic of iDCs was slightly down-regulated (fig11a). Trends over multiple experiments show that co-culture with unstimulated PMNs yielded DCs that were mostly phenotypically mature, however CD83 was not elevated compared to positive controls (iDCs treated with TNF-a, a known inducer of maturation in DCs), suggesting a semi-mature state [111]. Unstimulated PMNs were also more successful at elevating the functional maturation molecule HLA-DR than in positive controls (fig11b). Aa leukotoxin treated PMNs were the most potent inducers of maturation, suggesting that cell death may play a role in potency of DC responses. Treatment of PMNs with WT, MFB and minor fimbriate expressing DPG strains yielded similarly semi-matured DCs. In these treatments, CD86 expression was lowered compared even to untreated 1-day old immature moDCs, except with DPG-treated PMNs in which CD86 expression remained the same as these negative controls. In addition, WT-treated PMNs were able to up-regulate CD83 levels to those of positive controls, however MFB and DPG-treated PMNs showed significantly lower levels

of this molecule than those expressed by positive controls. This suggests that the major FimA may play a role in inducing changes in PMN phenotype that then affect DC expression levels, and as CD83 is thought to increase the robustness of the T cell response, this may produce an important functional outcome of T-cell priming by DCs [95]. LPS-treated PMNs however failed to make any difference in terms of CD83 or DC-SIGN expression (fig11b).

In similar experiments, adherent peripheral blood monocytes (PBMCs) were co-cultured with differentially treated, paraformaldehyde-fixed PMNs. While traditional differentiation of monocytes to a dendritic cell phenotypes employs cytokines GM-CSF and IL-4 and takes six days, 24 hours resulted in monocytes that were converted to semi-mature moDCs [52]. Compared to monocytes treated with growth factors, cells incubated with PMNs more rapidly down-regulated monocyte marker CD14, and up-regulated dendritic cell markers DC-SIGN, CD1c, and slightly increased levels of maturation marker CD83 (fig12a). Only DPG treated PMNs did not cause significant down regulation of CD83, and untreated PMNs actually caused a down-regulation of CD83 compared to untreated PBMCs (fig12b). After only 24 hours, the proportion of cells occupying the gate restraints for moDCs increased over the ratio of cells still gated as PBMCs, the greatest increase in moDCs being PBMCs cocultured with WT Pg-infected PMNs (fig12c). Conjugation of pre-DCs and PMNs can therefore influence the exact phenotype these cells acquire as well as how efficiently they differentiate.

Activated/Apoptotic PMNs cause DC migration

In order to determine the effects of PMNs on the migration of DCs, a series of boyden chamber assays were performed. PMNs activated with wild-type Pg381 for 90

minutes before loading were successful at recruiting primary isolated blood dendritic cells at a 5-fold higher rate compared to control conditions not containing PMNs (fig13ai). Wild-type treated PMNs were twice as effective at DC recruitment as PMNs treated with non-fimbriated MFB (fig13ai). In a follow-up experiment, supernatants harvested from PMNs treated with wild-type Pg381 attracted significantly more DCs than those from untreated PMNs and PMNs treated with MFB (fig13aii). These results suggest there is a soluble factor released from PMNs in response to exposure to fimbriated strains of *P. gingivalis* that may play a role in attracting DCs by affecting their migratory capacity.

Next we sought to determine the effect of cell-to-cell interactions between PMNs and DCs on the expression of DC migration markers. Overall, migration markers associated with the immature DC phenotype (CCR5, CCR6, and CCR2) were up-regulated, while maturation markers responsible for lymph node homing of DCs (CXCR4 and CCR7) were unaltered compared to negative controls or decreased (fig13b and c). Interestingly, TNF-α treatment of moDCs as a positive control also resulted in CXCR4 down-regulation (fig13c). This suggests that untreated but especially activated PMNs decrease propensity for moDCs to mature in terms of migration markers, keeping them in infected tissues for perhaps longer contract with PMNs. In addition WT and DPG Pg-infected PMNs and Pg LPS-treated PMNs prove to be especially adept at keeping CCR7 levels low (fig13c).

***DC-PMN conjugation may facilitate transfer of pathogens such as* P. gingivalis**

Because the survival of *P. gingivalis* seems enhanced by its invasion of DCs, while PMNs are effective killers of the bacterium, we attempted to determine if DC-PMN conjugation could be further manipulated by the bacterium by its ability to escape the

intracellular environment of the PMN and transferring into an associated DC. To determine this, PMNs were allowed to phagocytose the WT Pg381, DPG, and MFB strains. Antibiotic protection (metronidazole and gentamycin) and a series of washes were conducted to eliminate any bacteria not taken up by the cells within 30 minutes. Immature moDCs were then cultured with infected PMNs for 6 hours. Before co-culture either one or both cell types were fixed compared to viable DC-PMN controls. Transfer appears to depend on the viability of both involved cell types, as transfer of Pg is not seen in moDCs in conditions where either cell type was fixed (fig14a and c). Time zero images taken by confocal fluorescence microscopy show PMNs that have internalized CFSE-stained *P. gingivalis,* and immature moDCs stained for DC-SIGN, which contain no bacteria (fig14bi). After 6 hours of co-culture, WT and DPG Pg were observed intact inside moDCs (fig14bii and iv), while MFB was not internalized by either cell type (fig14biii). Microscopy results were confirmed by flow cytometry detection of cells that were DC-SIGN/CFSE double-positive indicating moDCs with intracellular Pg (fig14c). These results suggest the ability of *P. gingivalis* to invade DCs from within PMNs, perhaps manipulating machinery of the living host cells to improve the odds of its survival even after contact with PMNs.

***DC-PMN interactions with infection by* P. gingivalis**

These data tell a logical story of DC-PMN interactions and how these cells are independently and together affected by the presence of *P. gingivalis.* Fig15 summarizes these interactions in a proposed in vivo model. (1) PMNs as first responders engulf and clear much of the initial Pg infection within gingival tissues. Tissue resident DCs which survey gingival tissue encounter Pg via DC-SIGN (fig2, 3 and 4) and are thus inhibited

from interacting with PMNs by Pg (fig4 and 5) and their uptake of the pathogen may lead to its survival and persistence (fig1). (2) By uptake of Pg (fig1), they undergo apoptosis and become activated (fig 9 and 10), encouraging iDC and PBMC migration from peripheral blood by soluble factors (fig 13). (3) Conjugation of infected PMNs and DCs (fig2 and 3) results in pathogen transfer to DCs (fig14). (4) Contact-dependent changes in DC maturation (fig11) and migratory states (fig13) by Pg-infected PMNs result in semi-mature cells with no means of migrating to lymph nodes. (4a) Pre-DCs are encouraged to differentiation to Pg-salvaging iDCs by activated/apoptotic PMN (fig12). An elevated population of immature and semi-matured DCs is then available to house Pg and allow it access to distant sites by systemic circulation.

Chapter 5
Discussion

A. Survival of *Porphyromonas gingivalis* within DCs vs. killing in PMNs

These results further support the supposedly unique niche that *P. gingivalis* has adapted to fulfill, persisting in circulation in those with chronic periodontal disease [15]. The survival of *P. gingivalis* within human host cells has been assessed before, with studies showing that this bacteria was harbored and recoverable after uptake by epithelial cells [282], KB cells [283], and dendritic cells [15]. Furthermore, evidence that *P. gingivalis* is found in atherosclerotic plaques [284], lends to the idea that this bacteria's pathological mechanisms play a role in sites of inflammation throughout the body. At least one study has shown evidence that *P. gingivalis* survives transfer from being embedded within vascular tissue to other cell types where it resides in a recoverable state [282].

P. gingivalis survived uptake and 24 hours of internalization within moDCs (fig1). It has recently been shown that after engaging C-type lectin receptor DC-SIGN by bacterial minor fimbriae (mfa-1) [272], *P. gingivalis* is capable of evading autophagy with DCs by prohibiting fusion with lysosomes [225]. Coinciding with those findings, *P. gingivalis* was recovered after 24h of incubation with moDCs (fig1B). Furthermore, microscopic evidence shows internalization of intact *P. gingivalis* within DCs (fig1A), autophagy and breakdown of *P. gingivalis* within PMNs can be seen only 30 minutes post-infection, with obvious loss of integrity of the bacterial membrane (fig1D). It has long been known that neutropenia or deficiencies in neutrophil function predispose individuals to early-onset, aggressive periodontitis, suggesting that PMNs are the definitive immune cells in clearing periodontal pathogens [285, 286]. However, the collateral damage that

neutrophils cause from the release of ROS to inflamed and peripherally healthy tissues has been identified as part of the host response that contributes to the etiology of periodontal disease [287].

B. *P. gingivalis* alters surface receptor expression of PMNs

In the previously stated results, *P. gingivalis* was shown to exert changes in the surface expression profile of PMNs. The pathogen's fimbriae, shown to be unique as they share no significant homology to fimbriae from other bacteria [288], interact with β2 integrin, including the Mac-1 complex on the surface of PMNs [257]. Mac-1, comprised of β-subunit CD18 non-covalently bonded to α subunit CD11b, is the most prevalent integrin expressed by phagocytic immune cells [256, 257]. Specifically the major fimbriae of *P. gingivalis*, FimA, has been shown to associate with Mac-1, and it has been much debated whether ligation of this receptor is for the benefit of the pathogen, or host immunity [247, 254].

These results show that one subunit of Mac-1, CD11b, is elevated in response to fimbriated *P. gingivalis,* as well as the dual subunit activated form of Mac-1 (fig8B). While integrin activation primarily involves either changes in the conformation of receptors resulting in increased affinity for the ligand or changes in mobility that cause receptor clustering [289], quantitative up-regulation of integrin receptors on the cell surface may also be a mechanism for increased activation [290]. While this form of activation has not been shown to increase PMN adhesion to endothelium [290], it may be the mechanism our data points to for increased PMN-DC interactions. Increased CD11b (fig8 B) is seen with all *P. gingivalis* strains, including nonfimbriated strain MFB, perhaps demonstrating this third mechanism. However, activated Mac-1 is elevated only with

infection by fimbriated strains, suggesting that the primary mechanism of conformation changing, in this case the joining of both subunits of Mac-1 (CD11b and CD18), seems to be fimbriae-dependent. Additionally, studies have shown that encounters of neutrophils with bacteria within the bloodstream result in upregulation of CD11b, and as a result increased adhesion with endothelial cells but an impaired ability to extravasate and migrate into tissue [291, 292]. This may be another mechanism by which *P. gingivalis* that has entered the blood stream may impair neutrophil function and retain them in the blood stream to gain access to circulating mDCs.

CEACAM1 (CD66a) also increased on the surface of neutrophils infected with all fimbriated and non-fimbriated *P. gingivalis* strains, however the least amount of upregulation occurred with non-fimbriated MFB. While no literature exists that suggests this pathogen interacts with CEACAM1, other bacterial pathogens known to invade immune cells such as *Neisseria meningitides* both up-regulates and interacts with this receptor in vitro in a mechanism that results in immune evasion [275]. In this particular mechanism, ligation with CEACAM1 along with TLR2 activates the PI3k–AkT pathway and decreases the production of IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF), which regulate the differentiation, migration and activation of granulocytes. If further studies demonstrate that a similar mechanism exists for PMNs in response to *P. gingivalis* infection in addition to the upregulation shown here, this may prove another mechanism by which this pathogen successfully evades immunity. Upregulation of both Mac-1 and CEACAM1 with exposure to fimbriated *P. gingivalis* may be one aspect of the mechanism the leads to increased PMN-DC conjugation when co-cultures are performed with previously infected PMNs.

CEACAM8 (CD66b), while not identified specifically as a molecule responsible

for association of PMNs with DCs, is a molecule with an unknown function, but one that signifies PMN activation [293, 294]. It has been suggested that elevation of CEACAM8, which is usually independent of increases in Mac-1 expression, results in neutrophil aggregation [294] as well as lysosome priming and fusion to the cell membrane [295]. This marker was elevated in response to fimbriated *P. gingivalis,* and unique to the other molecules examined, increased markedly in strains which expressed the minor fimbriae mfa-1. It therefore may prove to play an important role in cell adhesion between neutrophils and with other immune cells.

C. PMN apoptosis and aponecrosis due to infection with *P. gingivalis*

The ability of *P. gingivalis* to elicit or prevent apoptosis in neutrophils is a complex issue as evidenced by the current literature. Multiple studies claim that this pathogen's lipopolysaccharide when directly added to isolated neutrophils or HL-60 derived neutrophils prevents or delays apoptosis, lending to the delayed clearance of activated neutrophils and subsequently increased collateral damage to tissues due to the host response, a hallmark of periodontal disease [296-298]. Other pathogenic elements of *P. gingivalis* such as its gingipains have been credited with elevating TREM-1, a cell surface receptor that is expressed by PMNs and monocytes and incites an intracellular response that leads to release of proinflammatory cytokines and initiation of apoptosis [258]. While whole *P. gingivalis* or its proteases and gingipains have been implicated in the initiation of apoptosis and aponecrosis of fibroblasts [299], autophagy of fibroblasts [300], and gingival epithelial cells [301], there are no reports of it inducing apoptosis in PMNs.

The results reported here are unique in that they point to a fimbriae-mediated apoptosis, with annexin-V positivity and poly-caspase activity occurring with both the isogenic single fimbriae mutant strains and the WT, but not the non-fimbriated mutant

MFB nor isolated *P. gingivalis* LPS. The MFI strain, expressing only the major fim (FimA) shows the greatest comparative right-shift in annexin-V (Fig9 a) and poly-caspase (Fig9b and c) and the highest amount of necrosis as evidenced by propridium iodide. While it seems counter-intuitive for a single fimbriae-expressing mutant to impose a more drastic effect than the double-fimbratied WT strain, both MFI and DPG have been shown to have differential effects from the WT, with MFI causing a less robust MMP-9 response from moDCs than the WT and a greater production of TNFα, and IL-1β than the WT [272, 302].

D. DC-PMN conjugation in the presence of *P. gingivalis*

These results reveal a relationship between DCs and PMNs mediated by the physical interaction of by receptors and integrins on the cell surfaces. Electron micrographs (fig2a) show close associations between the membranes of the two cell types resembling a "tight junction" morphology. It has been theorized with limited supporting data that DC-SIGN, a C-type lectin receptor expressed on immature, phagocytic DCs, is responsible for conjugation with neutrophils, as well as the clearance of effete neutrophils that have encountered pathogens [59, 197, 198]. These same studies credit Mac-1 and CEACAM1 on the surface of neutrophils with ligating to DC-SIGN by way of their glycosylation patterns, specifically repeating mannose moieties [59, 197, 198]. We confirmed the role of DC-SIGN in conjugation with neutrophils by experiments with Raji-DC-SIGN$^{-/+}$ cells (fig4) which demonstrate that DC-SIGN expression is necessary and sufficient for interaction with PMNs. As our lab has shown, similar mannose moieties on the minor fimbriae of *P. gingivalis* interact with DC-SIGN, perhaps explaining why infection of Raji-DC-SIGN^{+} cells with WT *P. gingivalis* as well as the mutant strains expressing either

fimbriae successfully block Raji-PMN conjugation. As Raji cells are not strongly phagocytic cells, the mechanism of this inhibition of cell associations may be a simple steric hinderance [303]. These results suggest that the affinity and avidity of DC-SIGN to fimbriated *P. gingivalis* may out compete that of DC-SIGN to PMN surface molecules.

Interestingly, the opposite is true if neutrophils are first pulsed with *P. gingivalis* before co-culture with Raji-DC-SIGN^{+} cells (fig6). Neutrophils pulsed with the three fimbriated strains cause greater association between cells, while there is no significant difference between PMN-infected first vs. Raji-DC-SIGN^{+} infected first setups in terms of the non-fimbriated strain, Pg-MFB (fig6d). This may occur due to the activation of neutrophils by fimbriated *P. gingivalis* as discussed above, and the increased expression of DC-SIGN ligands, Mac-1 and CEACAM1 on the cells' surfaces. It may also be due to the change in morphology and surface expression occurring due to the fact that PMNs exposed to *P. gingivalis* undergo apoptosis and there is much literature to support the premise that DCs "clean up" the debris of neutrophils, perhaps to sample their contents for detection of foreign antigen to be processed and cross presented by DCs to drive the T cell response [214, 304, 305].

The same pattern occurs for immature moDCs co-cultured with PMNs (fig5 and fig7). With moDCs infected prior to co-cultured with PMNs, fimbriated strains especially WT *P. gingivalis* and minor fimbriae- expressing mutant DPG diminish DC-PMN conjugation, while non-fimbriated mutant MFB demonstrates conjugation levels not significantly different from uninfected controls. In the case of moDCs, some steric hindrance may be occurring, however, because conjugation rates more dramatically lowered with co-cultures of moDCs and PMNs compared to Raji-DC-SIGN+ cells with PMNs, DC-SIGN may be diminished by uptake of *P. gingivalis* as the bacteria has been

shown to use this receptor as an invasion, being take up after surface biding in DC-SIGN-rich vesicles [253, 272]. In the case of first-infected PMNs co-cultured with moDCs, major fimbriae expressing strain MFI causes a great than 2.5 fold increase in PMN-moDC association compared to infected controls (fig7 b). This coincides with previously suggested results that this strain was most effective at causing upregulated levels of activated integrin Mac-1 and CD66a, the molecules on the surface of PMNs responsible for ligating DC-SIGN (fig8). This strain is also the strongest inducer of apoptosis in PMNs (fig9a), suggesting again that initiation of apoptosis in PMNs may cause them to be more readily engaged by DCs.

E. Transfer of *P. gingivalis* from infected PMNs to moDCs

Our data demonstrates that moDCs seem able to acquire whole *P. gingivalis* from previously infected PMNs after 6 hours of co-culture (Fig13). It has been shown that PMN may deliver antigenic molecules to DCs for presentation to T lymphocytes, making up for the fact that DCs generally lack efficient mechanisms for the breakdown of pathogens to presentable epitopes [214]. PMNs may also facilitate this transfer via NETs [306]. DCs also have the ability to cross present antigens present within effete apoptotic cells which they have cleared [305]. Transfer of a complete pathogen from PMNs to DCs as a mechanism by which the pathogen may remain viable has not been described as a mechanism of immune evasion. Here, we have *P. gingivalis* seemingly intact being transferred in less than 6 hours, also interesting the time by which no more viable *P. gingivalis* was recoverable from PMNs. In addition, viability of both cell types seem to play a role in this transfer, suggesting that this may be an active process on the part of the host cells, perhaps evidence of the living pathogen hijacking a physiologic antigen transfer

mechanism. Transfer still occurs even with the fixation of both cell types, granted at a much lower rate. Therefore, it is possible that *P. gingivalis* is capable of effecting this transfer itself. As previously described by Li et al., *P. gingivalis* seems capable of invading fresh cells in a viable state after being taken up initially by epithelial cells that diminished the bacteria to unrecoverable levels [247]. This cell-to-cell transfer suggests another pathogenic mechanism by which *P. gingivalis* that may survive initial uptake by PMNs, enter DCs and persist in a viable state within the host. However, no mechanism for this transfer has been elucidated by this study, nor is there any evidence that *P. gingivalis* remains viable after exposure to PMNs and uptake by DCs. These factors would need to be confirmed in future studies to validate the data presented here.

F. Effects of DC-PMN conjugation on moDCs

It has been proposed that physical association with PMNs causes immature DCs to undergo maturation and become fully antigen presenting [197]. In our experiments, PMNs were fixed after infection or activation and before co-culture to ensure that any changes in DC phenotype were due to cell-to-cell contact and not to soluble factors released by PMNs. Our results show that both unstimulated and Pg stimulated PMNs were able to affect DC phenotype, creating a semi-mature state of DCs (Fig11). Similar semi-maturity or interference of maturation has been seen with exposure to iDCs to neutrophil ectosomes, small vesicles released by degranulating neutrophils, as shown by variant expressivity of maturation markers such as CD40 and CD80 compared to LPS stimulated controls, as well as changes in morphology that differ from normally matured DCs [307]. Leukotoxin isolated from *Actinobacillus actinomycetemcomitans* was used as an additional positive control to reveal the DC response to rapidly degranulating, effete PMNs to

determine if the state of PMNs produced by *P. gingivalis* caused a different DC response. Our data demonstrates that the most significant difference in DC phenotype made by WT pulsed PMN and Aa leukotoxin was in terms of DC-SIGN (Fig11b). WT *P. gingivalis* infection of PMNs created a decrease in DC-SIGN on co-cultured moDCs, while leukotoxin-treated PMNs maintained levels of DC-SIGN even causing slight elevation. This discrepancy may be due to the changes in PMN phenotype that fimbriated *P. gingivalis* promotes, increasing DC-SIGN ligands CD11b/CD18, CD66a on PMNs, while Aa leukotoxin may inhibit Mac-1 interactions with moDCs as CD18 is the toxin's functional receptor [308].

The unique surface expression pattern induced by both unstimulated PMNs and infected PMNs may result from the DC-SIGN ligation differential created by the differences in surface expression of PMNs. DC-SIGN ligation by *Neisseria meningitis* has been shown to modulate DC maturation profiles, causing definitive traditional maturation when reacting with the receptor followed by a Th-1 type response [81]. Conversely, antibody-mediated ligation to DC-SIGN causes a decidedly immature phenotype [82]. Van Ginsbergen et al. demonstrated increased CD83 expression on DCs 18 hours after contact with FMLP-, TNF-α–, and LPS-activated PMN with evidence to support that cell-to-cell contact was achieved through Mac-1/DC-SIGN ligation, and suggesting that the state of PMN could modulate the exact expression profile of DCs and the nature of the immune response [197]. The present study shows a detailed maturation profile of DCs exposed to PMN under various activation states. Interestingly, while van Ginsbergen et al. claimed that inactive PMN were incapable of inciting DC maturation [197], this new data reveals that while full traditional maturation is not achieved, hallmark maturation molecules CD80 and HLA-DR are upregulated with unstimulated PMN even compared to

positive controls (Fig11).

We have also shown that unstimulated and *P. gingivalis* stimulated PMN affect the migration profile of DCs. Functional migration of DCs is greatly increased in response to PMN activated by fimbriae-expressing strains of *P. gingivalis*, and appears to be in response to soluble factors emitted by PMN (Fig14 a). In addition PMN cause a migration phenotype of DCs again not consistent with traditional maturation, with decreases in CCR7, the definitive marker that signifies end-stage maturation and is responsible for lymph node homing [119]. PMNs infected with WT *P. gingivalis* were also shown to increase CXCR4 receptor expression, a receptor responsible for neovascularization [309] and implicated in the survival of intracellular pathogens [252]. Both CCR5 and CCR6, receptors that normally guide DCs into sites of infected tissue [122] were increased compared to negative controls with both unstimulated and activated PMNs. CCR5 and 6 are usually downregulated in maturation, and CXCR4 usually increases on mature DCs [91, 111], demonstrating again that DCs pulsed with PMNs are relegated to a semi-mature, hyper-mobile state. This profile signifies one that may keep dendritic cells in the periphery for as long as possible, favoring the survival and dissemination of the *P. gingivalis* that has been phagocytosed by DCs or transferred from infected PMN.

P. gingivalis has been shown to ligate DC-SIGN by in minor fimbriae, and while this pathogen can cause differentiation of monocytes to iDCs [14] and adversely affect the ability of DCs to home to lymph tissues [224], it is only a weak inducer of maturation seemingly via its fimbriae [310]. While this pathogen may exert its own effects on the immune response directly, these results suggest a distinctive way in which it drives an immune response through manipulation of the most potent APCs through the most plentiful and aggressive of leukocytes.

G. Monocyte differentiation to moDCs by *P. gingivalis* infected PMNs

DC-SIGN ligation has also been shown to promote monocyte differentiation to immature moDCs, but results in cells in which certain transcription factors such as p38 MAPK, STAT1, STAT6 are inhibited as well as NF-kB activation upon priming with LPS, and therefore creates moDCs that are unable generate a robust CD4+ T cell response [80]. Our data demonstrates a definite shift of monocyte population toward an immature DC phenotype (Fig12). Both untreated and treated/infected PMNS were shown to be more potent inducers of differentiation that even conventionally given growth factors IL-4 and GM-CSF after only 24 hours of incubation instead of the normal 6 days (Fig12 b).

Untreated and treated/infected PMNs trended significantly in the opposite direction from the growth factor stimulated positive control only in terms of maturation marker CD83, demonstrating even a slight down regulation as compared to untreated monocytes. This can be interpreted as PMN-induce differentiation resulting in a more purely immature moDC population than one generated by growth factors. Interestingly, PMNs infected by the minor-fimbriae expressing DPG strain of *P. gingivalis* did not show a significant decrease in CD83 expression on differentiated monocyes as compared to negative (untreated) controls. DPG infection also resulted in significantly lower expression of activated Mac-1 and CD66a levels (Fig8b), as well as lower levels of apoptosis as compared to the WT (Fig9c). This may point to an ability of the minor fimbriae of *P. gingivalis* to alter PMN phenotype in a specific way distinct from both the WT and non-fimbriated MFB strains, and resulting in PMNs that in turn induce a unique immature moDC phenotype in which the CD83 levels of undifferentiated monocytes are maintained. In addition, leukotoxin-treated PMNs caused CD83 expression on par with monocytes

exposed to growth factors suggesting that a more drastic activation stimulus of PMNs may result in a DC phenotype closer to a state of terminal DC maturation.

WT *P. gingivalis* infected PMN co-cultures resulted in the highest ratio of moDCs to monocytes (Fig12c). By creating immature moDCs, cells that are inefficient at antigen processing, via infection of PMN first-responders may be a mechanism by which *P. gingivalis* ensures its overall survival and persistence within the host. This mechanism may also explain previously published clinical findings in which myeloid blood DCs in circulation are elevated in patients with chronic periodontitis with no other apparent source for these cells [15].

H. Effects of DC-PMN conjugation on PMN

While it has long been accepted that effete PMNs are engulfed and cleared by myeloid DCs, Gisbergen et al. theorized that the presence of DCs and their ligation with PMN surface molecules may increase the lifespan of PMNs and may perhaps reverse or prevent programmed cell death. Binding of Mac-1 by integrins such as ICAM-1 and fibrinogen and induce an intracellular signaling cascasde that promotes cell survival [311] [312]. However, the microenvironment at the time of Mac-1 ligation may instead result in cell death if apoptotic signals are present [313]. CEACAM1 (CD66a), the other DC-SIGN ligand on the surface of PMNs [314] mediates homophilic cell-to-cell interaction which has also been show to delay or offset PMN apoptosis [188]. Our data shows that while apoptosis still occurred in PMNs exposed to WT *P. gingivalis* and the minor fimbriae-expressing mutatnt straing DPG, the addition of moDCs 30 minutes post infection reduced levels of Annexin-V staining and caspase activity of PMNs, indicating reduced apoptosis. While the ligation of *P.gingivalis*-induced upregulated expression of Mac-1 and

CEACAM1 by DC-SIGN on moDCs as supported by the literature is a fair proposed mechanism for this decreased cell death, it is important to note that caspase activity in uninfected controls was reduced even more drastically with the addition of moDCs than in the WT infected setup (b). This along with the fact that PMNs treated with TNF-α, a potent inducer of PMN apoptosis, demonstrated the largest anti-apoptotic effect with the addition of moDCs (Fig10b and c) suggests that *P. gingivalis* may employ a mechanisms that adversely offsets the positive effects of the co-culture for PMNs, resulting in the still significant apoptosis of the cell type that kills this pathogen fairly efficiently.

I. Clinical importance of findings

The results described herein tell a unique story of immune modulation by a pathogen through its effects on and the interactions between two innate immune cells. In showing that DCs and PMNs associate with one another in the gingival tissue of patients with chronic periodontitis (Fig3), our data suggests that the relationship between these two immune cell types and the immunomodulation that occurs as a result of their conjugation can be an important element in understanding the etiology of host response in this disease process. Furthermore, the specific way in which *P. gingivalis* imposes control over this cell-to-cell interaction, affecting the nature of the immune response, and creating a niche in which it survives innate immunity and coerces host cells to promote its survival (outlined in Fig15) is a unique pathogenic strategy that may explain the bacteria's ability to succeed in causing low grade chronic infection locally as well as its systemic dissemination.

Chapter 6
Future Directions

The present study attempts to elucidate the interactions between DCs and PMNs in vitro and to determine the role *P. gingivalis* plays in interrupting this association as a means of modulating the innate immune response for its own benefit. The most intriguing aspect of these results is that innate immunity seems capable of more plasticity and potency in the overall immune response than historically assumed. The next logical step to follow up these findings would be creation of an in vivo animal model. Tracing PMN and DC migration in a living system challenged with infection by *P. gingivalis* will help us to unite the concepts discussed here to confirm a logical sequence of events that defines both the normal host response in terms of DC-PMN associations and the effect of these cell-to-cell interactions in the presence of disease. Knock-out organisms for one cell type, such as DC depletion with CD11c-Diptheria toxin receptor in transgenic mice, or for combinations of the identified receptors/molecules (Mac-1, CEACAM1, DC-SIGN) may serve to more exactly define the role of this physical cell conjugation.

In addition to the surface expression profiles shown in this study, more data needs to be collected to determine the soluble factors emitted by PMNs when infected with WT *P. gingivalis* versus single fimbriated or non-fimbriated mutant strains. The effect of these soluble factors on DC migration, maturation and differentiation from monocytes should be examined. As the focus of the present study was to determine the consequence of physical cell conjugation, soluble factors were not examined. However a full cytokine profile of DCs primed with *P. gingivalis* infected PMNs should be determined. In addition, the surface expression changes that DCs undergo in response to PMNs should be tested in

functional studies to determine the nature and robustness of the resulting T cell response.

The prospect that *P. gingivalis* is transferable from PMN to DCs also requires further study. The present data represented here only shows that seemingly whole bacteria are traveling from infected PMNs to moDCs after 6 hours, and that when both cell types are viable, moDCs containing *P. gingivalis* are increased. While this suggests the active involvement of these host cells, a more in depth study tracing the route of bacteria and then determining the viability of this bacteria is required to define a mechanism of transfer. Discovering the viability of the bacteria post transfer will also help to determine if this is an invasion tactic employed by the bacteria to achieve its survival or if it is simply a non-pathogen specific process by which DCs aquire antigens from more efficient phagocytic cells.

NETs may offer a mechanism by which pathogen is transferred from PMN to DCs. In some TEM images (not shown) some evidence of what may be NETs appears in cultures of PMN infected with WT *P. gingivalis.* More investigation is warranted to determine if these structures are comprised of histones and neutrophil elastase and are therefore NETs, and if these NETs and their trapping of *P. gingivalis* affect DC outcomes.

Figure 1

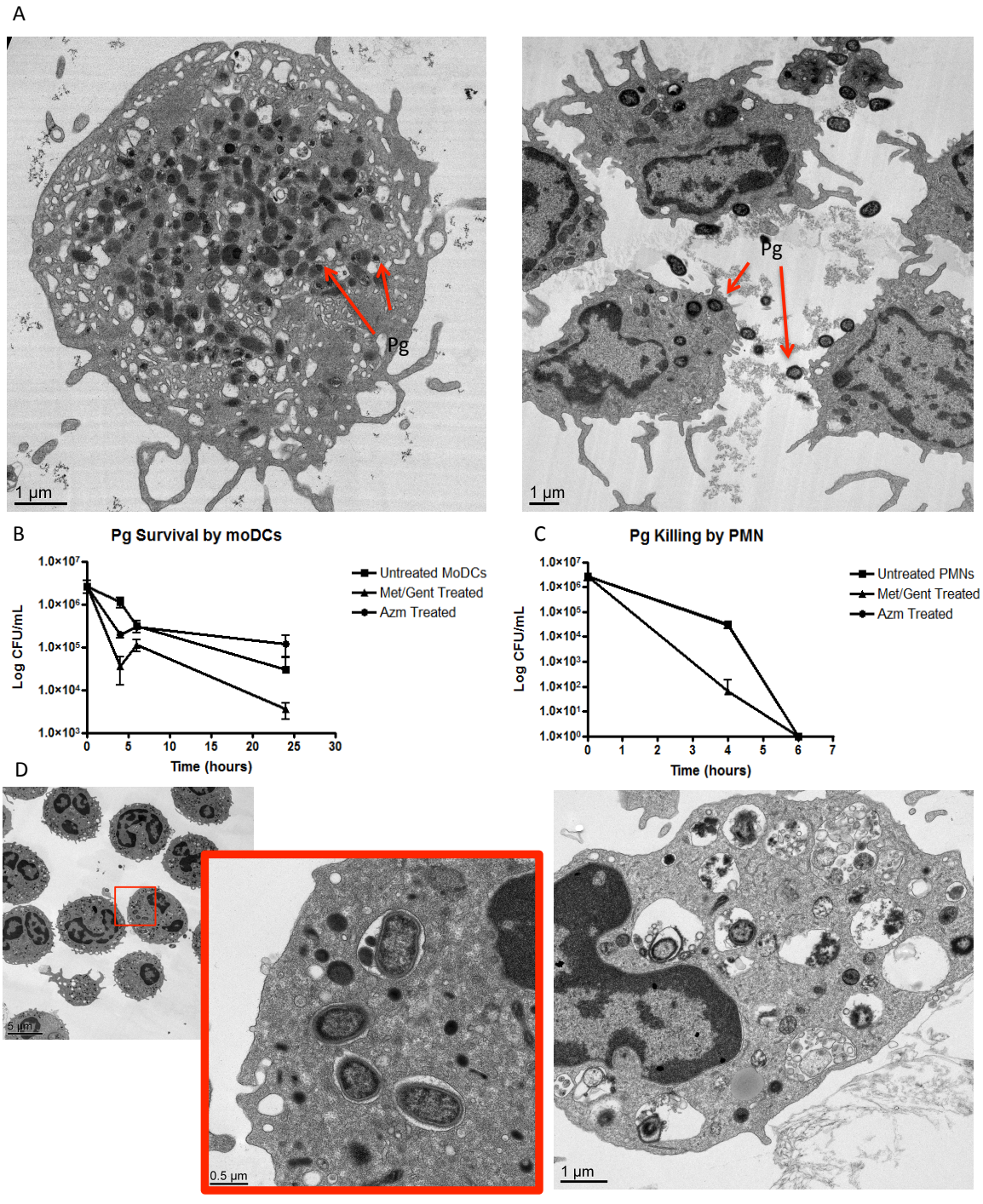
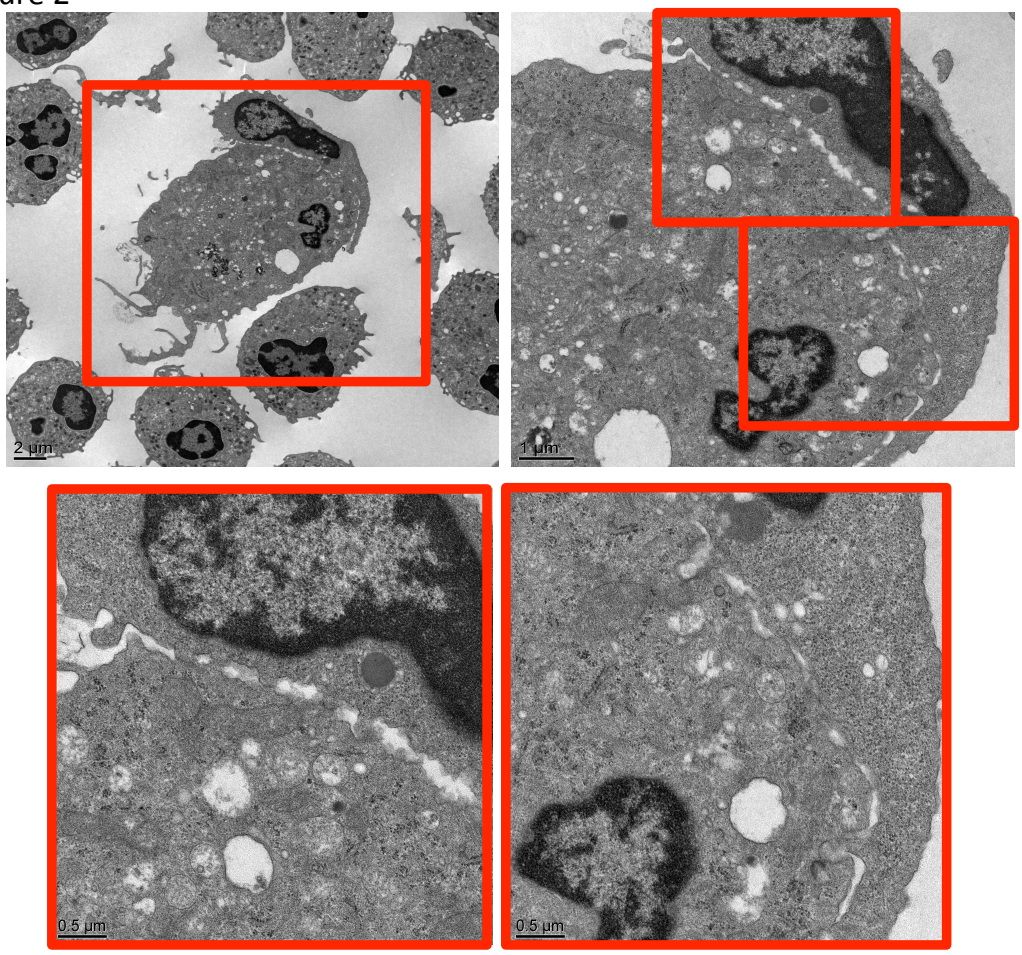

A
Pg
1 µm
Pg
1 µm
B
Pg Survival by moDCs
Log CFU/mL
Time (hours)
Untreated MoDCs
Met/Gent Treated
Azm Treated
C
Pg Killing by PMN
Log CFU/mL
Time (hours)
Untreated PMNs
Met/Gent Treated
Azm Treated
D
0.5 µm
1 µm

Fig1: **moDCs/mDCs uptake *P. gingivalis* and promote its survival while PMNs kill *P. gingivalis***

A. TEM images of moDCs (left) and primary isolated blood mDCs (right) 6 hours post-infection with *P. gingivalis.* Bacteria is seen intracellular and intact. B. Graph showing number of colony forming units (CFU/mL) as calculated from *P. gingivalis* colonies recovered on blood agar plates from moDC cell lysate. Bacteria was recovered after treatment of moDCs with metronidazole/gentamycin from cell lysate at 4, 6 and 24 hours. C. Graph showing number of CFU/mL calculated from *P. gingivalis* colonies recovered on blood agar places from PMN cell lysate. Each point on the graph represents an average CFU over three separate experiments and error bars signify SD. Bacteria was recovered after 4 hours, but none was recoverable by 6 hours, with or without antibiotic protection. D. TEM image of *P. gingivalis*-infected PMN isolated from the whole blood of a healthy volunteer. Cells were infected an MOI of 10 and uptake of bacteria is visible in the first image and in the close up (red outline). Image was taken 1.5 hours post infection and signs of bacterial degradation can be seen. E. Close up image of Pg infected PMN. Signs of autophagy are visible with large double membrane vacuoles enclosing Pg that appears heavily degraded. Cells were fixed for imaging 3 hours post-infection at MOI 10.

Figure 2

A



Merge DAPI blue Texas Red FITC

B

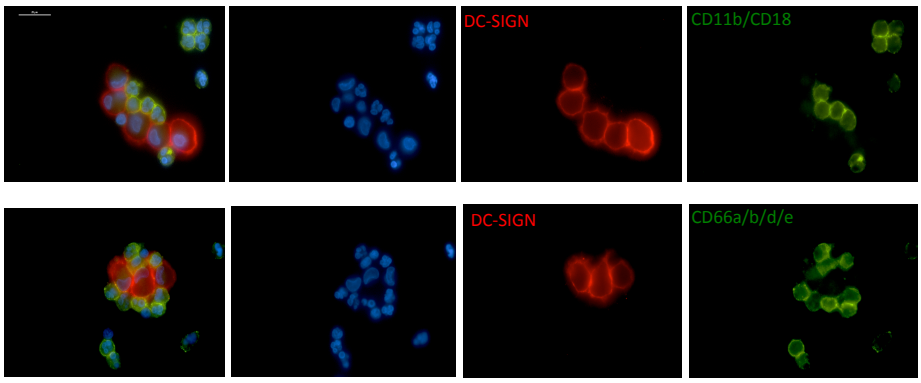


Fig2: **Intimate associations between membranes of moDCs and PMNs form in co-culture**

A) A series of TEM images demonstrating conjugation between a PMN and an moDC. Cells were co-cultured at a ratio of 5:1 PMNs:moDC for 60 minutes. Cells were prepared for microscopy by suspension in a fixative containing gluteraldehyde and paraformaldehyde to allow for cross linking and preservation of the cell membrane. Note the extremely close association of the membranes between cells, signifying deliberate physical association between these two cell types. Each image B-D are a higher magnification of the same field as shown in image A. Bars are 2um, 1um and 0.5um, respectively. B) Confocal fluorescence microscopy images (63x) showing moDCs stained for DC-SIGN conjugating with PMNs stained with either anti-Mac-1 (CD11b/CD18) (i) or a general granulocyte marker antibody for CEACAM molecules (CD66a/b/d/e) (ii). These images show co-localization between DC-SIGN and Mac-1, as well as DC-SIGN and CD66 both as yellow portions of overlap between Tx-red DC-SIGN and FITC Mac-1 or CEACAM, confirming that Mac-1 integrin and the CEACAMs play a role adhering PMNs to immature DCs via DC-SIGN.

Figure 3

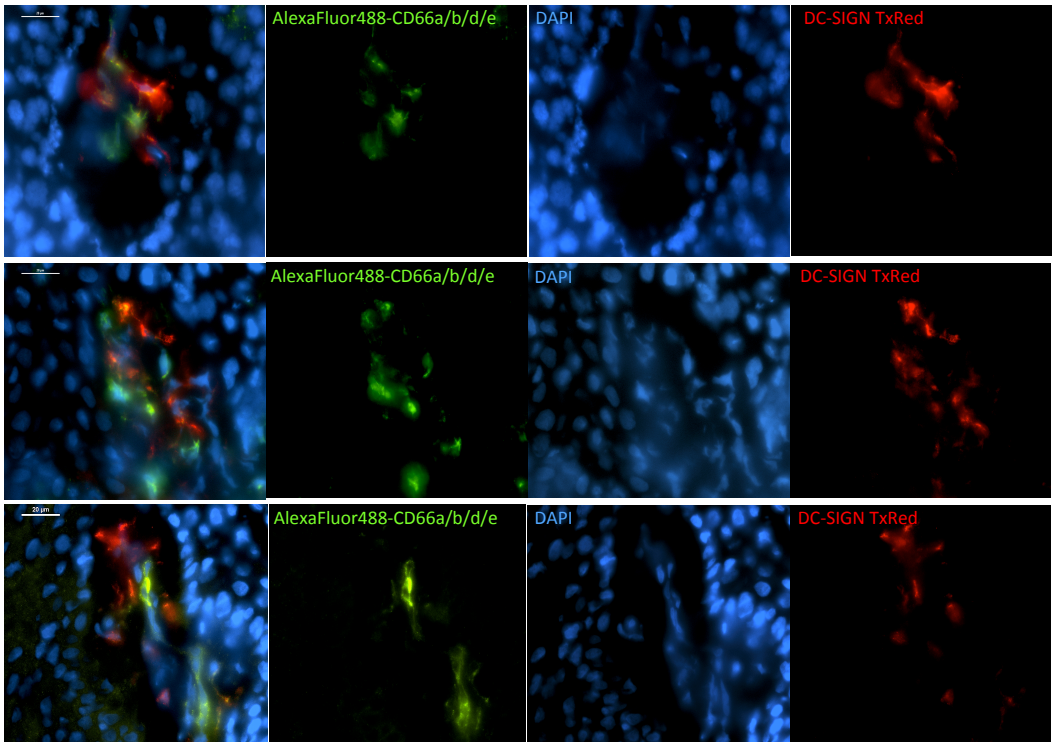


Fig3: **DCs and PMNs are found co-localized in gingival tissue of human subjects with periodontitis**

Immunohistochemical staining of three gingival samples taken from three individuals with at least moderate chronic periodontitis. A non-specific CEACAM family antibody conjugated with alexfluor488 stains PMNs in the green channel. Anti-DC-SIGN antibody conjugated with flouresen Texas-red stains immature DCs in the red channel. Nuclear staining is with Dapi Blue (UV channel). Both cell types were found co-localized in diseased gingival tissue in all three subjects. Samples of healthy gingiva showed no leukocyte infiltrate and therefore no evidence of PMN-DC co-localization (data not shown). Both cell types appear to be present together in a zone of the gingival epithelium deep to the rete pegs signifying the extent of the basement membrane, a zone typically reserved for immune cells handling the adaptive component of the immune defense.

Figure 4

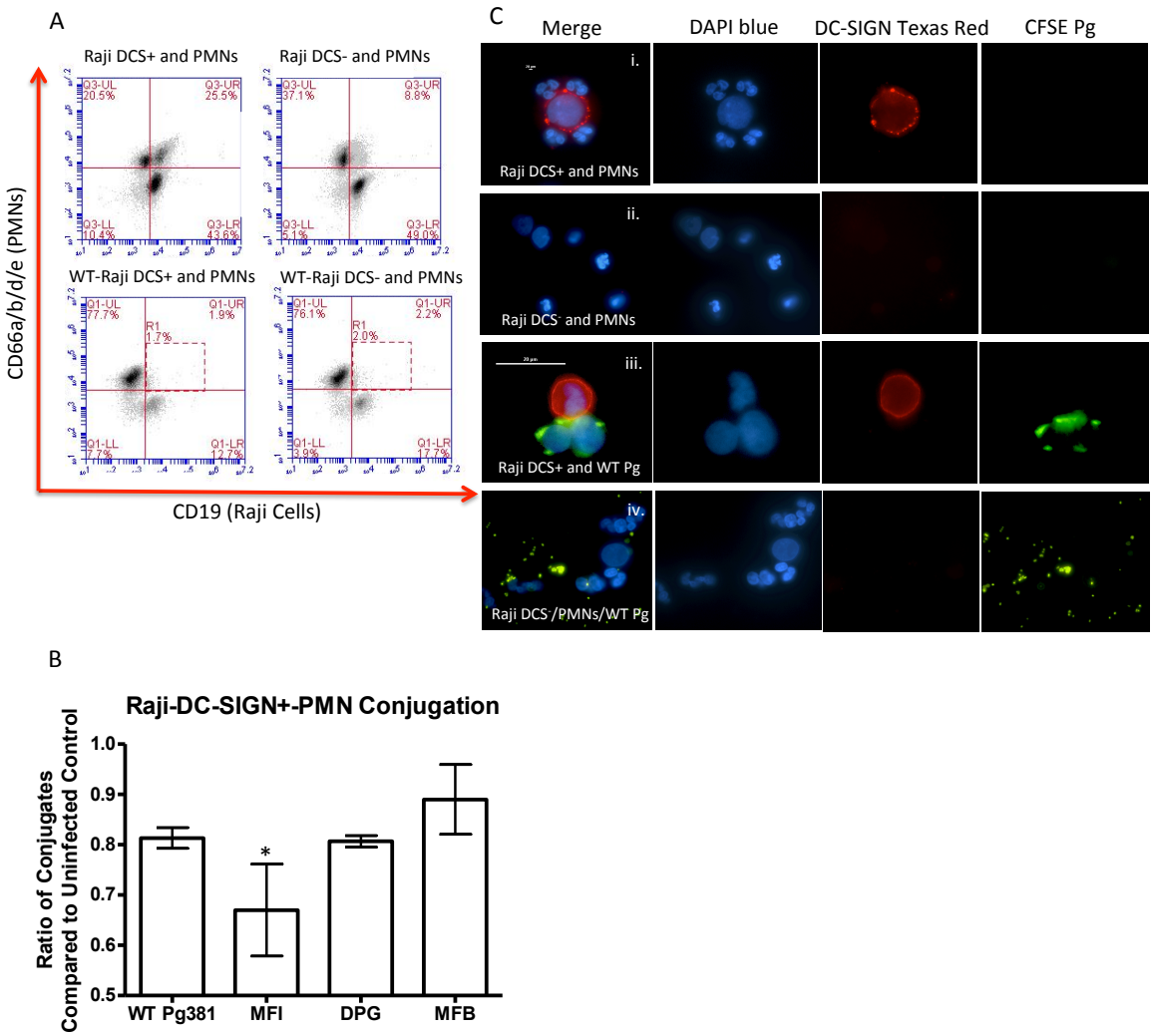


Fig4: **DC-SIGN is necessary and sufficient for forming cell conjugates with PMNs as evidenced by stably transfected Raji cell lines/ Raji-DCS+ cells pretreated with fimbriated strains of Pg381 were less able to conjugate with PMNs**

Raji DCS+/DCS- conjugation with PMNs: A. Flow cytometry scattergrams depicting FITC-CD66a/b/d/e-labled PMNs and PE-CD19-labled Raji cell conjugation by a double positive population in the UR quadrant. Conjugation with PMNs only occurs with Raji cells stably transfected to express DC-SIGN. Raji DCS+ cells treated for 30 min with wild-type Pg381 at MOI 10 were less likely to conjugate with PMNs ($p<0.001$). Raji DCS- cells treated with Pg381 were unaffected. WT-treated Raji DCS+ cells conjugate with PMNs in similar amounts as Raji DCS- cells with PMNs. B. Graph quantitatively showing the ratio of RajiDCS+-PMN conjugates with control co-cultures of uninfected PMNs and Raji cells. Bar heights indicate the mean of 3 identical experiments and error bars represent SD. Ratio of conjugates formed in the presence of WT, MFI, and DPG strains of Pg381 is all significantly less than 1 ($p<0.05$). Pretreating Raji-DCS+ cells with the nonfimbriated MFB reduced conjugate formation compared to control but not significantly. Major FimA expressing strain

MFI was more successful than the WT in blocking cell interactions ($p<0.05$). C. Confocal fluorescence microscopy images (63x): (i) PMNs clustered around Raji DCS+ cells in the absence of Pg, (ii) Raji DCS- cells co-cultured with PMNs in the absence of Pg. Note that no association between this sample of cells is apparent. (iii) Raji-DCS+ cells cultured with CFSE-stained WT Pg381 at MOI10, which blocks surface staining with an anti-DC-SIGN antibody on two of the cells shown, (iv) Raji DCS- cells treated with WT Pg381 and co-cultured with PMNs. Conjugation with PMNs does not occur in the absence of DC-SIGN, nor does accumulation of Pg on Raji cell surfaces.

Figure 5

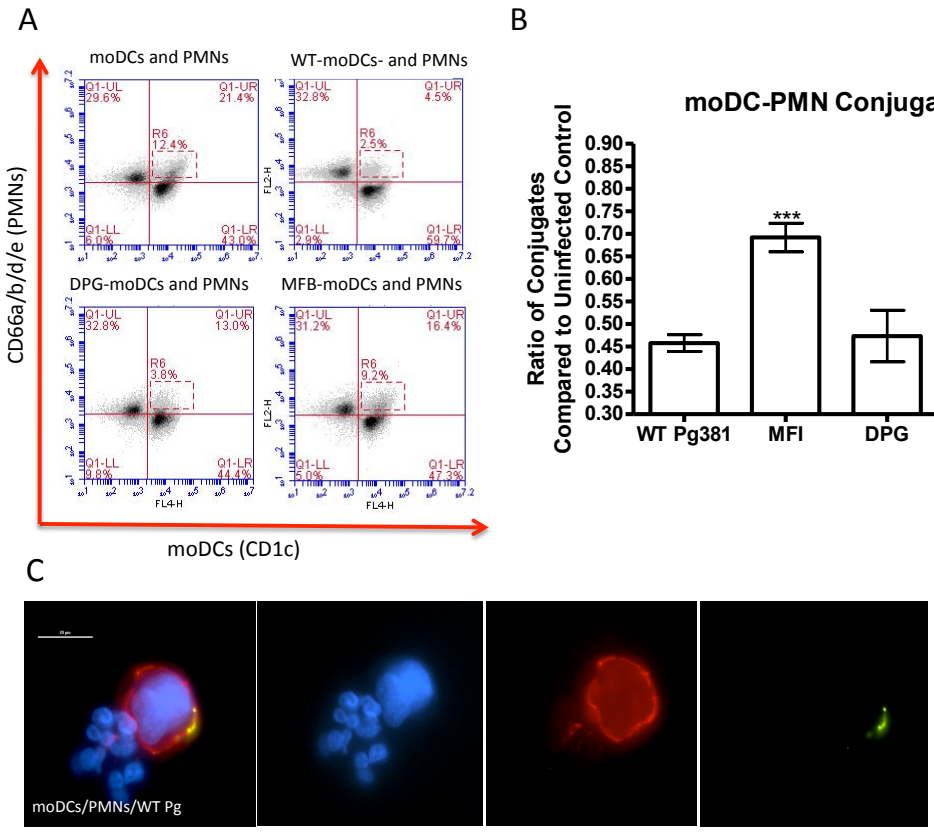


Fig5: **Fimbriated strains of Pg381 blocked conjugation of infected moDCs with PMNs**

A. Flow cytometry scattergrams depicting FITC-CD66a/b/d/e-labled PMNs and PE-CD1c-labled moDC conjugation by a double positive population in the UR quadrant. Conjugation with PMNs is highest with non-infected moDCs and is drastically decreased when moDCs are infected with the WT strain and minor fimbriated strain, DPG-3. Pg MFB fimbriae-deficient strain lowered moDC affinity for PMN but were significantly close to control conditions. MoDCs treated for 30 min with Pg at MOI 10. B. Graph quantitatively showing the ratio of moDC-PMN conjugates to control co-cultures of uninfected PMNs and moDCs. Bar heights indicate the mean of four identical experiments and error bars represent SD. All ratios are under 1, signifying conjugation occurred less with exposure to all strains than in controls. However MFB and major-fimbirated strain MFI are significantly closest to control conditions ($p<0.0001$). C. Confocal fluorescence microscopy images (63x): moDCs cultured with CFSE-stained WT Pg381 (green channel) and PMNs. Pg is co-localized with PE-conjugated anti-DC-SIGN and PMNs cluster at DC-SIGN positive sites not blocked by Pg.

Figure 6

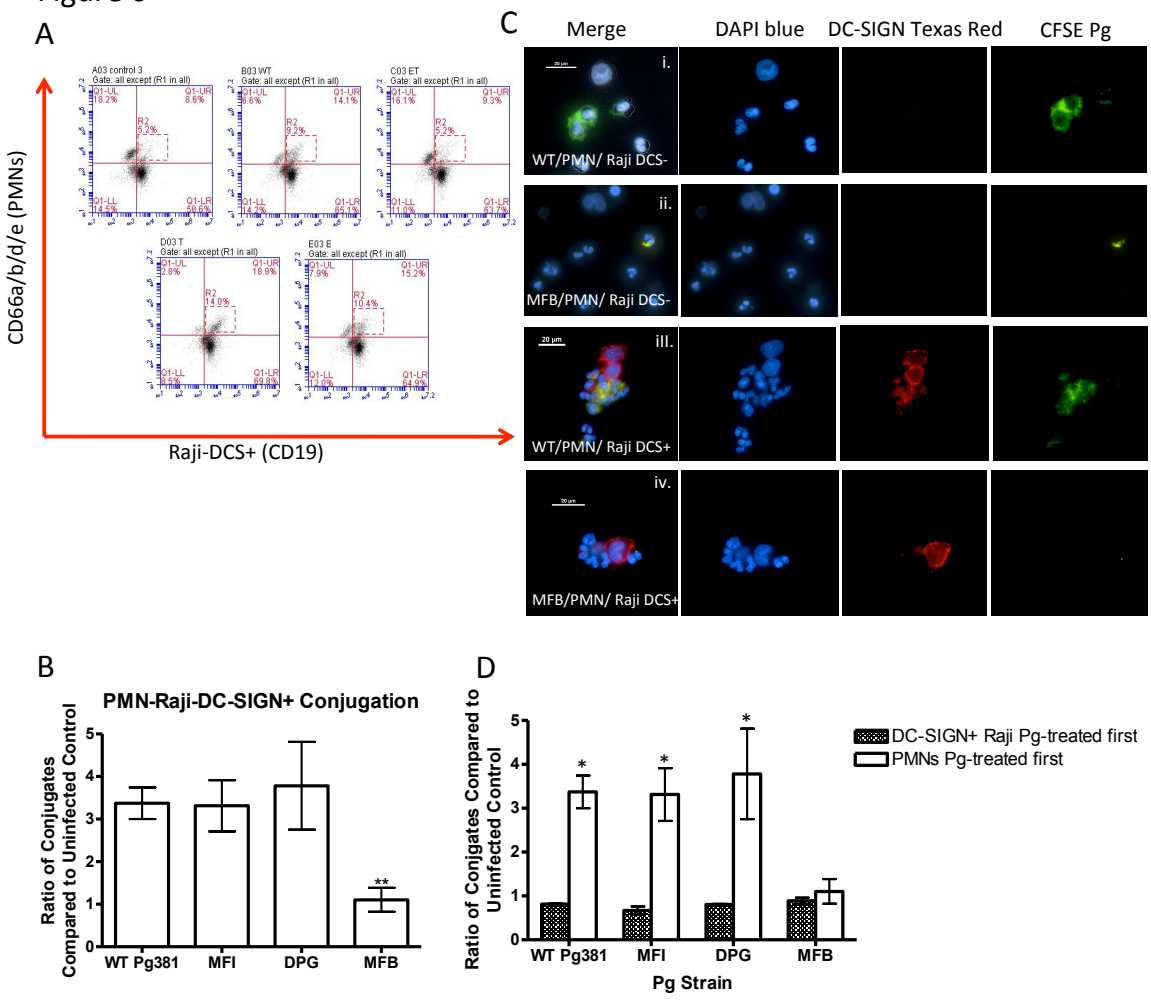


Fig6: **Fimbriated strains of *P. gingivalis*-treated PMNs increase conjugation with Raji DCS+ cells**

A. Flow cytometry scattergrams representative of one experiment out of 4 identical experiments showing that PMNs treated with wild type Pg381, DPG, a strain expressing only the minor fimbriae, and MFI, a strain expressing only the major fimbriae, and co-cultured with Raji DCS+ cells formed more conjugates than co-cultures with no bacteria added or with infection by MFB, a non-fimbriated Pg381 mutant. Double cell conjugates are in the UR quadrant of each scattergram. B. Graph summarizing flow cytometry data showing increased association of PMNs and moDCs when PMNs are treated with wild-type Pg381, or one of the fimbriated mutants in terms of fold increase compared to control ($p<0.01$ compared to control). PMNs treated with non-fimbriated strain MFB or PMNs that remain untreated associate less with moDCs (**=$p<0.0.1$ compared to other mutant strains) . PMNs are stained with FITC-conjugated anti-CD66a/b/d/e, and moDCs with PE-conjugated anti-DC-SIGN. Graph represents average conjugate formation as recorded in 4 identical experiments. C. Confocal fluorescence microscopy images (63x): (i) wild type Pg381 treated PMNs and Raji DCS- cells exhibit no conjugation. PMNs are able to take up a

significant amount of wild-type Pg. (ii) MFB-Pg381 treated PMNs and Raji DCS- cells also exhibit no conjugation. Unlike the wild-type, PMNs are unable to take up a large amount of the non-fimbriated mutant. (iii) wild type Pg381 treated PMNs and Raji DCS+ cells exhibit increased association. (iv) MFB-Pg381 treated PMNs conjugate with Raji DCS+ cells to the same degree as untreated controls. The non-fimbriated mutant is not taken up by PMNs. D. Graph summarizing the fold differences of RajiDCS+- PMN conjugates formed when Raji cells are infected with the four strains of Pg prior to co-culture with PMNs vs. when PMNs are Pg infected first, as obtained from conjugate detection by flow cytometry. The ratio of conjugates formed is greater when PMNs are infected before co-culture with all strains ($p<0.05$), however the non-fimbriated mutant Pg381MFB shows the smallest fold difference. Ratios were calculated from the average of 4 identical experiments. Error bars represent SD.

Figure 7

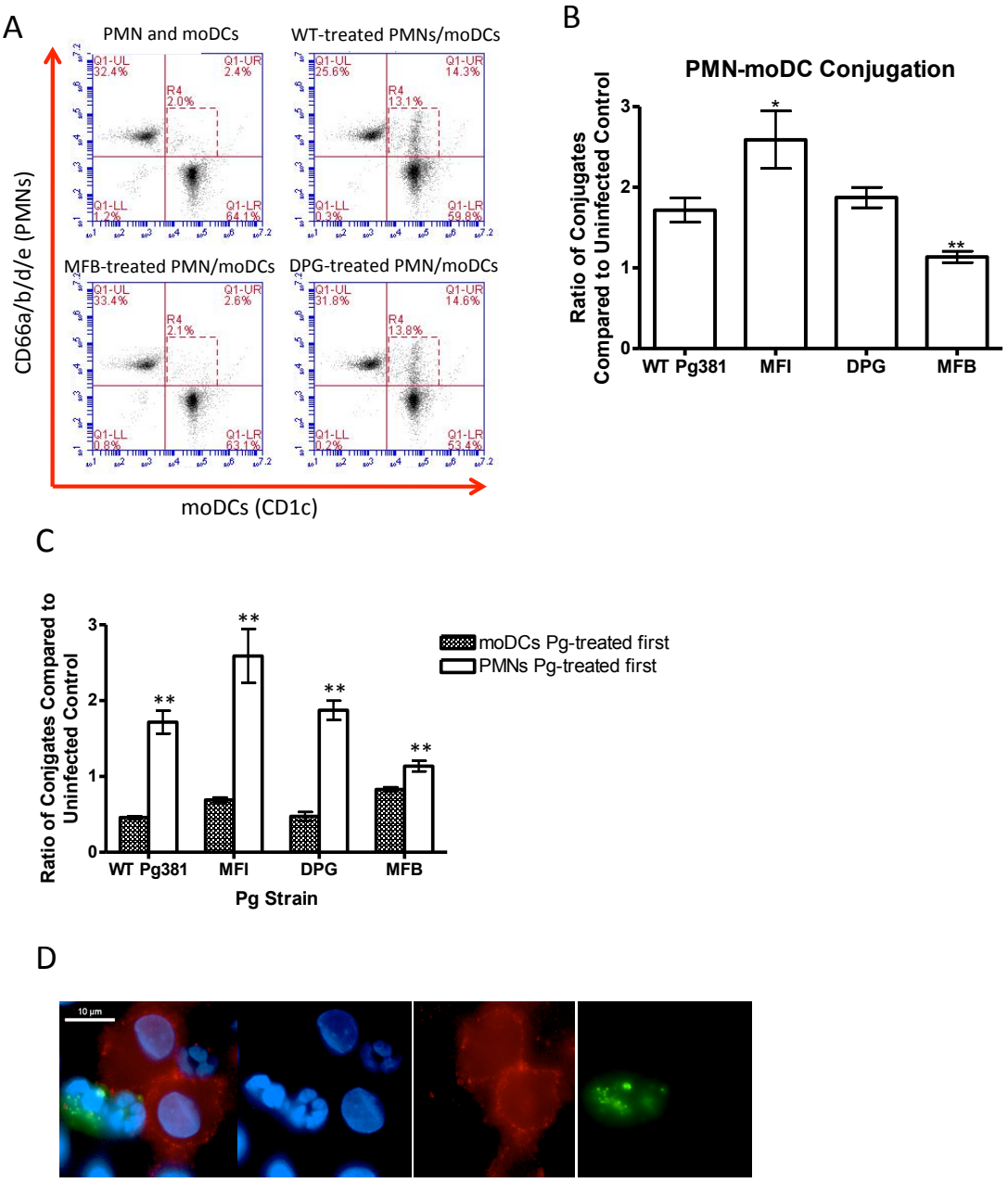


Fig7: **Fimbriated strains of Pg encourage moDC-PMN conjugation when PMNs are Pg infected before co-culture**

A. Flow cytometry scattergrams showing increased association of PMNs and moDCs when PMNs are treated with wild-type Pg381, or one of the fimbriated mutants. PMNs treated with non-fimbriated strain MFB or PMNs that remain uninfected associate less with moDCs . PMNs are stained with FITC-conjugated anti-CD66a/b/d/e, and moDCs with PE-conjugated anti-CD1c. B. Graph summarizing flow cytometry results of four identical experiments conducted with PMNs infected with four strains of Pg prior to co-culture with moDCs. Heights represent mean ratio of

conjugates compared to control (no Pg infection) and error bars represent SD. All strains except MFB show significant increase in conjugate formation as compared to control (**=$p<0.01$). Pg MFI (major fimbriated strain) shows significantly higher levels of conjugation than compared to WT Pg (*=$p<0.05$), and MFB infection demonstrates significantly lower levels of conjugation as compared to WT infection (***=$p<0.0001$). C. Graph summarizing the fold differences of moDC-PMN conjugates formed when moDCs are infected with the four strains of Pg prior to co-culture with PMNs vs. when PMNs are Pg infected first, as obtained from conjugate detection by flow cytometry. The ratio of conjugates formed is greater when PMNs are infected before co-culture with all strains (**=$p<0.01$), however the non-fimbriated mutant Pg381MFB shows the smallest fold difference. Ratios were calculated from the average of 4 identical experiments. Error bars represent SD. D. Confocal fluorescence microscopy images (63x): PMNs cultured with CFSE-stained WT Pg381 (green channel) and then co-cultured with moDCs. Pg is seen taken up by PMNs which are closely associated by moDCs stained with PE-conjugated anti-DC-SIGN.

Figure 8

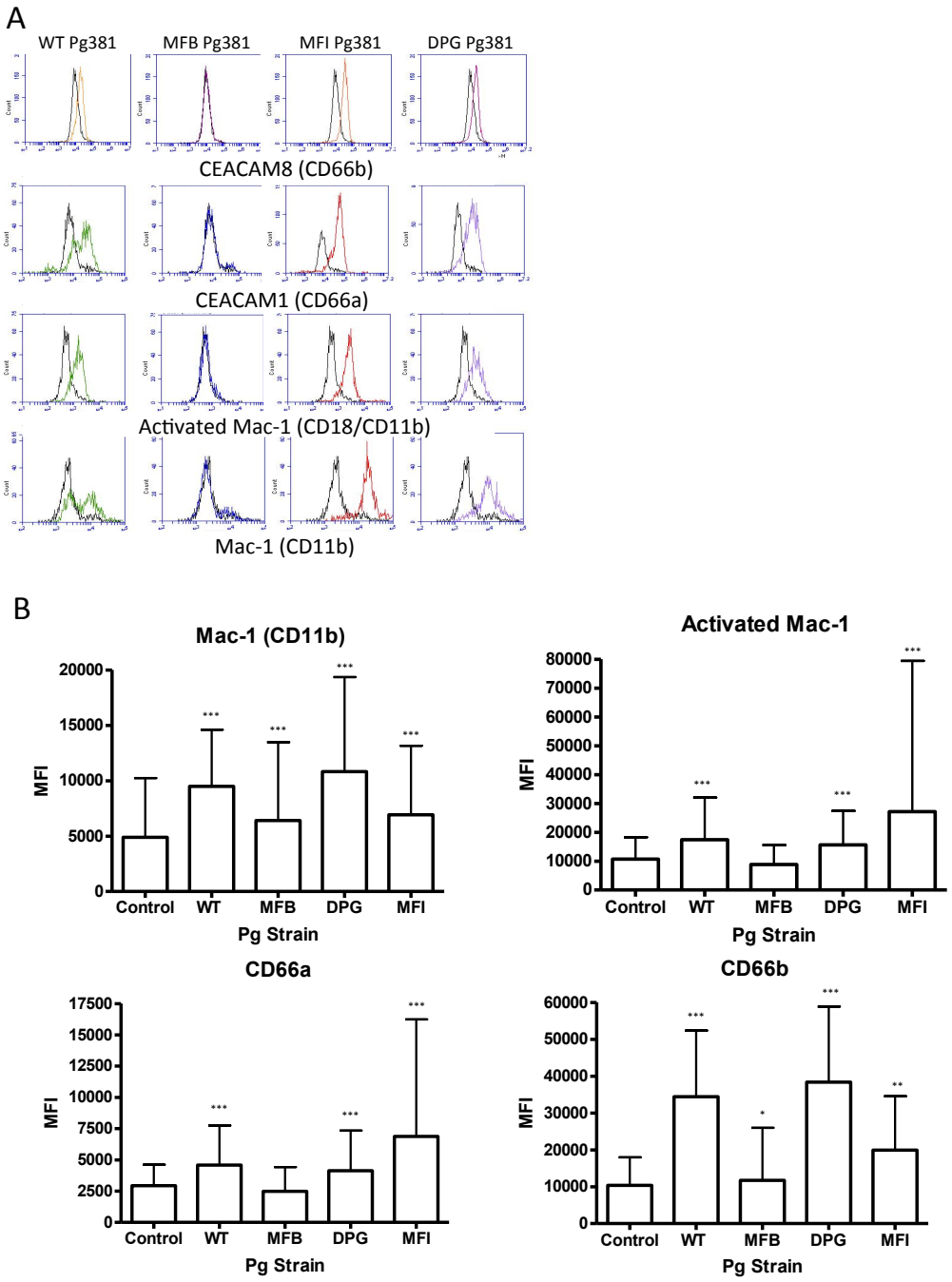


Fig8: **Fimbriated strains of Pg cause surface phenotype changes in PMNs**
A. Histograms showing increased expression of PMN surface molecules, CD66a, CD66b, CD18/CD11b, and CD11b when infected with WT, minor fim-deficient strain DPG, and major fim-decificient strain MFI. Non-fimbriated strain MFB did not result in any increase. All shifts are significant ($p<0.01$) by comparison of means and coefficients of variation. Black traces represent control levels with no exposure to bacteria. Cells were fixed 30 minutes post-infection. Results are from one representative experiment of four identical experiments all demonstrating similar

trends. **B.** Graphs summarizing average change in expression in four identical experiments, bar heights representing the average mean fluorescence intensity (MFI) and error bars represent SD. All markers show significant increases in expression of CD11b, CD11b/CD18 (activated Mac-1), CD66a, CD66b for all fimbriated strains of Pg as compared to control (***=$p<0.0001$, **=$p<0.01$, *=$p<0.05$). Non-fimbriated MFB infection causes significant upregulation of Mac-1 (CD11b) and CD66b (***=$p<0.0001$, and *=$p<0.05$, respectively) compared to uninfected control.

Figure 9

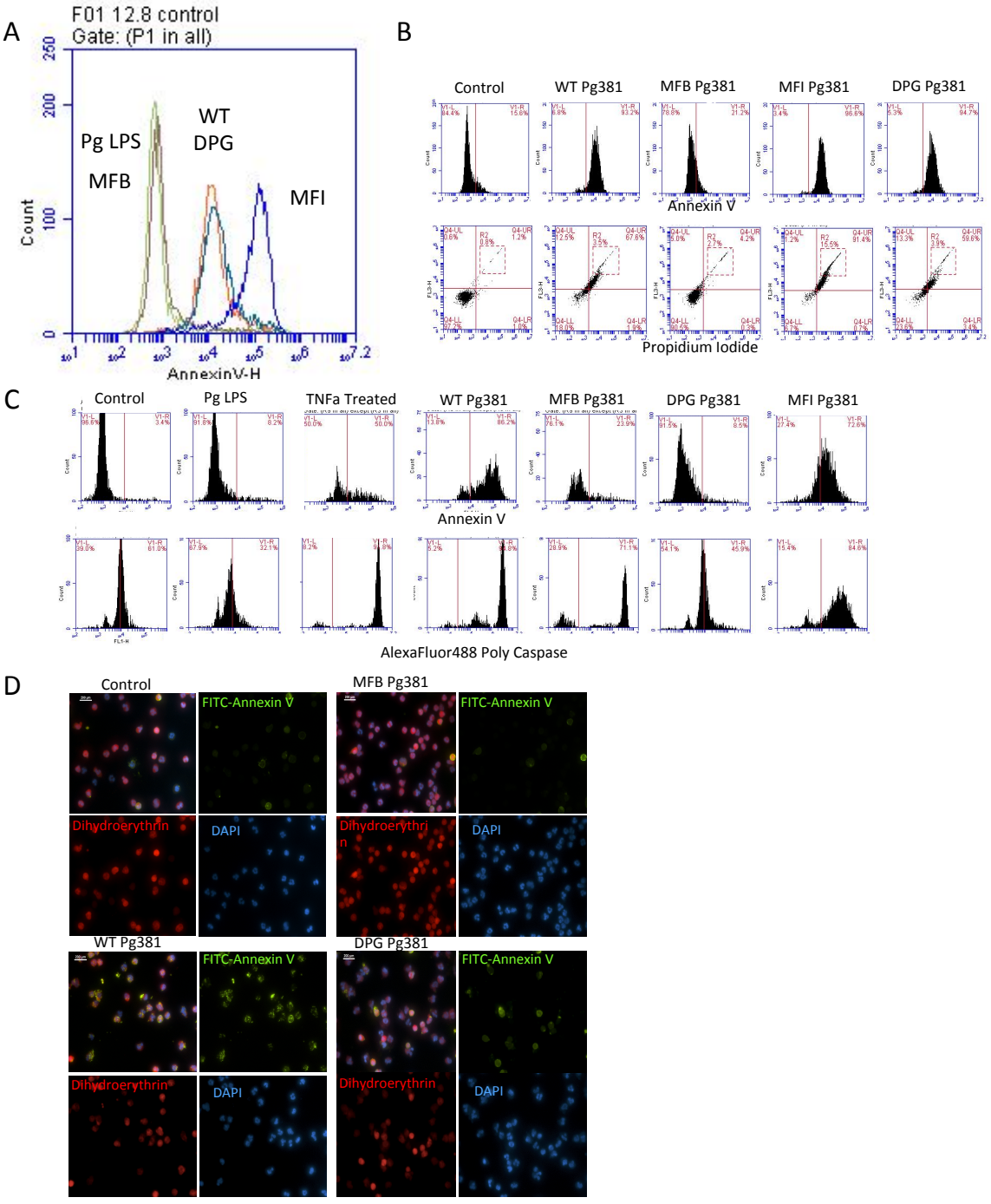
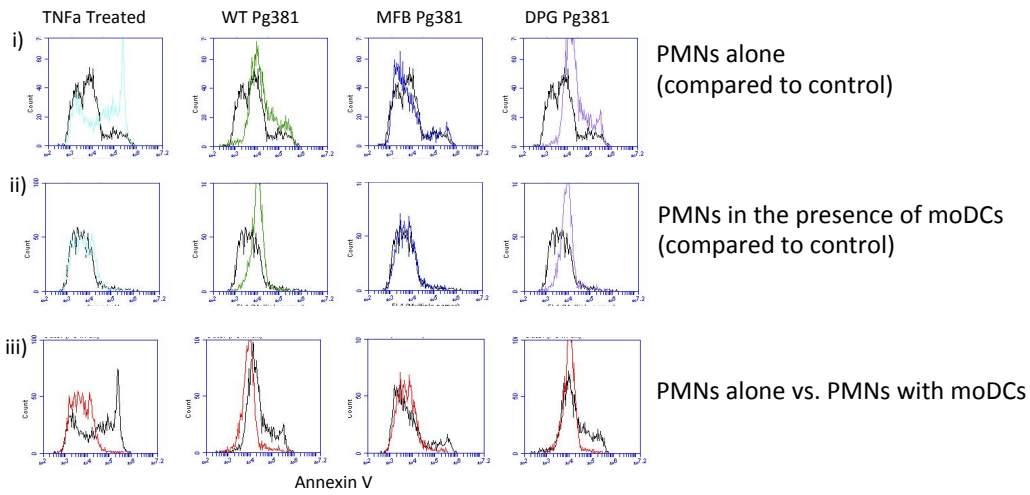


Fig9: **PMNs undergo cell death when challenged with fimbriated strains of *P. gingivalis***

A. Flow cytometry generated histogram showing relative expression of surface Annexin-V on PFM exposed to isolated Pg LPS, non-fimbriated strain MFB, isogenic mutants DPG and MFI, and WT Pg. Strain MFB and LPS do not elicit in increase in Annexin-V (FL-1), suggesting that apoptosis is not caused by these challenges. WT and strain DPG, expressing the major fim showed increased expression of annexin-V,

and major fimbriated strain MFI exhibits the strongest upshift of Annexin-V. B. Histograms showing levels of surface Annexin-V and propridium iodide uptake of PMNs as proxys for PMN apoptosis and end-stage necrosis, respectively, when exposed to WT, MFB, DPG, and MFI strains as opposed to uninfected controls. Increases in expression of surface Annexin-V as well as increases in propridium iodide uptake increase in the presence of all strains of Pg, least of all non-fimbriated MFB, and most drastically with infection of minor-fim expressing isogenic mutant MFI. Propridium iodide positivity shown in scattergrams as this dye is detected by the overlap of FL-1 and FL-2 channels. Histograms are representative of 4 identical experiments showing the same trends. Shifts are significant with $p<0.01$ as calculated from mean fluorescence intensity (MFI) and coefficient of variation. C. Histograms showing Annexin-V shifts in tandem with Polycaspase activiity, both parameters measured with a manufacturers kit. This experiment is representative of 4 identical ones all showing similar trends in expressivity. Positive control TNFa shows the highest caspase peak shift followed by WT Pg. MFB also causes a similarly intense caspase activation despite only slight expression of extracellular annexin V. Caspase activation with challenge of Pg LPS is similar to control. D. Confocal fluorescence microscopy images showing PMNs at 20x magnification. Green channel reveals extracellular AnnexinV positivity, red channel shows dihydroethidium staining signaling no loss of membrane integrity. Nuclear staining is with DAPI blue. Each set of images demonstrates one of four conditions: uninfected control, WT Pg, non fimbriated MFB, and minor fimbriated strain DPG. WT and DPG elicit the most fluorescence in the green channel signifying extracellular annexin V and therefore apoptotic activity.

Figure 10



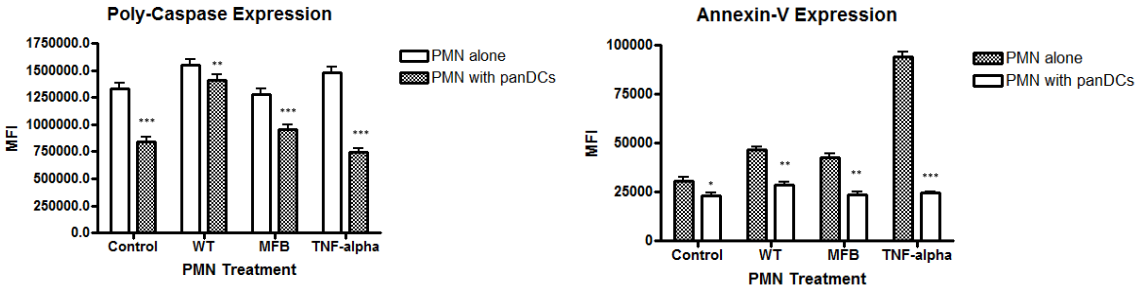


Fig10**: DCs cultured with PMNs infected with *P. gingivalis* reverse apoptosis of PMNs**

A. Histograms generated by flow cytometry experiments depicting expression of extracellular AnnexinV as a marker for apoptosis. Row (i) shows annexin V levels expressed by PMNs exposed to TNFa as a positive control as well as WT, MFB and DPG strains of Pg as compared to control levels in which cells were not exposed/infected (black tracing). Row (ii) shows annexin V levels expressed by PMNs under the same conditions as (i) except that moDCs were introduced to PMNs 30 minutes post infection as compared to a control of un-infected PMNs co-cultured with moDCs (black tracing). Row (iii) demonstrates annexin V levels comparing exposed/infected PMNs (black tracing) to those co-cultured with moDCs (red tracing). Histograms are representative of three identical experiments all demonstrating similar trends. B. Graph demonstrating poly-caspase activity in Pg infected PMNs either alone or with panDCs isolated from the blood of healthy volunteers. Bar heights represent average mean fluorescence intensity and error bars represent SD. Co-culture with panDCs significantly reduced levels of poly caspase activity under all conditions (**=p<0.001, ***=p<0.0001). The greatest effect can be seen in the positive control, in TNF-a treated PMNs with and without exposure to panDCs. C. Graph showing Annexin-V expression by PMNs alone infected with

either WT Pg, MFB strain Pg and positive and negative controls and infected/exposed PMNs cultured with panDCs isolated from the blood of healthy volunteers. Bar heights represent average MFI and error bars, SD. Positive control conditions, exposure to TNFa caused the greatest Annexin V positivity, and the greatest shift with the addition of panDCs. All shifts between PMNs alone and PMNs with panDCs are significant (**=$p<0.001$, ***=$p<0.0001$).

Figure 11

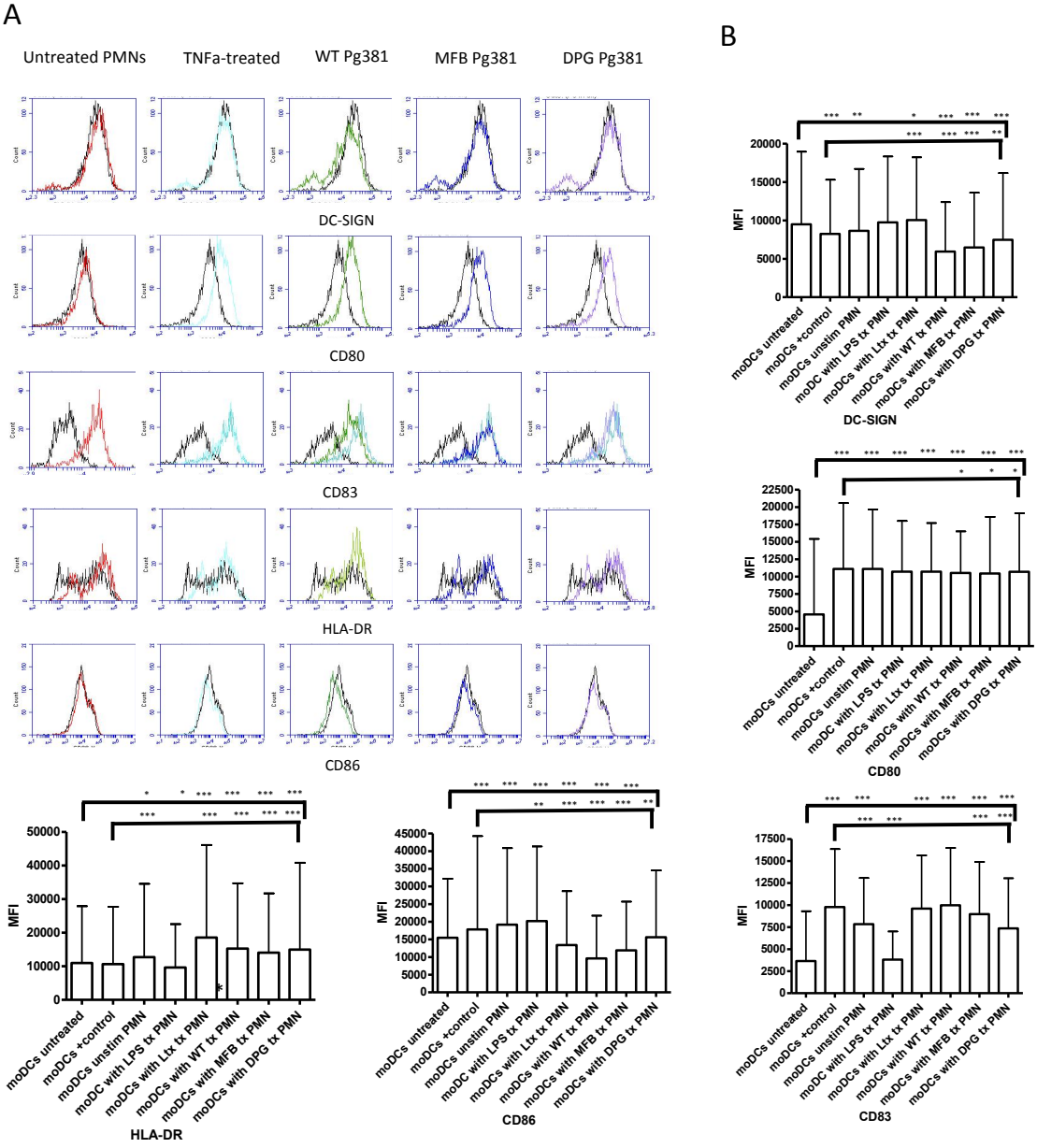


Fig11: **PMNs exposed to P. gingivalis can modulate the maturation profile of moDCs**

A. Representative histograms showing surface expression of various DC markers after 24-hours exposure to untreated PMNs and PMNs infected with WT Pg, non-fimbriated MFB Pg, minor-fimbriated isogenic mutant DPG Pg, and TNFa. Results are represented on four identical experiments all showing similar trends. Each histogram compares negative control, moDCs alone (black trace) to untreated PMNs (red tracing), PMNs exposed to TNFa (light blue trace), PMNs infected with WT Pg (green trace), PMNs infected with MFB (dark blue trace), and DPG major-fim deficient strain (violet trace). DC-SIGN, typically expressed on immature moDCs, is decreased with exposure to infected PMNs, while maturation markers CD80, CD83, HLA-DR are

increased. CD86, an end-stage maturation marker for moDCs remains the same or is downshifted with Pg infected PMNs. **B.** Graphic representation of four experiments with moDCs subjected to infected PMNs for 24 hours. Similar trends as seen in (A) are observable. These experiments included A.a. leukotoxin exposed PMNs as well as Pg LPS treated PMNs. Bar heights indicate average mean fluorescence intensity (MFI) and error bars signify SD. Statistical significance is denoted as compared to moDCs with no co-culture (negative control) and moDCs treated with TNFa to encourage maturation (positive control) (***=$p<0.0001$, **=$p<0.01$, *=$p<0.05$).

Figure 12

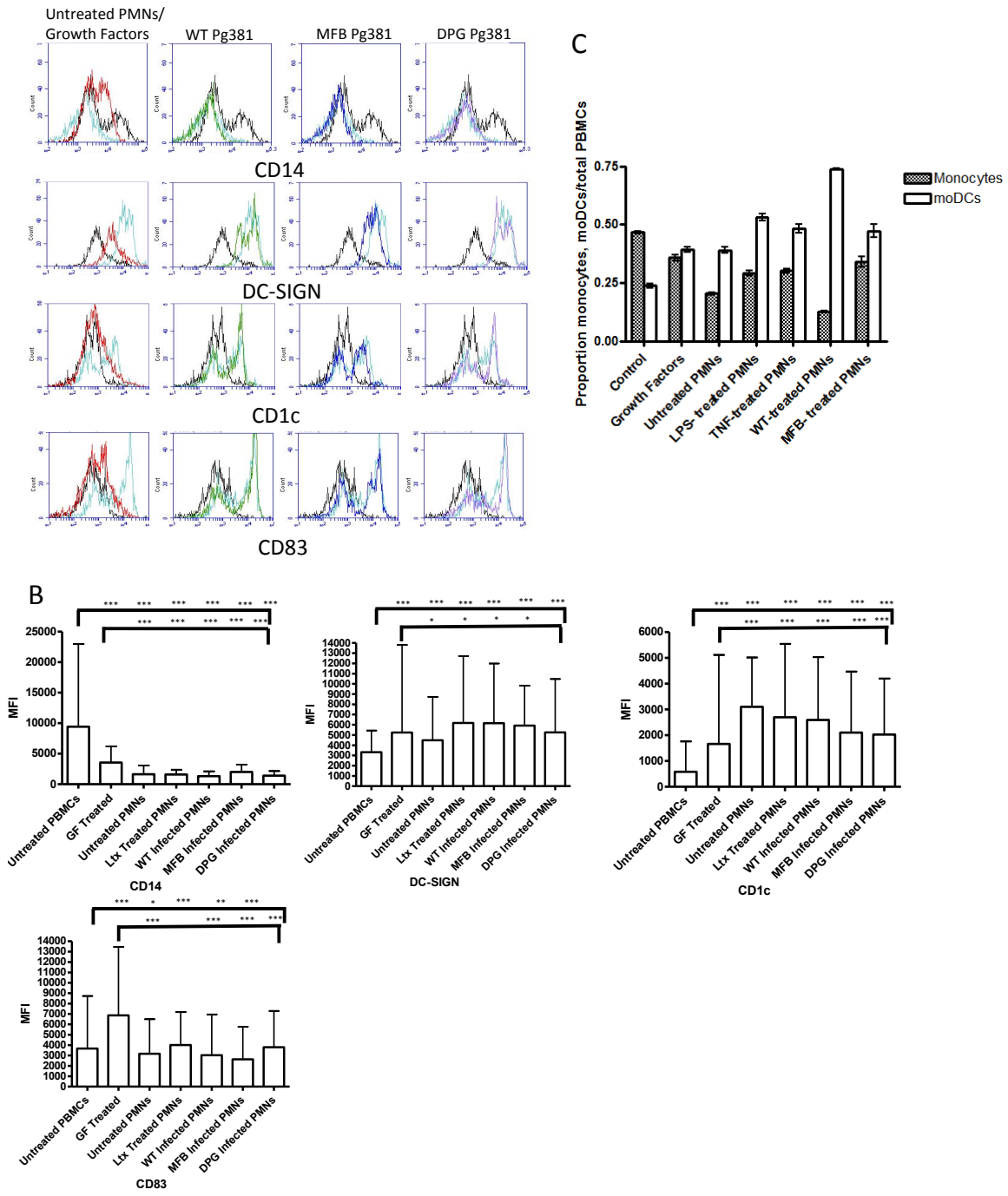


Fig12: **PMNs both untreated and exposed to Pg are able to cause peripheral blood monocytic cells (PBMCs) to develop an moDC suface phenotype**

A. Flow cytometry histograms showing shifts in surface expression of monocytic and moDC markers CD14, DC-SIGN, CD1c, and CD83. Each compares monocytes alone (black trace) to positive control monocytes subjected to GM-CSF and IL-4 growth factors to promote monocyte differentiation to moDCs (light blue trace), to

monocytes exposed to either untreated PMNs (red trace), WT infected PMNs (green trace), MFB non-fimbrated strain infected PMNs (dark blue trace), and DPG major-fim deficient mutant strain infected PMNs (violet). **B.** Graphic representation of four experiments with blood monocytes subjected to infected PMNs for 24 hours. Similar trends as seen in (A) are observable. These experiments included A.a. leukotoxin exposed PMNs. Bar heights indicate average mean fluorescence intensity (MFI) and error bars signify SD. Statistical significance is denoted as compared to monocytes with no co-culture and no added growth factors (negative control) and monocytes treated with IL-4 and GM-CSF to encourage conventional differentiation to immature moDCs (positive control) (***=$p<0.0001$, **=$p<0.01$, *=$p<0.05$). **C.** Graphical representation comparing ratios of monocytes and differentiated moDCs to total number of PBMCs based on gating for forward and side scatter characteristics of each cell type. Bar heights represent the average ratio over four identical experiments and error bars represent SD. MoDC proportion exceeds the proportion of monocytes to a statistically significant degree in all conditions with the exception of the negative control (***=$p<0.0001$, **=$p<0.01$, *=$p<0.05$).

Figure 13

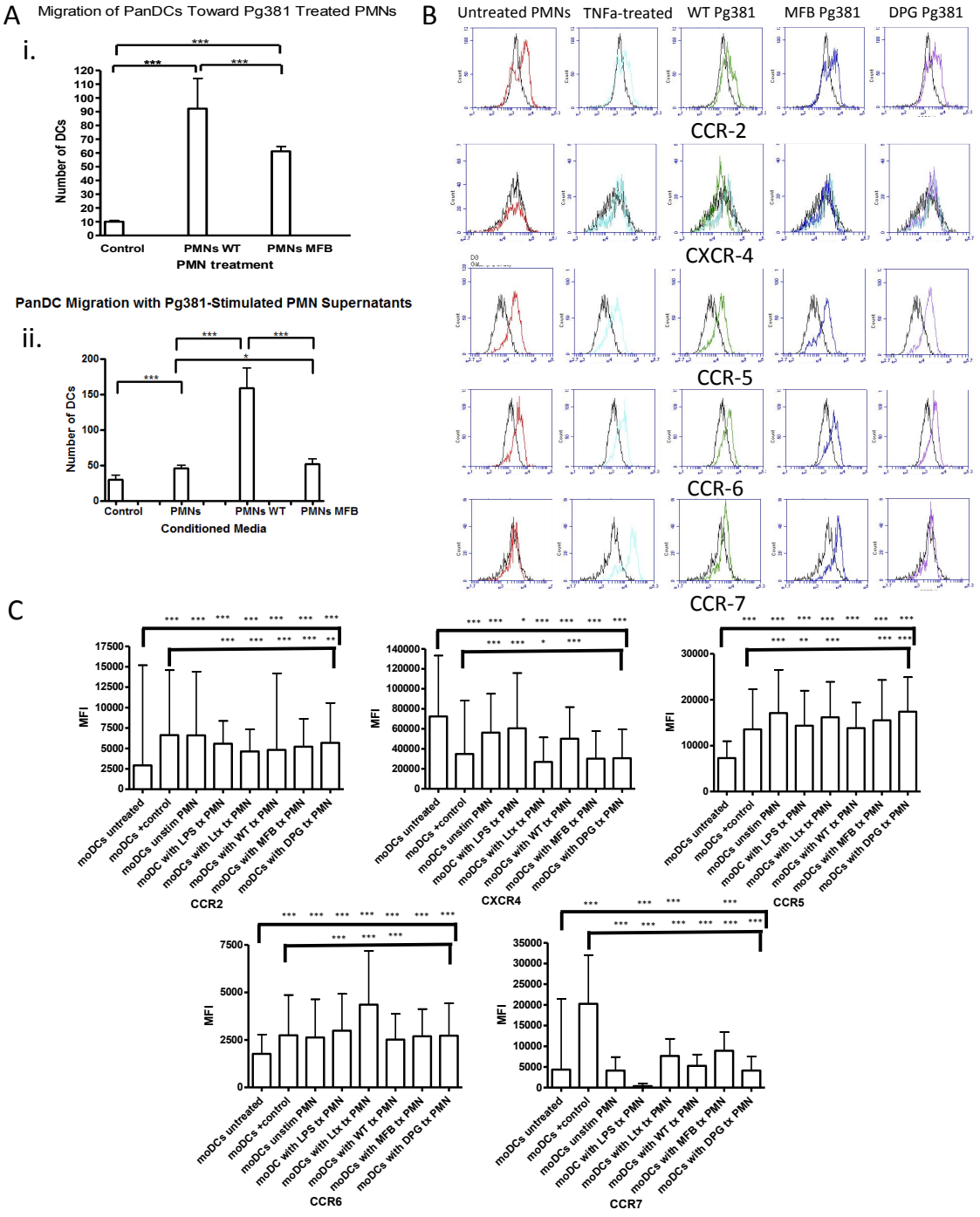


Fig13: **PMNs, especially PMNs infected with fimbriated strains of Pg, encourage moDC/panDC migration both functionally and phenotypically**

A. (i) Graph demonstrating number of panDCs isolated from whole human blood of healthy donors shown to be migrating toward uninfected PMNs and PMNs infected with either non-fimbriated MFB Pg strain or WT Pg in a boyden chamber assay. Negative control was media containing no PMNs (ii) Graphical results of the same

experiment conducted with supernatants collected after 30 minutes of PMN infection with Pg. Supernatant from uninfected PMNs was also included. Bar heights in both (i) and (ii) represent average number of panDCs counted systematically over three experiments, each experiment including triplicate wells for each condition. Error bars represent SD. *=p<0.05, ***=p<0.0001 **B.** Histograms generated by a representative flow cytometry experiment of four identical experiments all showing similar trends. Showing changes in expression profiles of migration markers on moDCs subjected to uninfected or Pg infected PMNs. Markers evaluated included CCR2, CXCR4, CCR5, CCR6 and CCR7. Each histogram shows frequency of expression of untreated immature moDCs (black trace) and moDCs co-cultured with untreated PMNs (red trace), TNF-a treated PMNs (light blue trace), WT Pg infected PMNs (green trace), MFB non-fimbriated mutant strain infected PMNs (dark blue trace), and DPG minor-fimbriated mutant strain infected PMNs (violet trace). **C.** Graphic representation of four experiments with moDCs subjected to infected PMNs for 24 hours. Similar trends as seen in (A) are observable. These experiments included A.a. leukotoxin exposed PMNs. Bar heights indicate average mean fluorescence intensity (MFI) and error bars signify SD. Statistical significance is denoted as compared to moDCs with no co-culture (negative control) and moDCs treated TNFa to encourage conventional maturation (positive control) (***=p<0.0001, **=p<0.01, *=p<0.05).

Figure 14

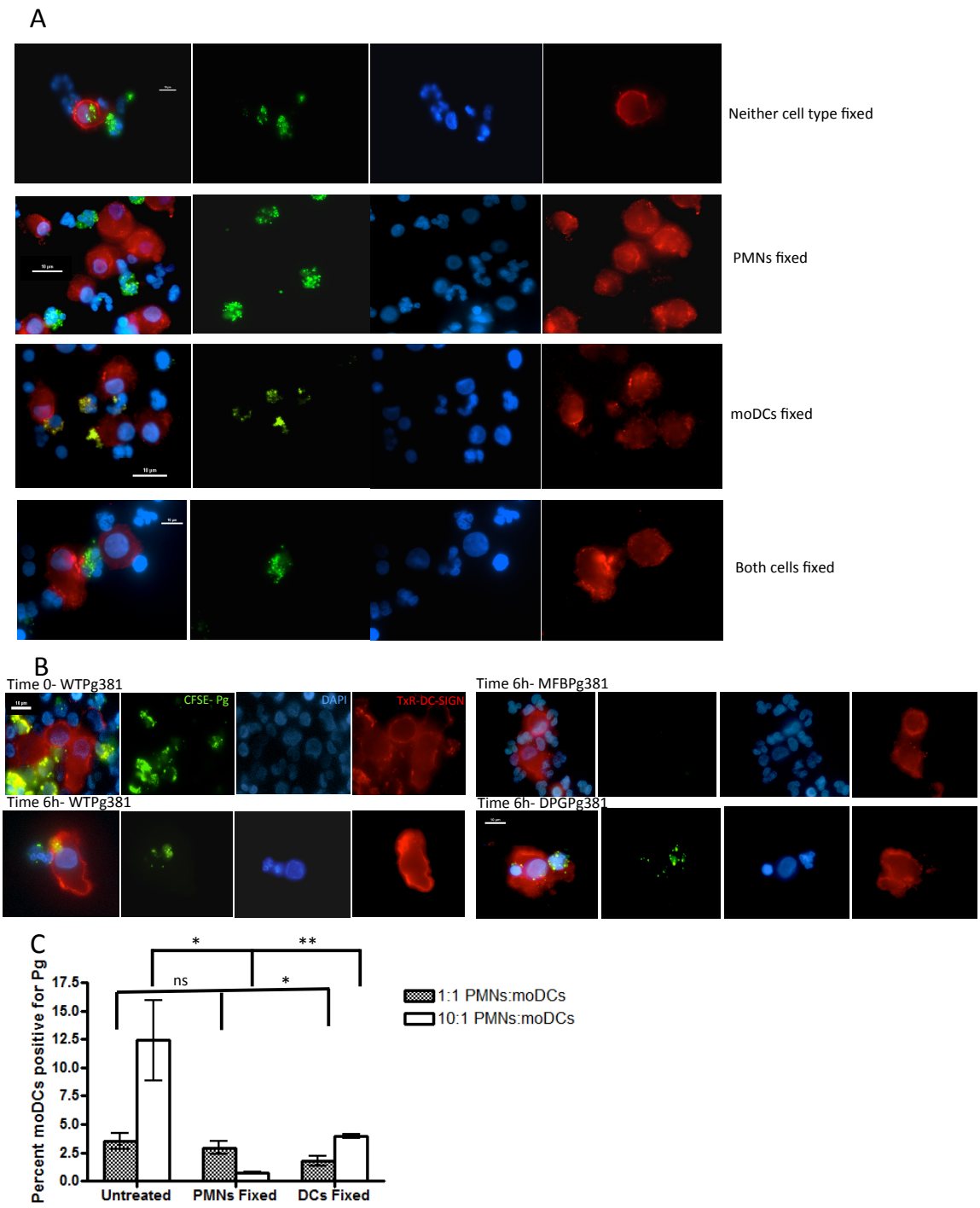


Fig14: **Contact between Pg infected PMNs and moDCs facilitates transfer of Pg to moDCs in a process that requires viability of both cell types**

A. Confocal microscopy images at 63x maginification. MoDCs are tagged with Texas Red conjugated anti-DC-SIGN anitbody. Nuclear staining is with DAPI blue. CFSE-stained WT Pg (green) was used to infect PMNs at MOI of 10 for a period of 30 minutes. Excess Pg was washed off cells and infected PMNs were cocultured with

moDCs at 1:1 PMN:moDCs ratio for a period of 6 hours. (1) moDCs and PMNs are both viable. (ii) PMNs were fixed after uptake of Pg and before co-culture with moDCs. (iii) DCs were fixed before co-culturing with infected PMNs. (iv) Both cells were fixed before co-culture but post-infection with Pg. B. (i) CFSE-stained WT Pg infected PMNs co-cultured with moDCs at time 0. MoDCs are tagged with Texas Red conjugated anti-DC-SIGN antibody, nuclear staining is with DAPI blue. (ii) MoDC and PMN co-culture after 6 hours, demonstrating WT Pg present inside the moDC shown presumably transferred from the associated infected PMN. (iii) Time 6 hours of co-culture with non-fibriated MFB strain Pg infected PMNs and moDCs. Note that non-fimbriated Pg is not acquired by PMNs. (iv) Time 6 hours of co-cultured with minor-fim expressing DPG strain infected PMNs with moDCs. Pictured moDCs are now positive for internalized Pg suggesting that PMN-moDC cell to cell transfer of Pg has occurred. If Pg uptake by PMNs and subsequent transfer to moDCs is fim-dependent, this would suggest the minor fimbriae is adequate for this process to occur. C. Graphic summary of flow cytometry data of experiments conducted in the same manner as in (A). Bar heights represent percent of moDCs double positive for CFSE signal and DC-SIGN indicated moDCs positive for uptake of Wt Pg as transferred by infected PMNs. Dark bars indicate an average ratio of PMNs:moDCs of 1:1 and white bars indicate an average ratio of 10:1 PMNs:moDCs as calculated from the data of four identical experiments. Error bars represent SD. Three experimental groups at each cell ratio are (1) untreated viable cells, (2) Pg-infected PMNs fixed prior to co-culture with moDCs, (3) moDCs fixed prior to co-culture with Pg-infected PMNs. Significance shown comparing fixed cell groups of the same ratio to untreated controls (**=$p<0.01$, *=$p<0.05$, ns=no statistically significant difference).

Figure 15

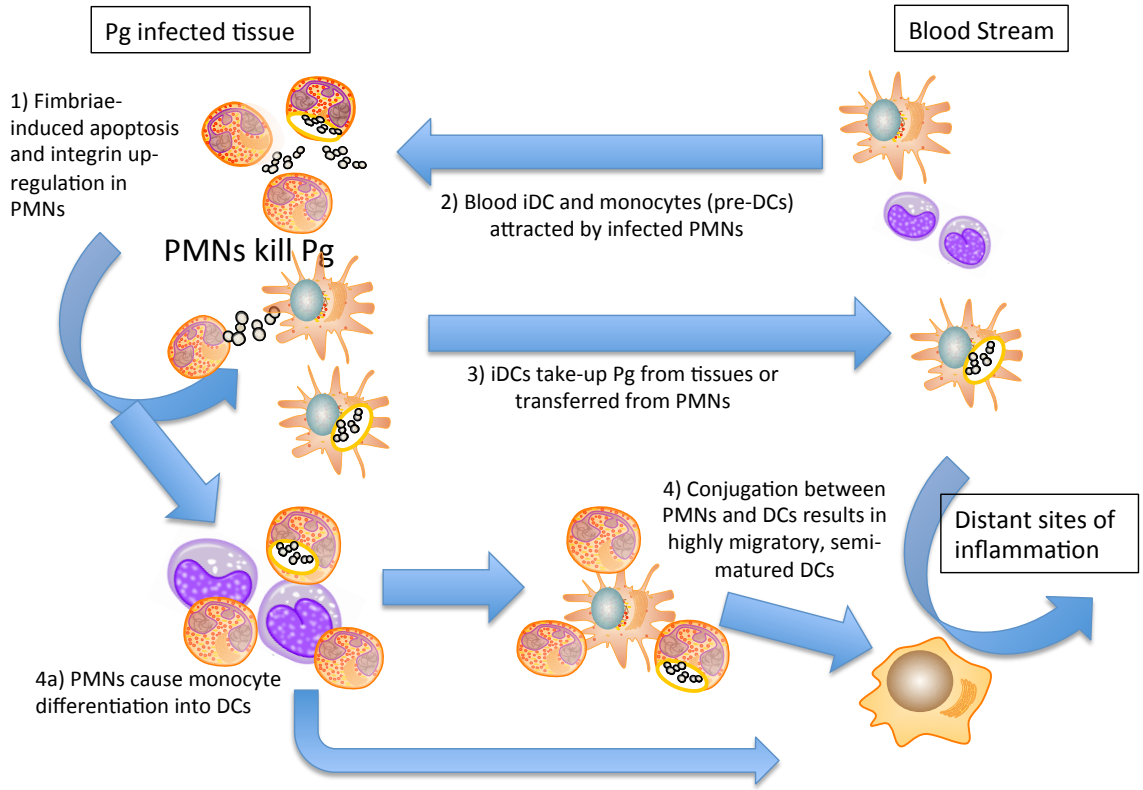


Fig15: **Summary of the Proposed interactions between PMNs and DCs in the presence of *Porphyromonas gingivalis* Infection**

1) PMNs as first responders to acute Pg infection engulf and destroy the bacteria, and in the process are activated by Pg in a fimbriae-dependent manner to upregulate activated surface B-integrins and CEACAM molecules and to undergo autophagy and subsequently apoptosis and aponecrosis. 2) Dying PMNs attract immature blood DCs and pre-DCs by means of soluble mediators to the site of infection. Associations between the two cell types are enhanced do the fimbirae-dependent priming of PMNs and physical conjugates are formed. 3) iDCs take up Pg from infected tissues and transferred from PMNs 4) Conjugation with infected PMNs causes DCs to develop a semi-mature, highly migratory phenotype that may cause them to persist in circulation, travel to distant sites of inflammation and survive harboring Pg safely inside. 4a) Infected PMNs also are able to influence monocytes to differentiate into moDCs, increasing populations of moDCs and therefore, enhancing Pg's odds of survival.

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