Stony Brook University

Pathogen Traffic by Dendritic Cells in Periodontitis: Links to Coronary Heart Disease

A Dissertation Presented

by

Julio Carrion

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Oral Biology and Pathology

Stony Brook University

May 2012

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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Chronic low-grade infections, such as chronic periodontitis (CP), are suspected of increasing Coronary Artery Disease (CAD) risk; however, the linking mechanisms are just emerging. *Porphyromonas gingivalis (Pg)*, commonly implicated in CP, along with multiple other pathogens, have been associated with the pathogenesis of coronary artery disease (CAD). However, it is unclear how these pathogens travel to and invade the arteries and their precise role in the disease process. *P. gingivalis* is known for invading epithelial, fibroblast, endothelial and myeloid dendritic cells (mDCs). Recent *in*-vitro evidence has demonstrated that *P. gingivalis* targets DC-SIGN+ (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) mDCs via its DC-invasin minor (mfa-1) fimbriae and uncouples dendritic cell maturation from the cytokine response, leading to intracellular bacterial persistence. Unlike epithelial and endothelial cells, mDCs are highly migratory. Thus *P. gingivalis* could potentially traffic to atherosclerotic plaques via mDCs. Moreover, DC-SIGN+ mDCs increase dramatically

in unstable atherosclerotic plaques, as observed in gingival tissue lesions from patients with CP. Thus we hypothesize that *Pg* infected mDCs in CP lesions redistribute to the peripheral blood. These along with blood mDCs that pick up Pg directly in the blood, contribute to the infiltrating pool of mDCs observed in patients with CAD.

The objectives of this study were to determine the frequency of blood and tissue mDCs in CP and CAD patients relative to healthy individuals. In addition, we began to establish the role of blood mDCs in dissemination of periodontal pathogens to atherosclerotic plaques. Accordingly, we examined the infection status of mDCs in gingival tissues and coronary artery atherosclerotic (ATH) plaque biopsies, as well as peripheral blood mDCs of a cohort of CP patients, with or without existing acute coronary syndrome (ACS). We initially focused on confirming that CP patients (n=26) were colonized by *P. gingivalis,* by analysis of the oral biofilm (i.e. subgingival dental plaque) by qRT-PCR of 16s rDNA; followed by genomic blast sequence analysis (qRT-PCR/GBS) of the 197 bp PCR-amplified product. The results indicate a 44% subgingival colonization rate with *P. gingivalis* in CP patients. Gingival biopsies from the *Pg*-infected CP patients were then analyzed by immunofluorescence-laser confocal microscopy (IF-LCM), focusing on colocalization of the DC-invasin of *P. gingivalis,* mfa-1 (AEZαMfa1), and on its target receptor on mDCs, DC-SIGN. The results reveal mfa-1 $^{+}$ DC-SIGN^{+} mDCs in CP lesions. These *Pg* "infected" DC-SIGN^{+} mDCs were also observed in post-mortem coronary artery ATH plaques. The presumed conduit for transfer of *Pg* to ATH plaques, migratory CD19^{-} DC-SIGN^{+} CD1c^{+} mDCs were then isolated from peripheral blood of CP and ACS/CP patients and analyzed by IF-LCM. The results confirm the presence of mfa-1^{+} *P. gingivalis* infected blood mDCs in the blood of CP and ACS/CP patients. The estimated CFUs (eCFUs) *of Pg* in blood mDCs equaled 1.32 ($\pm$ 1.21) X 10^5. Other pathogens identified in blood mDCs included

Pseudomonas spp., *Moraxella catarrhalis*, *Klebsiella pneumonia*, among other species. Overall, the findings support blood mDCs as a conduit for transport of infectious agents from gingival tissues to coronary arteries and possibly other distant sites in the human body.

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Acknowledgments

I would like to dedicate this work to all of those who have supported me since the beginning of this exciting scientific journey. First, I would like to acknowledge the assistance of Todd Rueb in the Stony Brook Flow Cytometry laboratory for the help and time provided in the cell sorting experiments. I would like to thank Emil Kozarov, M.S., and Ph.D. for providing the coronary artery samples. The completion of this work would not be possible without the help of the Stony Brook (SB) dental students Adam Bear, Erica Ravin, Lauren Jain, Tina Fan, and SB undergraduate student Liang Zhuo. Thank you for helping in performing the many experiments of the present work. I would like to acknowledge my previous (Amir Zeituni-development of *Pg* mfa-1: AEZαMfa1 monoclonal antibody) and current lab mates Brodie Miles and Yasvir Grewal, Ph.D. for helping with the ELISA experiments, Greg Savino, Ph.D. for technical assistant with all the immunofluorescence work, and Elizabeth Scisci for her time spent in the recovery of pathogens from circulating dendritic cells. I would like to extend my gratitude to all those within the SB School of Dental Medicine administration for the encouraging support over the years. I would like to specially acknowledge my research mentor Christopher W. Cutler, D.D.S., Ph.D. for believing in the accomplishment of this work and Vincent J. Iacono for his clinical mentorship over the years in the field of Periodontics and Implant Dentistry. Nevertheless, I would like to sincerely thank my wife Hilsha Carrion and my three beautiful daughters Faviola, Sofia and Natalia Carrion for always loving and smiling at me, and enduring with patience throughout the last 6 years of my education. Finally, this study was supported by a US Public Health Service grants from the NIH/NIDCR (K23DE018187-01 to Julio Carrion; R01 DE014328, R21 DE020916 to Christopher W. Cutler. and F31DE020014 to Amir Zeituni) and

by a Minority Dental Faculty Development (MDFD) Grant from the New York State Academic Centers (NYSAC).

Introduction

I. Overview

An estimated 80 million U.S.A. adults have one or more types of cardiovascular diseases (CVD) with the total cost to the U.S. for 2009 estimated to be $475.3 billion [1]. Coronary artery disease (CAD), also known as atherosclerosis (ATH) is the single most important contributor to CVD; however, only 50% of ATH patients have currently identified risk factors [2]. Chronic periodontitis (CP), a common inflammatory disease of the oral mucosa, is linked to an increased CVD risk [3].

Dendritic cells (DCs), potent antigen presenting cells of the immune system, have been identified infiltrating the coronary arteries of CVD patients [4]. Two subtypes of DCs with different functions have been identified: (1) myeloid dendritic cells (mDCs), which include epithelial Langerhans cells (LCs), and interstitial DCs (intDCs). IntDCs are present in all other tissues, expressing CD1c (BDCA-1), CD11c and CD33 and secreting IL-12 after stimulation with bacterial products; (2) plasmacytoid dendritic cells (pDCs), which express CD123, respond to viral expression and are major producers of interferon-α [5].

Although macrophages infiltrate atheromatous plaques in higher numbers and composed most of the atherosclerotic plaque core [6], recent studies have found elevated numbers of mDCs in atheromatous plaques susceptible to rupture [7]. In addition, animal studies have shown that mDCs infiltrate the arteries early in the atherosclerotic disease process, engulf lipids and become foam cells (atherogenic DCs), thus contributing to the formation/development of the atheroma [8]. Moreover, by priming naïve T-cells within the arterial wall, mDCs trigger and could potentially perpetuate inflammation in CAD, thus contributing to plaque instability and

possibly rupture. Many questions remain to be answered on the role of mDCs in atheromatous plaque formation and rupture; however, primary among these questions is to identify the source or origin of these atherogenic DCs in CAD. [9]

III. Coronary Artery Disease

A. Etiology and Risk Factors

Coronary artery disease, also known as coronary heart disease (CHD), is the leading cause of death for men and women in the U.S.A. [10]. CAD occurs by a condition known as atherosclerosis, which occurs when fatty deposits, immune cells and debris, accumulate within the innermost lining (intima) of the artery, leading to the formation of an atheroma [11]. Progressive deposition of lipids, along with smooth muscle cell proliferation can lead to partial or complete obstruction of the lumen of the artery. This can result in infarction of the myocardium (heart attack) due to reduced or obstruction of oxygenated blood to the heart muscle.

The formation of an atheroma is preceded the accumulation of lipid-laden cells (foam cells), also known as fatty streaks [12]. The fatty streaks are present since childhood and it may or may not progress to atheroma formation, depending on genetic predisposition, lifestyle and other factors. The atheroma is characterized by the presence of foam cells and lipids (low density lipoprotein, LDL) at its core and is surrounded by a layer of smooth muscle cells and collagen matrix. In response to this, the vascular endothelium becomes activated and induces the expression of leukocyte adhesion molecules. This promotes further arterial infiltration of leukocytes, such as macrophages, T and, B lymphocytes, neutrophils, dendritic cells, among others [13, 14]. In response to microbial pathogens, auto-antigens and other signals, these

leukocytes can become activated and secrete inflammatory cytokines that can compromise the stability of the atheroma, leading to plaque rupture. This is further enhanced and aggravated by matrix metalloproteinases (MMPs) within the atheroma, which undermine the supporting collagen matrix around the plaque, thus debilitating it and increasing risk of rupture. Moreover, prothrombotic factors are released which promote thrombus formation at the site of plaque rupture, leading to chest angina or more fatally myocardial infarction. Much is known about the role of macrophages in this process, but studies are just emerging addressing the role and contribution of DCs to the overall process of atheroma formation.

The risk of CAD increases in the presence of the following factors: increasing age typically after age 40; (2) hereditary/genetic predisposition: risk increases if there are pre-existing conditions in the family members or close relatives; ethnical/racial backgrounds: African Americans have higher risk due to higher blood pressure rates than Caucasians. The same holds true for Mexican Americans, American Indians, and others due to higher rates of obesity and diabetes. Additional risk factors includes gender: typically males possess a greater risk than females, but once they reached their menopause the risk is similar to men; diabetes mellitus: seventy-five (75%) of people with diabetes currently die of some form of CAD; smoking: tobacco users have two to four (2-4x) times greater risk than non-smokers; abnormal cholesterol levels: generally, people with low levels of high density lipoprotein (HDL) and high levels of low density lipoprotein (LDL) are at greater risk of CAD; metabolic syndrome: involves patients with high triglyceride levels, high blood pressure, excessive body fat around the waist line and increased insulin resistant. Other significant risk factors include, but are not limited to: high blood pressure (hypertension), physical inactivity, stress, substance (cocaine) and alcohol abuse, chronic kidney disease, obesity, and chronic periodontitis. More recent evidence

has also implicated DCs infiltration as a risk factor for CAD [15]. It is generally accepted that leukocytes infiltration into the coronary arteries is necessary for the initiation and development of CAD [16].

B. Role of Leukocytes

1. Granulocytes

A. Neutrophils

Neutrophils, also known as polymorphonuclear phagocytes (PMN), are the most abundant type of blood leukocyte in mammals; they account for approximately 70% of all white blood cells. Their main feature is the multilobulated shape nucleus that distinguishes them from other blood leukocytes. Neutrophils are the host first line of defense against invading microbial pathogens, such as bacteria and viruses. Migration and infiltration of neutrophils into infected tissues is directed by chemical signals, through a process known as chemotaxis. In response to the antigenic challenge these cells secrete reactive oxygen species (ROS) and myeloperoxidases aimed at eliminating the invading microbial pathogen. Prolonged release of these and other neutrophil substances can be detrimental and result in localized tissue destruction. The presence of neutrophils in atheromas, in particular at sites of plaque rupture, in patients with acute coronary syndrome (ACS) indicates that these cells may play a role in the atherosclerosis process [17]. In fact, knockout animal models of atherosclerotic disease (ApoE-/- and LDLr-/-) have shown the accumulation of neutrophils and its byproducts at aortic lesion sites [18, 19]. Evidence points to a disruption in the interaction between CXCR4 and CXCL12, as a causal molecular mechanism. Circulating neutrophils and plaque formation was attenuated in a CXCR4 deficient animal model, in which atherosclerosis was induced via a high fat diet [20]. Furthermore, human

studies have shown positive correlations between circulating neutrophils and coronary artery disease [21]. Recruitment of neutrophils to sites of atheroma formation, along with their capability to secrete pro-inflammatory cytokines and other mediators (ex. MMP-9), might promote plaque instability and progression of CAD lesion.

B. Mast cells

Mast cells are most commonly associated with allergies and anaphylaxis reactions. They are also involved in wound healing and host defense against microbial pathogens. Mast cells are characterized by their granule content of histamine and heparin. The notion that mast cells are involved in coronary artery disease stems from studies that have found them in coronary and carotid plaques at sites of endothelial erosion and rupture [22, 23]. Mast cells enhance plaque development via the release of mediators that promotes lipid accumulation, degradation of high-density lipoprotein, and reduce cholesterol efflux [24, 25]. Mast cells also produce inflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor (TNF) that contributes to plaque development [26]. In addition, serine proteases released by mast cells such as, chymase and tryptase activates MMPs leading to collagen degradation and plaque instability [27]. Recent evidence has shown that mast cells increase vascular permeability, thus facilitating further leukocyte infiltration. This is mediated, in part, through the release of CXCR2 ligands [28]. Mast cells have also being implicated in aneurysm development by inducing smooth muscle cell apoptosis, extracellular matrix degradation and vascular wall remodeling [29].

C. Eosinophils and basophils

Eosinophils and basophils are usually associated with parasitic infections. In addition, they play a role in allergic and inflammatory reactions. The frequency of eosinophils and

basophils in the human circulation ranges from 1-6% and less than 1%, respectively. Unlike neutrophils and mast cells, the role of eosinophils and basophils in CAD remains unclear. To elucidate the function of these cells in atherogenesis is further complicated by their short half-life and rapid apoptosis. Recruitment of eosinophils and basophils appears to be mediated through CCL2 and CCL11 ligands into atherosclerotic plaques [30]. CCL2 and CCL11 are produced by smooth muscle cells in response to TNF-α. Studies in humans have found increase expression of CCL11 and its receptor CCR3 in carotid plaques, but in the absence of eosinophils. CCR3 might be involved in the recruitment of mast cells and macrophages, which also express CCR3 [30]. More studies are needed to track these cells in atherosclerosis. This will validate or exclude their significance in the pathogenesis of coronary artery disease.

2. Monocytes and macrophages

Monocytes represent about 2-10% of the total blood leukocyte count. Monocytes are the precursors of tissue resident macrophages and DCs. Monocytes infiltrate tissues in the periphery to maintain homeostasis in order to replenish dying or apoptotic cells. They also migrate to the periphery in response to inflammatory signals, such as in infection or injury. In response to chemokines, monocytes infiltrate sites of infection/inflammation and differentiate into macrophages/DCs depending on the environmental cues and stimuli. Macrophages' main function involves phagocytosis of cellular debris and microbial pathogens, and stimulation of lymphocytes to respond to the invading pathogen. In CAD, macrophages also engulf oxidized low-density lipoproteins (ox-LDL) via scavenger receptors leading to the development of foam cells, one of the key features of early atherosclerosis. Animal studies have shown that monocyte infiltration into atherosclerotic plaques is, in part, mediated by CCL2, CCR2, CCL5 and CCR5 [31]. Moreover, monocyte accumulation in the atheroma appeared to be correlated to lesion size,

in an $ApoE^{-/-}$ animal model [32]. This suggests that monocytes play an important role during the early phases of atherosclerosis. Two monocyte subsets have being identified in human atherosclerosis: resident $CD14^{+}CD16^{-}$, which patrol healthy arterial tissues in response to CCR5, and $CD14^{low}CD16^{+}$, which are preferentially recruited to inflamed tissues via CX_3CX1 and CCR2 [33, 34]. Resident monocytes contribute to the atherosclerosis process, in part, via the production of pro-angiogenic factors, whereas the role of the inflammatory monocyte subset is associated to proteolytic and phagocytic activity. CCR5, CX_3CX1, CCR2 are overexpress in atherosclerotic tissue samples, further supporting the role of monocytes in atherogenesis via recruitment into the arteries [35].

3. B and T Lymphocytes

B and T cell lymphocytes are the major components of the adaptive immune system. B cells are involved in the humoral (antibody) response, whereas T cells are associated with a cellular mediated immune response. The main function of B cells involves antigen presentation to T cells and differentiation into plasma cells that produce neutralizing antibodies against microbial pathogens. T cells, on the other hand, express T cell receptors specific for antigens and produce inflammatory cytokines that regulate the overall inflammatory response when challenge by bacteria and viruses. A unique feature of B and T lymphocytes is the development of memory, that is their ability to recognize previously encountered pathogens and being able to mount and faster and stronger immune response against the microbial pathogen, leading to a more efficient elimination. The lymphocyte content in the systemic circulation ranges from 28-33% of the total blood leukocyte count. Lymphocytes are also present in atherosclerotic plaques, but in lower numbers than monocytes and macrophages. One of the initial studies to shown that lymphocytes may play a role in atherogenesis found that immunodeficient mice have less plaque

formation when hypercholesterolemia was induced by knocking out the apolipoprotein E gene [36]. Further investigations have indicated that the lymphocyte role in atherogenesis varies according to the T_H-subset that is involved in the process. Moreover, a dynamic equilibrium between T cell subsets has been reported during atherogenesis [37]. The TH1 cell subset, secretes IFN-γ, helps to prolonged the inflammatory response by inducing MHC class II molecule expression in antigen presenting cells and the stimulation of smooth muscle cells proliferation within the artery [38]. Furthermore, knockout of T-bet, a transcription factor of the T_H1 cell subset, dampens atherosclerosis and illustrates the important role of this T cell subset in the overall atherogenic process [39]. The role of T_H2 cell subsets have being generally associated to a reduction of vascular inflammation and thus atherosclerosis [40]. Moreover, hypercholesterolemia mice experiments have shown that the T_H2 immune response reduces atherogenesis [41]. This T cell subset is characterized by the production of IL-4, IL-5 and IL-10 which induces B cells to produce protective antibodies, particularly against oxidized LDL antigens [39]. Other T cells subtypes have being implicated in atherosclerosis, such as $CD8^+$ and $CD4^+$ naïve T cells. Experiments have shown that $CD8^+$ T and $CD4^+$ naïve T cells mediate lysis and proliferation of smooth muscle cells in mice. T regulatory (Treg) cells subset produced transforming growth factor-β which has anti-plaque development capabilities and anti-inflammatory properties [42]. Natural killer T cells and T_H17 subsets have also been found in atheromas and it is suggested that they have an important role on the overall process of CAD initiation and development [43, 44]. The implication of B cells in CAD stems from their presence in human atherosclerotic plaques [45]. B cells appeared to infiltrate arteries during the early and late stages of the disease process [46] and their role appeared to be protective against

atherogenesis via antibody production against ox-LDL antigens [47]. Together these data imply a role for T and B lymphocytes in the pathogenesis of CAD.

4. Dendritic cells

Dendritic cells (DCs) are the sentinels of the immune system and their main function is the surveillance of the peripheral tissues for invading microbial pathogens. In addition, upon antigen encounter DCs migrate to lymphoid organs where they present the antigen to lymphocytes and initiate and perpetuate adaptive immune responses, through the initiation of inflammatory immune responses. DCs are also involve in immune tolerance to self-antigens [48]. DCs in human blood represent about 1% of the total peripheral blood monocytes (PBMCs) and are characterized by the lack of lineage specific markers and have high expression of MHC-Class II [49, 50]. Two subtypes of DCs with different functions have been identified: (1) myeloid dendritic cells (mDCs), which include epithelial Langerhans cells (LCs), and interstitial DCs (intDCs). IntDCs are present in all other tissues, expressing CD1c (BDCA-1), CD11c and CD33 and secreting IL-12 after stimulation with bacterial products; (2) plasmacytoid dendritic cells (pDCs), which express CD123, respond to viral expression and are major producers of interferon-α [5]. MDCs are responsible for antigen capture in peripheral tissues and migration to lymph nodes for antigen presentation [51]. Myeloid derived DCs (CD11c+) are further subdivided into CD16+, BDCA-1 (CD1c) and BDCA-3 (CD134) and of these BDCA-1 is the most prevalent and the most potent allostimulatory cell population [52]. DC-SIGN, a common marker for the identification of mDCs on peripheral tissues, have being identified in BDCA-1+ blood mDCs, albeit in low levels. DC-SIGN is also targeted by bacterial and viral pathogens for entry and immune suppression.

In 1995, mDCs were first identified in coronary arteries and since then evidence has accumulated supporting an important role for DCs in CAD. The fact that a vascular network of mDCs have also being found in the arterial intimal layers of healthy young individuals [53], perhaps suggests that mDCs have a role in CAD development early in the disease process. Animal studies have also found that mDCs infiltrate and accumulate in atherosclerosis-prone regions. This is, in part, mediated by the vascular cell-adhesion molecule 1 (VCAM1) and CX_3CR1 [54, 55], and possibly via DC-SIGN interactions. DC-SIGN+ mDCs have been found in unstable atherosclerotic plaques [4] and their infiltration might be mediated by interactions with intercellular adhesion molecule 2 (ICAM-2) [56]. More relevant experimental studies have shown higher frequencies of mDCs in rupture-prone regions, relative to sites within the artery that are either disease free or have early signs of plaque formation. Furthermore, DCs appeared in clusters with T cell lymphocytes, thus possibly implicated in initiation of immune responses, inflammation and plaque instability [57]. An interesting hypothesis suggests that the increased numbers of mDCs in athero-prone regions is due to a reduction in systemic circulation of blood DCs. This was evidenced by a study with patients with acute coronary syndrome, which demonstrated fewer circulating levels of mDCs, possibly recruited into atherosclerotic plaques [58]. Recent studies have also shown that infiltrating DCs phagocytose oxLDL and contribute to the overall foam cell pool formation with the arteries [59].

The main function of MDCs in the atherogenic process just has begun to been elucidated. One proposed mechanism involves INF-γ production by $CD4^+$ naïve T cells upon DC stimulation by oxLDL antigens; breaking tolerance to self-antigens and initiation of inflammation within the vascular wall [60]. Another study shows that in the presence of hyperlipidemia, DC migration to lymph nodes is impaired, leading to persistence in peripheral

arterial tissues and possibly more likely to infiltrate the coronary arteries and contribute to the inflammatory process [61]. DCs are also well known for the production of chemokines, such as CCL19 and CCL22, which are responsible for recruiting T cells into atherosclerotic plaques and further contribute to the atherosclerotic process by DC-T cell priming interactions. These and other mechanisms are being actively explored, but as mentioned earlier the original sources of infiltrating arterial DCs remains to be determined.

5. Progenitor cells

Progenitor cells originate from the bone marrow where they infiltrate peripheral tissues through the systemic circulation. Progenitor cells are able to differentiate into specific cells types according to the host requirements. The role of vascular progenitor cells in the CAD process is controversial. Some studies argue for a protective and reparative effect, while other has implicated them in disease progression and plaque instability. Two types of vascular progenitor cells have being identified in the circulation of mice and humans: endothelial progenitor cells (EPC) and smooth muscle progenitor cells (SMP) [62, 63]. EPCs are found in the bone marrow, blood and adventitia [64, 65] and they play a role in vascular remodeling, and regeneration of damaged endothelial cells, as well as neovascularization of ischemic arterial tissue [66]. Recruitment of EPCs to coronary arteries is mediated, in part, by hypoxia, vascular endothelia growth factor (VEGF), granulocyte/macrophage colony-stimulating factor (GM-CSF) and CXCL12 [64, 66], where they can exacerbate atheroma progression and instability. SPCs differentiate from bone marrow, circulating or tissue resident precursors and express markers of the smooth muscle cell lineage [67]. A recent study showed improvement in plaque stability after infusion of SMPs into an immunodeficient Apoe$^{-/-}$ mice [68]. Moreover, circulating SMPs are found in increased numbers in patients with stable CAD, but decrease in patients with a history

of ACS, suggesting their possible protective role in atherosclerosis [68], and perhaps by preventing rupture of the fibrous cap. Further research about the role of vascular progenitor cells is warranted to clarify their protective or pathological implications in CAD.

C. Role of microbial pathogens

It is now generally accepted that infection/inflammation have a significant role in CAD progression and plaque instability. Infection by microbial pathogens and its associated antigens can induce inflammation within the arterial vessel and thus contribute to atherosclerosis. Four molecular mechanisms have been proposed for how bacterial pathogens may induced or accelerate CAD [69]. These include, but are not necessarily limited to, (i) direct invasion of the vascular endothelium by pathogens in the systemic circulation, (ii) immunological sounding, (iii) molecular mimicry, and (iv) pathogen trafficking of microbes within circulating leukocytes in the peripheral blood [69].

Viruses and bacterial microorganisms have been implicated in the atherosclerotic disease process [70]. The first evidence of viral infection related to CAD was shown in 1968 [71]. In this animal study acute coronary arteritis (inflammation of the arterial wall) was induced by viral infection with *Coxsackie B* virus [71]. *Herpesviridae* is another group of viruses that have been implicated in the pathogenesis of CAD. For instance, Marek's disease virus, a herpes virus, have been shown to be involved in the progression of CAD in chickens, and this effect was independent of cholesterol levels [72]. Moreover, herpes viruses appeared to contribute to atherosclerosis by inducing smooth muscle cell proliferation within the arterial wall [73]. Further evidence comes from studies involving *cytomegaloviruses* (*CMV*) in CAD. One of the most important studies showed the presence of *CMV* in coronary arteries by DNA based methods [74,

75]. The presence of *CMV* genomic DNA in early atherosclerotic lesions, in young cardiovascular trauma victims [76], suggests that this pathogen may be involved in the initial stages of CAD development. Further studies were less encouraging as they showed the presence of *CMV* at a similar rate in healthy and disease coronary arteries [77].

Bacterial infections are also implicated in the pathogenesis of atherosclerosis. *Chlamydia pneumoniae*, *Helicobacter pylori*, among others were among the first bacterial microbes to be involved in the pathogenesis of atherosclerosis. Presently, the strongest available evidence showing an association between CAD and infectious agents comes from *C. pneumoniae* [78]. *C. pneumoniae* is known for causing upper and lower respiratory tract infections, asthma and endocarditis [79]. Early epidemiological studies in the 1980's, showed an association between angina pectoris and AMI and increased serum antibodies to *C. pneumoniae* [80]. In this study an association remained after adjusting for confounding variables such as smoking. A more recent meta-analysis of the literature, however, found no association, after adjusting for traditional risk factors including socioeconomic status [81]. *C. pneumoniae* has also been identified in atherosclerotic plaques (up to 80%) from humans with the aid of PCR techniques and electron microscopy [82]. The presence of *C. pneumoniae* in healthy arteries is uncommon [83, 84]. Cultivating this organism from aortic samples has been a very difficult task, but a recent study showed 38.5% *C. pneumoniae* recovery from abdominal aortic aneurysms [85]. *In vitro* studies have shown that *C. pneumonia* is also capable of infecting human endothelial cells, smooth muscle cells and macrophages [86]. This is important because the *C. pneumonia* lipopolysaccharide (LPS) enhances LDL accumulation in macrophages and thus enhances foam cell formation [87]. Moreover, the heat shock protein 60 (HSP-60) from *C. pneumoniae* have been shown to induce LDL oxidation, one of the key events that leads to macrophage-derived

foam cell formation [88]. Induction of pro-inflammatory cytokines and matrix metalloproteinase by *C. pneumoniae* is just another important factor that contributes to CAD progression and development [89].

H. pylori, a bacterial pathogen most commonly implicated in peptic ulcerations, has also been found to be associated to CAD. Similar to *C. pneumoniae, anti-H.pylori* serum antibodies appeared to be associated to an increased risk for CAD [90]. The increased risk conferred by elevated serum antibodies against *H. pylori* persisted after adjusting for common risk factors, such as smoking, high cholesterol, hypertension and others [90]. Perhaps the strongest evidence linking *H. pylori* DNA to atherosclerosis is its presence in atheromatous plaques [91]. However, independent studies have failed to find it in similar vascular tissues [92].

Porphyromonas gingivalis (*Pg*), one of the main bacterial pathogens implicated in chronic periodontitis, have also been associated with CAD [93]. *P. gingivalis,* along with other periodontal pathogens, (*Prevotella intermedia, Bacteroides forsythus [now Tannerella forsythia], and Actinobacillus actinomycetemcomitans [now Aggregatibacter actinomycetemcomitans*), have been found in human atherosclerotic plaques by means of 16s rDNA PCR detection [94, 95]. Animal models of atherosclerotic disease have shown that *P. gingivalis* accelerates atherosclerosis and smooth muscle cell proliferation resulting in thickening of the intimal layer of the vascular wall [96]. Moreover, using a heterozygous apolipoprotein E-deficient (ApoE(-/-) mice model, showed that repeated injections of *P. gingivalis* in the tail vein for 6 months, resulted in accelerated atherosclerosis, greater aortic lesion size and macrophage infiltration, relative to controls [97]. These results were later validated by independent investigators by using a similar mouse model, but this time they challenged the animals intraorally; this model resembles more closely the disease process in human subjects. Results

demonstrated that while *Pg* accelerates atherosclerosis, *Pg* immunization prior to oral challenge prevented it [98]. This indicates that chronic exposure to the periodontal pathogen *P. gingivalis,* as seen in humans with CP, may accelerate CAD progression.

The adhesion of bacterial pathogens to the vascular endothelium is another important factor that has relevant implications in atherosclerosis development. For instance, among the multiple virulence factors that *P. gingivalis* has in its arsenal, fimbriae functions in invasion and colonization [99]. Two fimbrial types have been identified in *Pg*, that is, the major fimbriae, encoded by the FimA gene, and the minor fimbriae, encoded by the mfa-1 gene. While the major 41kDa fimbriae targets cell surface integrins [100], the Cutler laboratory has recently demonstrated that the minor 67kDa fimbriae targets DC-SIGN (CD209) on monocyte-derived DCs for entry into the host. DC-SIGN is also targeted by other microbial pathogens, such as *H. pylori* [101], *HIV* [102] and *Mycobacterium tuberculosis* [103]. Oral injection of fimbriae deficient *Pg* into an ApoE (-/-) mice model, failed to accelerate atherosclerosis relative to wild-type *Pg* strain [98]. This study also demonstrated that while *Pg* fimbriae mediates attachment and colonization into the host cell, it also induced endothelial adhesion molecules (cell-associated intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], which may further contribute to *Pg* attachment and invasion into the vascular wall [98]. Interaction between DC-SIGN+ mDCs and ICAM-2+ endothelial cells in the vascular endothelium could well be a mechanism that leads to DC infiltration.

More recent investigations have shown that *Pg* mediated inflammatory responses within the arterial wall of a ApoE (-/-) mice model was mediated, in part, via Toll-like receptor 2 (TLR-2) [104]. Furthermore, acceleration of atherosclerosis in the ApoE (-/-) mice model appears to be pathogen specific [105]. In this interesting animal study, ApoE (-/-) mice were challenged with

P. gingivalis and *H. pylori*, and it was found that atherosclerosis only developed in *Pg* infected mice alone. *Pg* infected animals also induced systemic inflammation relative to the controls and those infected with *H. pylori* [105]. This further supports the notion that while many pathogens have been found in CAD, very few organisms contribute to the initiation and progression of atherosclerosis.

D. Resolution of inflammation and treatment modalities

Current modalities for the treatment of CAD focus on the modification of traditional risk factors, such as hypertension and hyperlipidemia, but these therapies sometimes failed to address the host inflammatory responses, elicited by microbial pathogens. Treatment with statins in patients with CAD leads to inhibition of cholesterol synthesis, reduction of systemic LDL and also has anti-inflammatory properties [106]. Statin therapy also improves endothelial function [107], and reduces systemic high-sensitivity C-reactive protein (hsCRP), a risk factor for atherosclerosis [108]. Other established and common regimens for the treatment of CAD includes, but are not limited to, aspirin (inhibits platelet aggregation) [109], β-blockers and renin-angiotensin system inhibitors, both of which are anti-hypertensive in essence [110]. Emerging therapeutic approaches involves the use of artificial HDL-like apolipoprotein complexes which promote cholesterol efflux from the arterial vessel and promotes regression of coronary atherosclerosis [111]. Reduction of infarction and heart failure has been shown with Thiazolidinediones (TZDs), a more recent drug therapy for CAD, but this results required further testing and validation [112]. Other promising therapies are currently under intense investigation and some are at the levels of clinical trials [113]. In lieu of previous animal studies showing that immunization prevents development of atherosclerosis, vaccination is another attractive approach to develop protective immunity and thus prevent CAD [114].

Systemic antibiotics have also been considered for the treatment of CAD, due to previous evidence implicating microbial bacterial species in the pathogenesis process [115, 116]. This idea was borne out of evidence showing an association between *C. pneumoniae* and cardiovascular disease [80], as previously stated. The first small clinical trials used azithromycin, a macrolide antibiotic that inhibits bacterial protein synthesis, and found that it conferred protective cardiovascular effects [117]. This evidence led to validate this hypothesis in larger randomized placebo-controlled clinical trials. The major studies were the WIZARD [118], PROVE-IT [119] and ACES [120] human clinical trials, all conducted in North America. In these trials, the primary endpoint was based on a composite of death due to CAD, nonfatal myocardial infarction, coronary revascularization, or hospitalization for unstable angina. Results from these studies were discouraging, because antibiotic treatment (ranging from 3 months to 2 years) did not affect the primary endpoint relative to the placebo controlled patient population. Failure of azithromycin to reach the microorganisms, in the chronic lesions of atherosclerosis, was suggested as one explanation for the negative results

Moreover, being that CAD is a slowly developing chronic disease, the delayed or long term effects of systemic antibiotics in the prevention of cardiovascular events remains unknown. Of particular relevance is the fact that in these trials the effect of azithromycin on other bacterial species, known to be associated to CAD, was not investigated. A recent trial examined the effect of a 3 month course of clarithromycin, a similar macrolide antibiotic to azithromycin, at reducing recurrent cardiovascular events (CV) in patients with ACS and chronic periodontitis (CP) [121]. One of the most important finding of this study was that patients with CP had a five-fold increase of recurring CV in spite of treatment with systemic antibiotics [121]. The authors of this study proposed the idea that CP infection may be, in part, one of the reasons for the disappointing

results of these clinical trials. One explanation to this may be that many periodontal pathogens in CP, including *P. gingivalis*, are intracellular pathogens [115, 116]. *In-vitro* [122] and clinical studies [123] showed that azithromycin is effective against extracellular *P. gingivalis*. However, *P. gingivalis* species, resistant to azithromycin, have been identified in patients with CP; these possibly represent intracellular strains of *Pg*. *In-vitro* studies have also shown that *Pg* is able to infect [124] and replicate [125] in endothelial and epithelial cells. Moreover, recent evidence demonstrated that *P. gingivalis* is capable of spreading from infected epithelial, endothelial and smooth muscle cells to new host cells where it multiplies and leads to bacterial persistence [126]. *P. gingivalis* also targets DC-SIGN+DCs and uncouples DC maturation from the cytokine response [127]; yet another mechanism *Pg* uses to evade the host response. *Pg*-infected DCs, in patients with CP, could be a mechanism this and other pathogens use to traffic to atherosclerotic lesions, and thus contribute to the progression and development of CAD [105].

III. CHRONIC PERIODONTITIS (CP)

A. Etiology and Pathogenesis

Periodontal disease is a common and complex inflammatory disease with variable clinical presentation. The most common form is chronic periodontitis (CP), also known as "gum disease". CP is a disease of the supporting tissues of the teeth that results in loss of connective tissue attachment and of alveolar bone. It is initiated by Gram-negative bacteria, organized in a biofilm that induces an inflammatory response in the individual or host. Gingivitis represents the initial and reversible inflammatory reaction to the biofilm, characterized by reddened and bleeding gingiva. If left untreated, gingivitis may progress to chronic periodontitis due to persistent bacterial infection, leading to soft tissue and progressive bone destruction, tooth

mobility and subsequent tooth loss [128]. CP is categorized by its extent (number of affected sites) and severity (degree of clinical attachment loss). There is no particular age at which onset is more likely, but it is clear that CP is mostly considered a disease of middle age [129]. By a conservative estimate about 31% of the United States population exhibits mild forms, 13% display moderate severity, and 4% have advanced disease symptoms [130].

One of the major bacterial components that can elicit an inflammatory host response is lipopolysaccharide (LPS), among many others. In response to the bacterial challenge in the gingiva (i.e. LPS) the host secretes inflammatory cytokines such as, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), prostaglandin E2 (PGE-2), tumor necrosis factor alpha (TNF-α) and matrix metalloproteinases (MMPs). This event results in the initial recruitment of neutrophils, followed by infiltration of monocytes, macrophages, DCs, T and B cells and others to the affected periodontal tissues. The initial immune response is aimed at eliminating and combating the bacterial challenge in the gingiva. The clinical manifestation of this stage is primarily gingival inflammation, which can be effectively treated with appropriate professional or oral home care. However, lack of treatment leads to sustained gingival inflammation that can potentially turn into CP in susceptible patients. It is not clear what factors determine the transition from gingivitis to periodontitis, but cytokine dysregulation in the periodontal tissues may be, in part, responsible for this event. For instance, IL-1β is found to be elevated in inflamed periodontal tissues [131] and the gingival crevicular fluid (GCF) in experimental gingivitis [132]. Increased levels of IL-1β leads to increased osteoclastic bone resorption, in part mediated by activation of the RANK-RANK-l pathway [133], and increased production of prostaglandins and matrix metalloproteinases [134]. In fact, studies have shown that IL-1β synergizes with TNF-α in the destruction of alveolar bone [135]. Another important cytokine present in high

levels in periodontal inflamed tissue is IL-6 [136]. IL-6 promotes bone resorption alone and acts synergistically with IL-1β [137]. Moreover, IL-6 production induces the liver to produce hsCRP, a known risk factor for CAD. Altogether, changes in the microbial biofilm, local dysregulation and over expression of inflammatory cytokines in the gingiva leads to periodontal tissue destruction with possible systemic implications, such as increase risk for atherosclerosis.

B. Role of Innate and Adaptive Immunity

Innate and adaptive immune defenses play an important role in shaping the host response to changes in the microbial biofilm. Small infiltrates of inflammatory leukocytes are present in states of gingival health [138], presumably to maintain homeostasis of the periodontal tissues through removal of unwanted debris and foreign microbial antigens. Accumulations of plaque or biofilm in the gingival sulcus leads to gingivitis [139] and in susceptible individuals will progress to CP [140]. Transitioning from gingival health to periodontitis has been previously described through a series of stages, based on clinical and histological findings, as: initial, early, established and advanced lesions [138]. The initial lesion takes place within 2-4 days after biofilm accumulation around the gingival margin. The histopathological features of this stage involves primarily the accumulation of PMNs [141], increase in GCF and loss of connective tissue. Bacterial enzymes and byproducts are responsible for increasing the permeability of the junctional epithelium and the ingress of complement components, such as C3a and C5a. These components lead, in part, to the secretion of vasoactive amines from mast cells, which are responsible for vascular permeability and edema formation. In addition, activated mast cells secretes TNF-α, which in turns leads to the activation of adhesion molecules on the endothelial cells, leading to further infiltration of leukocytes to the gingival tissues. Moreover, release of

enzymes from activated neutrophils into the connective tissues can lead to collagen degradation and unwanted tissue destruction.

During 5-7 days of biofilm accumulation, the dominant cell type in the early lesion now shifts to monocytes/macrophages and lymphocytes [142]. The predominant lymphocyte at this state is the T cell with a CD4:CD8 ratio of 2:1. T cells become activated by antigen presenting cells (DCs) via MHC-II class antigens (HLA-DR). Langerhans cells (immature epithelial DCs) express high levels of MHC-II and their frequency increases during this stage of disease as well. Another significant function of immature DCs is that they migrate to the peripheral lymph nodes where they sensitize T cells to the bacterial antigen encountered. This in turn results in migration of those sensitized T cells to the inflamed periodontal tissues in an attempt to control the bacterial insult and achieve a balance of the biofilm. Vascular changes are more pronounced, noticed by activation of capillary beds and the development of inflammatory infiltrates around the peripheral vasculature. There are significant increases in endothelial cell leukocyte adhesion-molecules (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), along with induction of IL-8 by gingival epithelial cells, which are chemoattractive of PMNs [143]. The massive infiltration of neutrophils to the gingival sulcus helps build a barrier against bacterial pathogens and prevents their further migration [144]. In addition to neutrophils, various DCs subsets appear to infiltrate the gingival tissues at distinct stages of disease. Langerhan cells (LCs), immature dendritic cells primarily involve in antigen capture and processing, infiltrate the gingival epithelium during this stage. This is followed by their migration to the connective tissues (lamina propia) where they begin to undergo maturation, loose their capture-processing functions to become antigen presenting cells [145]. Moreover, dermal DCs (DC-SIGN+) also increase in the lamia propia in CP lesions and localized towards the lymphatics and vasculature [146, 147].

Inappropriate plaque removal leads to bacterial persistence in the gingival sulcus and a pronounced shift from T to B (plasma) cells, which is the characteristic feature of the established lesion [148]. Other important changes includes further edema, secretion of pro-inflammatory cytokines (IL-1, IL-6) and greater soft tissue destruction [149]. Clinically, the junctional epithelium now is displayed further apically leading to the formation of a periodontal pocket, where periodontal anaerobic pathogens thrive and survive. The cellular infiltrate in the advance lesion is very similar to the established lesion, but the main difference of the presence alveolar bone loss, which is the hallmark of CP. Destruction of the connective tissues is aggressively performed by MMPs and serine proteases [150], while alveolar bone resorption is mediated by osteoclast activation via the RANK-RANK-L pathway [151].

Immunoregulation, during the transitioning from gingival health to disease, is a key determinant for the development to CP. T cells are central in the regulation of this response and in this context, a fine balance between the so-called Th1 and Th2 is crucial. T helper responses were first identified in 1986 [152]. Th1 cells are involved in cellular mediated immune responses, typified by delayed-type hypersensitive response (DTH), and primarily secretes IL-2 and Interferon gamma (INF-γ). In addition Th1 cells suppress B and plasma cell responses. In contrast, Th2 cells mediates the B cell humoral response by secreting IL-4, IL-5 and IL-10, while suppressing T cell proliferation responses [153]. It is generally accepted that gingivitis is associated with a cellular mediated Th1 response, while the progressive CP lesion is dominated by Th2 cells [154]. Recently, another lineage of T cells has been described, that is the so-called Th17 cells. Th17 produce IL-17, which in turn induces the secretion of IL-6, IL-8 and PGE2 and play a role in inflammation and osteoclastic bone resorption. [155, 156]. Conversely, another T

cell subset (Treg) has been described as well. T regs have an important function in suppressing autoimmune response, and perhaps protective against bacterial infections [157].

Other important features of the innate responses involve the presence toll-like receptors (TLRs). These receptors are present on DCs, neutrophils and macrophages, among other cells, and function to recognize highly conserved structures (pathogen associated molecular patterns [PAMPs]) on microbial pathogens. Common examples of PAMPs includes LPS, peptidoglycan, double stranded RNA, lipoproteins and bacterial DNA [158]. It is thought that TLRs play a role in determining the nature of the host response to periodontal pathogens, for instance TLR-2 and TLR-4 activation results in markedly different immune responses, evidenced by the resulting cytokine profile. TLR-4 activation promotes the expression of IL-12p70 and INF-γ inducible protein 10 (IP-10), which drives a Th1 immune response. However, TLR-2 induction of IL-12p40 which is characteristic of a Th2 response [159]. Overall, the innate and adaptive immune responses play a significant role in susceptibility to CP.

C. Linking evidence to CAD

The idea that CP is associated with CAD is supported from analysis of data from NHANES 1, which demonstrated that periodontal disease is a risk factor for cerebrovascular accidents (CVAs), and in particular non-hemorrhagic stroke [160]. CAD is more prevalent in patients with a CP history and is more common in patients with elevated antibody levels to periodontal pathogens, such as *P. gingivalis* [161, 162]. Several studies found correlations between oral disease and both myocardial infarction (MI) [163] and CAD [164]. A seven-year prospective study showed a positive relationship between the incidence of MI and deterioration of oral health in subjects with pre-existing CAD [165]. The NHANES study followed subjects

for 17 years and showed the presence of periodontitis increased the risk of CAD by 25% when compared to those subjects with minimal periodontal disease [166]. Recently patients with CP have been shown to exhibit systemic inflammation, evidenced by elevated plasma hsCRP, fibrinogen, IL-6, and other such biomarkers [136, 167-171] . Additional supports comes from meta-analysis showing an increased incidence of CAD among patients with CP [3]. Results from other meta-analysis have concluded that periodontitis is a risk factor for CAD with a relative estimate risk ranging from 1.24 to 1.35 after adjusting form classical confounding factors [172]. More recently, it was demonstrated that failure to maintain a good level of oral health and biofilm control, results in an increase risk to CAD [173]. Moreover, tooth loss was also recently associated to increase cardiovascular disease mortality [174].

According to a 2009 consensus between the American Journal of Cardiology and the Journal of Periodontology, two biologically plausible mechanisms have been proposed to explain the association between CP and CAD [175]. First, patients with moderate to severe CP have increased levels of systemic inflammation determined by human serum C-reactive protein (hsCRP) [176]. Recent evidence has shown that poor oral hygiene results in higher CRP and fibrinogen serum levels [173]. High levels of hsCRP predicts the future of coronary artery disease complications, such as myocardial infarction (MI) and unstable angina pectoris [177]. This idea has been further supported by interventional trials showing that the treatment of periodontitis results in reduce systemic inflammation in patients with a previous history of atherosclerotic disease [178].

The second mechanism stems from the idea that CP causes a systemic bacteremia. Periodontal pockets in patients with CP harbors around 10^8-10^{12} gram-negative bacteria. Periodontal pathogens have been found in the atheromas of patients with CAD [94]. This has

been further support by animal studies demonstrating that in the presence of periodontitis, atheroma formation is further enhanced [179]. Other non-periodontal bacterial microorganisms, such as *C. pneumoniae*, have being also found in the atheromas of patients with CAD [180], as previously discussed. During the late 1990's, studies began to examine the effect of antibiotics in CAD. Although the results were initially promising, recent clinical trials have shown no effect at reducing CAD complications, such as MI [119]. Altogether these data suggests that the immune response triggered by these microorganisms may have a more significant impact in CAD complications.

Recent studies have also implicated, in addition to hsCRP, elevated levels of MMP-2, -8 and -9 are associated with CAD and CP. For instance, patients were more likely to have a cardiovascular event when had elevated serum levels of MMP-8. Further they also reported that MMP-8, and -9 was also elevated in the GCF in patients with CP [181]. This has important implications because MMP-9, for instance, have been positively correlated with the incidence of MI [182]. Recent data appears to suggest that mDCs are a significant contributor of MMP-9 [183]. In this *in-vitro* study MMP-9 was highly induced by monocyte-derived dendritic cells when challenged with *P. gingivalis*, relative to controls. MMP-9 is also involved in periodontal tissue destruction, thus facilitates leukocyte extravasation into the disease tissues.

D. Host-pathogen interactions: *Porphyromonas gingivalis*

P. gingivalis, along with two other periodontal pathogens, *Tannerella forsythia and Treponema denticola*, form the so-called red complex [184]. Among these pathogens, *P. gingivalis*, a gram-negative anaerobic and asaccharolytic rod, is the most prevalent in patients with CP [185, 186]. *P. gingivalis* relies on its virulence factors (polysaccharide capsule, fimbriae, proteases against complement and antibodies, gingipains and LPS, toxins and hemagglutinins) to

induce and modulate the immune host response [187, 188]. The capacity of *P. gingivalis* to invade, colonize and grow in CP gingival tissues is mediated, in part, by mayor and minor fimbrial adhesins [189, 190]; adhesive hair-like appendages originating from the bacterial cell surface [191]. While the major fimbriae, a 41kDa protein, targets cellular β-integrins (CD29, CD18) [100], the minor fimbriae, a 67kDa protein, targets DC specific intracellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN or CD209) on monocyte-derived dendritic cells for entry [127]. DC-SIGN is a type II membrane protein with C-terminal carbohydrate domains (CRD) that belongs to the C-type lectin superfamily [192]. DC-SIGN selectively recognize endogenous high-mannose oligosaccharides; it also mediates the initial interactions between DCs and T cells. In addition to *P. gingivalis*, other important pathogens such as *Neisseria gonorrhoeae* [103], *Mycobacterium tuberculosis* [101], *Mycobacterium leprae*, *HIV* [102], among others targets DC-SIGN in mDCs for entry and host immune modulation.

An intense infiltrate of DC-SIGN+DCs [145, 193], among other leukocyte subsets, are noticed in the inflamed gingival tissues in patients with CP. After DCs antigen encounter in the gingival epithelium, they appear to mobilize toward the capillary-rich connective tissues [145], in order to exit towards the peripheral lymph nodes for induction of adaptive immune responses. Alternatively, *Pg*-infected DCs, in CP lesions, may re-enter the blood circulation where they can reach sites of activated vascular endothelium, such as the coronary arteries in CAD [105]. This idea is further supported by evidence that DC-SIGN+DCs infiltrate rupture prone unstable plaques [4], but the original sources of these cells are presently unclear. Possible sources include, but are not limited to, DCs from peripheral inflamed gingival tissues [145], peripheral blood monocytes [194], and bone marrow progenitors. DCs that infiltrate atherosclerotic plaques express atherogenic markers, that include C1q (classical component pathway involved in

apoptotic cell clearance) [195]; Hsp 60/70 (chaperone proteins involved in autoimmune responses) [196]; chemokine receptors CCR2, CCR5, CX3CR1; and chemokines CXCL16, CCL19, and CCL21 (mediate DC migration and homing) [194]. In addition evidence that infiltrating DCs have matured are noticed by the expression of CD40, CD80, and CD86 [197]. Moreover, fimbriated *P. gingivalis* not only invades and infects DCs, but also induces the expression of these and other atherogenic markers [127]. This evidence further supports the notion that *Pg*, along with other pathogens, evades the host response and chronically persists in CP lesions by invading and manipulating the normal DC functions to their own advantage. This results in altered DC homeostatic migration patterns and redistribution through the circulation to the periphery, such as the coronary arteries.

E. Immune evasion strategies by *P. gingivalis*

Microbial immunoevasion, by multiple attack strategies, is an evolving behavior required for survival and persistence in the host. *Mycobacterium tuberculosis* [198], *Listeria monocytogenes* [199], *Legionella pneumophila* [200], *Coxiella burneti [201]* are examples of bacterial species that have developed evasive strategies. These bacteria parasitize host cells by arresting or reprogramming phagosomal maturation, by escaping maturing phagosomes or by withstanding the microbicidal properties of the phagolysosome. Inhibition of complement activation [202], TLR-4 evasion and antagonism [203], degradation of TLR co-receptors [204], inhibition of phagocyte killing [205] and promotion of intracellular survival [206] are among one of the may immune evasion strategies *P. gingivalis* uses to subvert host defense mechanims and avoid elimination.

Gingipains, encoded by rgpA, rgpB, and kgp genes, are cysteine proteases with proteolytic activity involved in disruption of host proteins, such as antibodies and the

complement system. In a recent *in-vitro* study it was demonstrated that gingipains from *P. gingivalis* confers protection against bactericidal activity, mediated by digestion of C3 component [202]. The authors of this study also found that early in the infection process, *P. gingivalis* actually activates the complement system to favor its colonization and nutrient needs. This is followed by C3 complement inactivation, once *Pg* has significantly grow within the biofilm and thus possess a much greater amount of proteases to prevent bactericidal killing.

Another immune evasion strategy *P. gingivalis* exploits is by inhibiting antimicrobial peptides, such a β-defensins from human epithelial cells [207]. β-defensins confers resistant to epithelial surfaces from the invasion by bacterial pathogens. *Pg* inhibits β-defensins by secreting heterogeneous and atypical forms of LPS that acts as TLR-4 antagonists [203]. Induction of TLR-4, results in β-defensins secretion by epithelial cells, which leads to pore formation in the bacterial cell wall and thus cell lysis. *In-vitro* and *in-vivo* studies have shown that TLR-2 also senses *P. gingivalis*, promotes pathogen clearance and reduces alveolar bone loss [208, 209]. *P. gingivalis* also targets leukocyte pattern recognition receptors, such as complement receptor (CR3) and induces phagocytic uptake [210]. CR3 mediated pathway is primarily involved in the phagocytosis of apoptotic cells, which most often does not represent a danger signal to the host. *Pg* targets this pathway in order to promote its intracellular survival by preventing phagocytosis [206]. CR3 binding by *Pg* suppresses the release of IL-12, involved in T cell differentiation, leading to persistence by preventing bacterial clearance [205]. Ligation of CR3 in dendritic cells also leads to poor antigen presentation and suppression of their stimulatory and migratory capacity [211]. Thus, *Pg* fimbrial ligation of CR3 in DCs, may not only lead to intracellular

persistence in CP lesions, but to may allow relocalization to systemic tissues where it can infect new host cells, such as arterial endothelial cells [126].

Previously we have shown that *Pg* associates with DCs *in-situ* [212] and *in-vitro* [127]. *Pg* targets DC-SIGN via its minor fimbriae and this interaction results in a dampening of the maturation status and the inflammatory cytokine profile of DCs [127]. Expression of DC-SIGN is one indicator that DCs are not fully mature. However, absence of the minor fimbriae results in marked DC maturation and proinflammatory cytokine response. Furthermore, while the major *Pg* fimbriae induce DCs to prime naïve T cells into a Th1 effector response, the minor fimbriae elicits an immunosuppressive Th2 phenotype. *Pg* not only has evolved to evade host immune responses, but appears to also regulate DCs immunogenic functions to promote its persistence.

Previous evidence have demonstrated *Pg* association with immature DCs (Langerhans cells) in CP lesions [212]. Moreover, immunofluorescence studies have shown that DC-SIGN+DCs appear to be mobilized to the highly vascular lamina propria in patients with chronic periodontitis [145], probably in response to periodontal pathogens, such as *P. gingivalis*. *In-vivo* DC-SIGN ligation by *Pg*, might disrupt DC maturation in CP lesions and possibly prevent DCs migration to peripheral lymph nodes. Thus *Pg*-infected DC-SIGN+DCs in CP lesions, may have the opportunity to relocate into the peripheral blood, where it can either promote systemic inflammation or reach areas of activated vascular endothelium, infiltrate and thus contributes to the increase levels of DCs noticed in unstable atherosclerotic plaques. This process might enhance the progression and instability of arterial plaque in patients with CAD.

Aims of the Research

1. To characterize mDCs in the blood and the gingival tissues of human subjects with chronic periodontitis, relative to periodontally healthy individuals. We hypothesized that the number of blood mDCs, containing *Pg* transcripts, will increase in CP relative to health. CP patients will have the higher numbers of blood mDCs containing *Pg* 16s rDNA. *Pg* will also be identified in association with DC-SIGN+ mDCs in gingival tissues.

2. To determine whether mechanical debridement elicits a change in the number or phenotype of blood mDCs in CP patients. We hypothesize that mechanical debridement (i.e. scaling and root planing [S&RP]) will induce a detectable increase in the number of mDCs containing *Pg* 16s rDNA in the peripheral blood of CP patients.

3. To characterize mDCs in the blood and post mortem-atherosclerotic plaques of human subjects with coronary artery disease (CAD) and CP. We hypothesize that the number of blood mDCs containing *Pg* 16s rDNA will be greater in patients with CAD and CP as compared to CP alone and healthy controls. We further hypothesize that *Pg* will be identified in association with mDCs in atherosclerotic plaques.

Material and Methods

1. Study Population and Design

The Committee on Research Involving Human Subjects (CORIHS) at Stony Brook University approved all protocols involving human subjects. Informed consent was obtained from all subjects before commencement of the study. The cohort of subjects with chronic periodontitis (CP) consisted of 40 subjects with moderate to severe CP as determined by the presence of greater than 20 teeth, of which at least 8 exhibited: probing depth > 4mm, attachment loss > 3mm, bleeding on probing, alveolar bone crest > 3 mm from cemento–enamel junction (CEJ). Demographic data and clinical parameters of the study subjects are shown in Table 1. Exclusion criteria included: steroidal anti-inflammatory agents, smoking, periodontal treatment within the past 6 months, pregnancy, diabetes, heart disease, or cancer. A subset of CP subjects included those with acute coronary syndrome (ACS) (n=15), diagnosed as reported (70) and shown in Table 1. Healthy controls (CTL) consisted of 25 age and gender-matched subjects, non-smokers without CP; who had no history of ACS, diabetes, cancer or other reported systemic disease.

This is an observational (longitudinal-4 weeks duration) study that examined if there is a correlation between the frequency of blood mDCs in CP (study's primary outcome), relative to periodontally healthy individuals (Fig. 1). Secondary outcomes included: the presence of *Pg* (16S rDNA) in gingival, coronary arteries and blood mDCs, relative to healthy controls. In addition, colonization of *P. gingivalis* in subgingival dental plaque was also examined. After the initial exam, all CP patients were subjected to scaling and root planing (local debridement of the root surfaces and pockets) under local anesthesia and the blood mDC response evaluated at 24

hours. Healthy controls were not subjected to scaling and root planing because there is no clinical need and it can be detrimental to clinical attachment levels. In addition, the kinetics of blood mDC response (after SRP) was also examined in a small subset of CP (N=5) patients at the following intervals: 1, 3, 6, 24, 48 hours, and 4 weeks. A similar intervention was performed in a CTL (N=1) patient after a supragingival prophylaxis (sham control). All samples were obtained under non-fasting conditions.

2. Blood mDC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from 30ml of whole blood and a nucleated cell suspension was prepared using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare). Myeloid DCs (mDCs) were isolated by positive immunoselection as described previously [213]. Briefly, PBMCs labeled with CD1c-PE+ (BDCA-1., Miltenyi Biotec Cat. no. 130-090-508), CD209-APC+ (BD Cat. No. 551545), CD19-FITC+ (BD Cat. No. 557697) were FACsorted (FACS ARIA-BD Biosciences) then sorted again to remove CD19+ (B-) cells from: (i) CP (ii) ACS/CP, (iii) CTL. These procedures routinely yielded mDC (CD1c+CD209+CD19-) preparations of >95% purity (Fig. 4).

3. Generation of monocyte-derived dendritic cells (MoDCs)

To serve as *in-vitro* model of DC infectivity and *P. gingivalis* survival, MoDCs were generated as we have described previously [127, 147, 193]. Briefly, monocytes were isolated from mononuclear cell fractions of the peripheral blood of healthy controls and seeded in the presence of GM-CSF (100 ng/ml, PeproTech Inc. Cat # 300-03) and IL-4 (25 ng/ml, R&D Systems Cat# 204-IL-010) at a concentration of 1–2 X 10^5 cells/ml for 6–8 days, after which

flow cytometry was performed to confirm the immature DC phenotype (CD14-CD83-CD1a+CD1c+DC-SIGN+ (all antibodies from BD Biosciences, except CD1c [Miltenyi Biotec]).

4. *In-vitro* infection and mDC differentiation model

After PBMC isolation and plastic adherence of monocytes, monocytes were scraped off plates and collected. The total numbers of monocytes were counted using Accuri's C6 Flow Cytometer® System and baseline levels of CD14, CD1c, DC-SIGN, and CD86 expression were obtained. Monocytes were then divided equally into 6-well plates and treated either with GM-CSF and IL-4 alone, wild-type *Pg*381 alone, GM-CSF and IL-4 plus wild-type *Pg*381 or RPMI alone. Bacterial multiplicities of infection (MOIs) chosen were 0.1, 0.5, and 1. Each experimental condition was performed in triplicate. Cells for each condition were collected on subsequent days 1, 2, and 3 for analysis of MoDCs present and changes in receptor expression. Cells were gated on scattergram plots based on size characteristics for both monocytes and MoDCs. Flow samples were collected based on total number of events rather than volume, so differentiation of monocytes into dendritic cells is represented as MoDCs per microliter of sample. Triplicates were averaged and assembled onto a line graph (Fig. 7b-c) to show the differentiation of monocytes to MoDCs with and without growth factors. Intensity profiles of CD14, DC-SIGN, CD1c, and CD83 expression were compared with baseline monocyte levels to show downregulation of CD14, upregulation of DC-SIGN and CD1c and immature state of MoDCs for each condition.

5. Flow cytometric phenotyping

MDCs were labeled with combinations of PE-, FITC-, allophycocyanin (APC) and PerCP mouse anti–human lineage Ab (CD14, CD19, CD11b/Mac-1, CD1c [BDCA-1, Miltenyi Biotec],

CD80, CD83, CD86, HLA-DR, CD209 (DC-SIGN), CD1a or isotype controls (all from BD-Biosciences, San Jose, CA); and Goat Anti-Mouse IgG (H+L) [Invitrogen]. Analysis was performed with a FACSCalibur flow cytometer (BD). Marker expression was analyzed as the percentage of positive cells in the relevant population defined by forward scatter and side scatter characteristics. Expression levels were evaluated by assessing mean fluorescence intensity indices calculated by relating the mean fluorescence intensity noted with the relevant mAb to that of the isotype control mAb for samples labeled in parallel and acquired using the same setting.

6. Blood mDC response after treatment

Blood PBMCs were obtained from all study subjects at baseline and 24 hours after a single intensive bout of scaling and root planing (S&RP), as reported [214]. This treatment has previously been shown to result in acute, short-term bacteremia [215, 216], as well as systemic inflammation [214]. In a small subset of CP patients (N=5) the kinetics of blood mDCs response was examined at 1, 3, 6, 24, 48 hours and 4 weeks after SRP (Fig. 6a-b). Similar intervention was undertaken in a healthy control individual after supragingival prophylaxis. The mDCs were labeled with fluorescent conjugated antibodies (as described above), sorted (FACS Aria-BD) and analyzed (FACS Diva v.6.1.3) as described above.

7. Derivation of monoclonal antibody to DC invasin, mfa-1 minor fimbriae

MAb 89.15 against the DC-invasin, mfa-1 (minor fimbriae) was generated by the Cell Culture/Hybridoma Facility at Stony Brook University [217]. Briefly, three female 6-8 week old BALB/c mice (Charles River) were immunized intraperitoneally with three 50 μg doses of native minor fimbriae (Mfa-1) in Sigma adjuvant (Sigma-Aldrich Co., St. Louis, MO) at two-week

intervals, following which sera was drawn and tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antigen-specific antibodies. The mouse selected for splenectomy had a titer of >1:1000 to the protein. Prior to fusion, the mouse was boosted intraperitoneally with 1 μg of Mfa-1 in PBS (Gibco-Invitrogen, Carlsbad, CA). Four days following the booster, the mouse was sacrificed, the spleen cells isolated aseptically and fused with mouse myeloma cell line Sp2/0 (ATCC), as described [217]. Clones were screened by ELISA against native minor fimbriae. Clones were then further screened using a whole bacteria ELISA against MFI, which expresses only the major fimbriae (*Pg* min-/maj+), and DPG3, which expresses only the minor fimbriae (*Pg* min+/maj-). Clone 89 was determined to be positive both by native minor fimbria ELISA and whole bacteria ELISA and thus was selected for sub cloning by limiting dilution. Sub clone 89.15 was selected by ELISA for further study. MAb 89.15 was determined to be of the IgG1 isotype having a κ light chain, by use of the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science, Indianapolis, IN). Antibodies from this sub clone are referred to as AEZαMfa1 (Fig. 2a).

8. Immunofluorescence staining of gingival and coronary artery tissues

Gingival tissues from the human gingiva were collected from untreated CP patients using a biopsy technique previously reported [218]. 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). Coronary artery tissues were obtained from human post-mortem patients with atherosclerosis (ATH) and CP (kind gift from Dr. Emil Kozarov, Columbia University, NY) and cryosectioned as described above. 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 block reagent (Miltenyi Biotec, Auburn, CA) for 1 h, and washed. Sections were incubated for 30 min. 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., Burlingame, CA). To identify DC-SIGN within arterial plaques and gingival tissue sections, fluorescein isothiocyanate (FITC)-conjugated (BioLegend, San Diego, CA.) or RPE-conjugated mouse anti-human CD209 (AbD Serotec, Raleigh, NC) were used. FITC-conjugated mouse anti-human CD1c was used (BD Biosciences) to identify CD1c. DyLightTM microscale antibody labeling kit (Thermo Scientific, Rockford, IL) was used to conjugate Alexa Fluor 594 to mfa-1 antibody AEZαMfa1 according to manufacturer's protocol. Controls included isotype matched antibodies and pre-immune antibodies. Images were acquired with a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System coupled with image processor software for image processing. In addition, sections were stained with hematoxylin and eosin stain (H&E) to examine cell and tissue morphology. The H & E images were analyzed by image enhanced light microscopy (Nikon E600).

9. *In-situ* association of mDCs with *P. gingivalis* by immunofluorescence

The mDCs from blood of CP and ACS/CP patients were analyzed by immunofluorescence cytometry to determine infection with *P. gingivalis*. Briefly, FACS-sorted CD19-CD1c+Dc-SIGN+ mDCs were permeabilized and fixed in Shandon Cytospin Collection Fluid (Thermo Scientific Cat. No. 6768315). The cytological specimen was deposited on Ultrastick slides (Thermo Scientific Cat. 3039) and cytocentrifuged (Shandon Cytospin 4, Thermo Scientific, Inc). After blocking human FcR, as described above, mDCs were probed with

PE-AEZαMfa1 and Vectashield TM mounting media with DAPI (VWR Cat. No. 101098-044) was added to the specimens and then slides were analyzed by conventional epifluorescence (Nikon E600) and by confocal microscopy as above.

10. Quantitative RT-PCR

To detect and quantitate *P. gingivalis*, genomic DNA (gDNA) and total RNA was isolated from subgingival plaque and mDCs with the RNeasy Micro kit (QIAGEN) according to the manufacturers' instructions, but with a slight modification. Briefly, gDNA Eliminator spin columns were not used during the isolation protocol. This was done to collect, in addition to total RNA, gDNA for *P. gingivalis* 16S rDNA detection in the mDCs. cDNA was synthesized using STR1 Enhanced Avian First Strand Synthesis Kit (Sigma-Aldrich Cat. No. STR1-1KT). The cDNA template was standardized to a concentration of 0.1µg/µl using a NanoDrop 3300 Fluorospectrometer (Thermo-Scientitific Cat. No. ND3300) with Quant-iTTM dsDNA HS Assay Kit (Invitrogen Cat. No. Q32851). Quantitative real time-PCR (qRT-PCR) was used to detect the presence of *P. gingivalis* (16S rDNA) in MoDCs, mDCs and dental plaque samples. The 16S rDNA consisted of a forward *Pg-specific* primer (5'-TGT AGA TGA CTG ATG GTG AAA ACC-3'and a universal reverse primer (C11R), 5'-ACG TCA TCC CCA CCT TCC TC-3'sequence as previously described [219]. The universal reverse primer was also used to detect non-*P.gingivalis* amplification products, which were then subjected to genomic blast sequencing. PCR reactions were performed using QuantiTect SYBR Green PCR Kit (Qiagen Cat. No 204145) on an iCycler Thermal Cycler (Biorad). The thermal cycling conditions were an initial incubation step of 15 minutes at 95^{o}C to activate HotStartTaq DNA polymerase. This step prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. This initial

incubation step was followed by 45-50 cycles at 94^{o}C for 15s, 54^{o}C for 30s and 72^{o}C for 30s. *P. gingivalis* 16s rDNA primer was a kind gift of Dr. Stephen Walker, SUNY-Stony Brook, NY. As a quantitative standard for qRT-PCR analysis of mDCs, MoDCs were spiked *in vitro* with *P. gingivalis* 381 at a range of multiplicity of infections (MOI) and colony forming units per ml (CFU/ml). This yielded a linear regression curve consisting of log10 CFU/ml vs. amplification cycles (Fig. 2e). The infection status of mDC from the blood was then expressed as estimated CFU/ml and MOI relative to our MoDC standard.

11. DNA Sequencing

In order to confirm the specificity of the PCR amplified product using the *P. gingivalis*16S rDNA primer, (or the non-P. *gingivalis* specific PCR amplified products) the resultant amplicons were sequenced. Briefly, the amplified product was run in a 1% agarose gel electrophoresis (Bio-Rad Cat. No. 162-0102), along with a 0.1-10.0kb DNA ladder (New England BioLabs Cat. No N3200S). The resultant band was extracted and purified from the agarose gel using QIAEX II Gel Extraction Kit (Qiagen Cat. No. 20051), according to the manufacturer's instructions. The DNA template (3.0 ng) were combined with 3.2 pmol 16S rDNA primer (197bp) and sequenced using a 3730 DNA Analyzer (Applied Biosystems). The sequenced product was then aligned against all human, prokaryotic and eukaryotic known database genome using Genomic BlastSequence (BLASTN 2.2.24+) (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

12. Quantitation of serum DC-poietins FLT3-L, sTNF RI, sTNF RII

Serum analysis of DC-poietin levels was conducted with colorimetric sandwich ELISA using respective Quantikine Immunoassays: Human Flt-3 Ligand (Quantikine DFK00), sTNF RI

(Quantikine DRT100), sTNF RII (Quantikine DRT200) (R&D Systems). Briefly, standard dilutions and recommended serum sample dilutions were incubated in pre-coated microplates containing a specific monoclonal antibody. Unbound debris was washed and an enzyme-linked polyclonal antibody specific for the target molecule was added. Unbound antibody was removed and a substrate solution was added to give a proportionate color change relative to the amount of DC-poietin in the well. The substrate reaction was stopped and microplates were read immediately at 450nm on a microplate reader with a correction wavelength of 540nm (EMax, Molecular Devices).

13. Determination of human anti-*Porphyromonas gingivalis* IgG titers

Serum levels of anti-*P.gingivalis* IgG antibodies were determined by ELISA as previously described [220]. In brief, 96-well ELISA plates were coated with *P. gingivalis* strain DPG-3 (1 x 107 cells/ well), followed by one hour incubation at 37^0C. The wells were blocked for 2 hours at 37^0C with 2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA; Cat. No. A3912) in PBS-tween20 (Sigma Chemical Co., St. Louis, MO, USA; Cat. No. P1379). Appropriate dilution of serum samples as determined by checkerboard titrations using pooled sera was added and the plates were incubated for 2 h at 37^0C. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H+L) antibodies (Promega, Madison, WI, USA; Cat. No. W4031) were added and incubated for 1 h at 37^0C. TMB (3,3',5,5'-Tetramethyl-benzidine Liquid Substrate for ELISA, Sigma Chemical Co., St. Louis, MO, USA; Cat. No.T4444) was used as a substrate. The reaction was stopped by the addition of 3N hydrochloric acid (Lab. Chem Inc., Pittsburgh, PA, USA; Cat. No. LC15360-2), and the optical density were read using an E-max microplate reader (Molecular Devices, Palo Alto, CA, USA) at 450 nm.

14. Intracellular Survival

Wild type *Pg*381 was used to infect MoDCs at a multiplicity of infection of 100. Uptake of the bacteria by human cells was confirmed by observing complete internalization of CFSE-stained *Pg* via epifluorescence microscopy, aided by deconvolution imaging software 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% CO2 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 per mL were determined.

15. ELISA assay for MMP-9

Isolated mDCs from healthy controls were pulsed with 100 ng *P. gingivalis*, *E. coli* LPS or *P. gingivalis* strains at a 1:25 MOI for 24 hrs were analyzed in triplicate for MMP-9 levels in pg/ml, by quantitative sandwich enzyme immunoassay technique (ELISA), as described by the manufacturer (Quantikine®, R&D systems, Minneapolis, MN).

16. Zymography

Isolated mDCs from healthy donors were mixed with Tris-Glycine SDS sample buffer (2x) (Millipore) and allow to stand for 10 min at room temperature. Samples normalized by volume according to protein concentration were loaded on a 10% zymogram (gelatin) Gel. Samples were run with 1x Tris-Glycine SDS running buffer at 125V for approximately 90 min, or when the Bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, gel was

removed and incubated in 1X Zymogram Renaturing Buffer (Triton × –100, 2.5% (v/v) in water) (Millipore) for 30 minutes at room temperature with gentle agitation. After decanting the Zymogram Renaturing Buffer, 1X Zymogram Developing Buffer (50mM Tris base, 0.2M Nacl, 5mM CaCl2, 0.02% Brij 35) (Millipore) was added. Gel was equilibrated for 30 minutes at room temperature with gentle agitation, buffer was decanted and fresh 1X zymogram developing buffer was added. Gel was incubated at 37°C for at least 4 hours or overnight for maximum sensitivity. The optimal result was determined empirically by varying the sample load or incubation time. Gels were stained with Coomassie Blue R-250 for 30 minutes, then destained with an appropriate Coomassie R-250 destaining solution (Methanol: Acetic acid: Water (50: 10: 40). Areas of protease activity appeared as clear bands against a dark blue background, where the protease has digested the substrate.

Results

1. Infection of DC-SIGN^{+} gingival tissues mDCs by mfa-1^{+} *P. gingivalis* in CP patient

Our previous *in-vitro* work established that the minor mfa-1 fimbriae of *P. gingivalis* is a DC invasin, with which it binds to DC-SIGN (CD209) on mDCs to gain access to the intracellular milieu [127, 221]. To determine if mfa-1 is involved in infection of mDCs in CP lesions, dual immunofluorescence staining was performed. Initial Western blotting analysis confirmed the specificity of monoclonal antibody AEZαMfa1 for the DC-invasin mfa-1 (Fig. 2a). The results indicate that *P. gingivalis* mfa-1 colocalizes with DC-SIGN+ mDCs in CP lesions from gingival tissues (Fig. 3a). Identical staining of healthy control (oral) tissue revealed trace mfa-1 and some DC-SIGN positivity, but no mfa-1/DC-SIGN colocalization was detected.

2. *P. gingivalis* in subgingival plaque: a source of mDC infection

To determine the source of the *P. gingivalis* that is infecting the mDCs in CP lesions, subgingival biofilms were sampled and analyzed for 16s rDNA of *P. gingivalis* (Fig. 2b). The identity of each amplified product was confirmed by genomic blast sequencing (GBS) (Fig. 2c). The results indicate that ~ 50% of the CP patients (including the source of the gingival tissue biopsy in Fig. 3a) yielded the ~197-200bp amplified product in their biofilm, with >90% sequence match with *P. gingivalis* (Fig. 2c, Table 5).

3. Blood mDCs carriers of *P. gingivalis* and other pathogens

To determine whether blood mDCs may also be *Pg*-infected, we isolated blood mDCs and analyzed the content of *P. gingivalis* 16s rDNA by qRT-PCR-GBS (Fig. 2d, Table 5). The presence of whole intact *P. gingivalis* within blood mDCs was determined by immunofluorescence-confocal microscopy (Fig. 3b). Representative amplified products from

mDCs of CP and ACS/CP patients, along with *in-vitro* controls are depicted (Fig. 2d). Overall, 72% of CP patients orally colonized by *P. gingivalis* were also positive for *P. gingivalis* 16s rDNA within blood mDCs (Table 5), while no healthy control mDCs yielded an amplified product. The correlation between oral carriage state and mDC infection rate in CP subjects was positive and significant (Spearman r = 0.5192, p = 0.0078). Although the oral carriage rate of ACS/CP patients was unknown, 37.5% were positive for *P. gingivalis* 16s rDNA in blood mDCs. To estimate in tangible terms the level of infection of blood mDCs in these patients, an *in vitro* MoDC-infection model was implemented. The results (Fig.2e, Table 5) estimate CFUs (eCFU) of *P. gingivalis* in blood mDCs from CP and ACS/CP patients equal to 132,623 eCFUs (+ 121,484 S.E.), while estimated MOIs were all below 1. Other species identified and sequenced from 16s rDNA of mDCs included *Helicobacter pylori*, *Pseudomonas spp*., *Moraxella catarrhalis*, *Klebsiella pneumonia* and *Salmonella enterica* and others. Live bacteria recovered on blood agar from mDCs included *Burkholderia cepacia* from two ACS/CP patients (Table 5). Cytocentrifuged mDCs confirmed the typical morphology of blood mDCs [213] and indicated the presence of mfa-1^+ *P. gingivalis* within blood mDCs at low MOI (Fig. 3b). The ability of *P. gingivalis* to gain entry into DC-$SIGN^+$ blood mDCs was further corroborated by pulsing *ex-vivo* isolated mDCs with CFSE-labeled *P. gingivalis in-vitro* and counter labeling with DC-SIGN. The results show intact *P. gingivalis*-CFSE within DC-$SIGN^+$ blood mDCs (Fig. 3c).

4. Infection of blood mDCs influences mDC frequency, in CP patients, *in-vivo*

To determine whether the frequency of mDCs in circulation is altered by the systemic bacteremia, we analyzed the mean number and percentage of mDCs in CP patients who are at increased CAD risk, and ACS patients, who have existing CAD. The mDC numbers were compared to the lowest risk group (healthy CTL). Representative results (Fig. 4a) illustrate an

apparent increase in blood mDCs in order of increased CAD risk. The mean number (Fig. 4b.1) and percentage (Fig. 4b.2) of blood mDCs in CTL, CP and ACS+CP are shown, indicating elevated blood mDCs ($p<0.05$, Students t-test) in CP vs. CTL and ACS+CP vs. CP and CTL. This was not due to an increase in total PBMCs, as PBMCs were decreased in CP and ACS/CP (Fig. 4b.3). The pathogen burden of the ACS and CP mDCs is shown in Table 5. As a previous correlation was made between blood DC frequency and serum levels of FLT-3L and soluble TNF receptors [222, 223], we analyzed these cytokines in the same patient sera. The results show no difference in FLT-3L, TNFr1 or TNFr2 in CP vs. CTL subjects. However, a significant increase was noted in all three cytokines in ACS/CP, relative to CP and CTL ($p <0.05$, Students t-test) (Fig. 4c). Due to the relationship between infectious seropositivity and cardiovascular disease mortality [224] we analyzed in CP and CTL cohorts the serum IgG antibody titers against DPG-3, a strain that solely expresses mfa-1 fimbriae [127]. The results demonstrate higher anti-*P. gingivalis* DPG-3 IgG antibody titers ($p<0.05$, Students t-test) in CP sera vs. CTL sera (Fig. 2f). Moreover, serum antibody titers to *P. gingivalis* DPG-3 were linearly related to blood mDC frequency in CTL vs. CP ($r2 = 0.56$, $p<0.0001$) (ACS+CP sera were not available) (Fig 2g).

5. Induction of short-term bacteremia increases mDC infection and mDC frequency

Mechanical debridement or scaling and root planing (S&RP) is the standard initial therapy for CP, and well documented to drive bacteria such as *P. gingivalis* and others into the bloodstream [214, 215, 225, 226]. To determine if this influences mDC infection and frequency, the CP patients were subjected to S&RP, mDCs isolated 24 hours later and analyzed for *P. gingivalis* content and blood mDC frequency. The results (Fig. 5a) indicate a significant increase in *P. gingivalis* content of blood mDCs. Accompanying this increase in mDC infection was a

~25% increase in blood mDC frequency (Fig. 5b). This response was statistically significant when comparing pre- and post-treatment (24 hours) in all the CP subjects ($p<0.05$, paired students t-test) (Fig. 5c). There was no increase in total PBMCs after therapy, nor were levels of DC-poietins FLT-3L, TNFr1 or TNFr2 significantly altered by therapy.

6. MDCs provide a protective niche for *P.gingivalis*

Viable *P. gingivalis* [227] and *Chlamydia pneumonia* [228] have been recovered from atherosclerotic plaques. To determine whether the obligate anaerobe *P. gingivalis* survives within mDCs under aerobic conditions, *in-vitro* culture studies were performed. Our results (Fig. 7a) showed that *P. gingivalis* survives for 24 hours within mDCs, while it dies rapidly in the absence of mDCs. Pre-treatment of mDCs with cytochalasin D reduces the protective effect of mDCs ten-fold at 6 and 24 hours. We further show that professional phagocytes PMNs kill *P. gingivalis* rapidly.

7. Mechanism of increased mDC frequency involves *de-novo* differentiation

As antibody ligation of DC-SIGN enhances DC differentiation [229], it was hypothesized that the DC-SIGN-targeting pathogen *P. gingivalis* would also promote DC differentiation. Therefore, monocytes were cultured with the growth factors GM-CSF/IL-4 and *P. gingivalis* added at low MOIs in select wells. The results confirmed that GM-CSF/IL-4 alone promotes differentiation of CD1c+ DC-SIGN+ mDCs, after an apparent one day delay. Moreover, mDC differentiation was enhanced ~ 28% by *P. gingivalis* at an MOI of 0.1, but was retarded by MOI of 0.5 and 1 in a dose dependent manner (Fig. 7b). Interestingly, in the absence of GM-CSF/IL-4 (Fig 7c), *P. gingivalis* alone at 0.1 MOI induced differentiation of CD1c+ DC-SIGN+ mDCs, and this continued for three days. When the MOI dose was increased to 0.5, the effect was

diminished, while at an MOI of 1 caused no enhancement of MoDC differentiation relative to controls.

8. Conversion of mDCs to atherogenic phenotype by DC invasive *P. gingivalis*

Matrix metalloproteinases (MMPs) have been implicated in extracellular matrix destruction, in plaque rupture and myocardial infarction [230]. MMP-9 is particularly important in mDC migration [231]. We therefore analyzed secretion of MMP-9 by mDCs pulsed with P. gingivalis strains that express the DC invasin mfa-1 (i.e. Pg381, PgDPG-3) or not (PgMFI). The results support mfa-1-dependent secretion of MMP-9 by mDCs (Fig. 7d). To determine the gelatinolytic activity of the MMP-9 secreted by MoDCs, we performed gelatin zymography (Fig. 7e), and titered the LPS doses from 100 ng to 800 ng. We show that *P. gingivalis* LPS indeed induces higher levels of enzymatically active MMP-9 than E.coli LPS (Fig. 7e). Other indicators of risk of plaque rupture, including C1q [232], HSP60/HSP70 (reviewed in [233], CCR2 [234] and CXCL16 [235] were then measured in mDCs in response to *P. gingivalis*. The results indicate upregulation of transcripts for C1q, HSP60, HSP-70, CCR2 and CXCL16, in response to *Pg*381, relative to untreated control (Fig. 7f). Moreover, *Pg* 381 challenged mDCs upregulates C1q, CXCL16 and HSP-70, relative to the fimbriae-deficient mutant (*Pg* MFB).

9. Mfa-1+ *P. gingivalis* infected mDCs infiltrate atherosclerotic plaques

To determine whether *P. gingivalis*-infected mDCs gain access to atherosclerotic plaques, consistent with pathogen trafficking function, we probed post-mortem coronary artery biopsies from patients with CAD and CP *in-situ* (Fig. 8). A representative sample is shown. Note erosion and vascular inflammation of the intimal subendothelial layer (box) (Fig. 8a). Evident is infiltration of atherosclerotic plaque with CD1c+ (Fig. 8b) and DC-SIGN+ (Fig. 8c) mDCs, as well as colocalization of *P. gingivalis* mfa-1 with its receptor DC-SIGN (Fig. 8d).

Discussion

Epidemiological and interventional studies have demonstrated that chronic periodontitis (CP) is a risk factor for coronary artery disease (CAD), but the mechanisms are unclear [165, 166, 172] *Porphyromonas gingivalis (Pg)*, routinely found in CP gingival lesions, promotes atherogenesis in animal models [105] and has been found in human CAD lesions [95]. *Pg* has evolved to invade host cells in order to avoid the harsh inflammatory immune response, typical in progressive CP. Endothelial and epithelial, are among the cells targeted by *Pg* for survival and bacterial persistence [124]. Unlike these cells, mDCs, also targeted by *Pg*, are highly migratory and can potentially traffic bacterial pathogens from the site of initial infection to peripheral distant locations [236]. This work provides evidence in humans for infection of mDCs as a significant route for pathogen dissemination to atherosclerotic plaques.

I. Low-grade chronic inflammation CP contributes to the pool of blood mDCs

Recent work has demonstrated that altered frequencies of blood DCs are an independent predictor for the presence of CAD [15]. Two types of blood DCs have been identified in the human circulation: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). In healthy young individuals blood DCs constitutes around 0.3% of the total peripheral blood circulating mononuclear cells. It is generally accepted that mDCs infiltrate tissues on the periphery in response to bacterial challenge, while pDCs migrate to lymphoid organs and are specialized in antiviral immune responses. In this study, we investigated the levels of blood mDCs in patients with low-grade chronic infection CP, primarily a bacterial triggered disease. Assessing the levels of blood mDCs in patients with CAD is important for two reasons: first, unstable atherosclerotic plaques in CAD patients are heavily infiltrated by mDCs, but the sources of origins are unclear;

secondly, bacterial infections have been implicated in the progression of CAD and the role of mDCs, in response to the bacterial challenge, is unclear.

The available evidence regarding the frequency of blood mDCs in patients with stable and unstable CAD, relative to health, is currently controversial; it is not clear whether blood DCs increase or decrease and the underlying implications [58, 237, 238]. Initial studies reported decreased circulating mDCs (21% decrease) in patients with CAD relative to healthy controls [58, 223]. Independent studies further demonstrated greater reductions in circulating mDCs in patients with ACS, which was attributed to myocardial necrosis, rather the atherosclerosis per se [239]. Although several studies appear to support decreased circulating blood DCs in CAD and ACS patients, all of these investigations excluded patients with low-grade chronic infections; it is unclear whether the presence or absence of chronic periodontitis, a known risk factor for CAD, was considered in the above studies. Our results demonstrate higher absolute frequency of mDCs in patients with CP and a further increase in patients with a history of ACS plus CP, relative to the healthy controls. This observation has been validated by an independent study that demonstrated elevated levels of peripheral blood mDCs in men with coronary artery disease [237]. Other factors that may have affected the relative mDCs frequencies in these studies includes DC surface markers and techniques used for DC isolation, and time at which blood samples were collected. Timing is important because percutaneous coronary intervention (PCI), a common treatment for CAD, results in increased frequency of blood mDCs.

We initially suspected that DC poietins (i.e. FLT3-L) levels were altering blood mDC frequency in CP, as reported in a number of diseases in humans [222, 240], [15, 223, 237, 239, 241-243] including CAD [223]. FLT3-L is a major cytokine involved in both pDC and mDC development from haematopoietic stem cells and their release from the bone marrow. Previous

reports have found a direct correlation between the reduced frequency of blood mDCs and FLT3-L and suggested that the reduced blood DCs in CAD might be caused by impaired DC differentiation from bone marrow progenitors. In this study the levels of the FLT3-L were unchanged between the CP and healthy controls, but an increase was observed in the ACS/CP population which might, in part, account for the mDC increases noted in this population. As stated above, the time period at which the blood samples were obtained might have also influenced the differences observed between FLT3-L levels and mDCs frequencies. Previous studies have obtained patient blood samples immediately after admission to the hospital for a cardiovascular event, such as myocardial infarction, while our ACS/CP population blood samples were obtained 1-2 years past their acute coronary event. Our ACS/CP cohort had been on long-term cardiovascular care, including lipid-lowering drugs, aspirin, beta-blockers, angiotensin converting enzyme (ACE) inhibitors and calcium channel blockers. Presently unclear is the effect of CAD treatment on circulating blood DCs, but a recent report suggest that the above medications increased the frequency of circulating blood mDCs [242]. For instance, aspirin promotes bone marrow progenitor differentiation to immature DCs, and strongly maintains DCs in an immature state. This would certainly lead to an increase in circulating DC numbers, rather than a decrease. Statins prevents or inhibits DCs adhesion and transmigration through dysfunctional endothelial cells, leading to less recruitment to atherosclerotic plaques and retention in the circulation [243]. Recent work has demonstrated that statins are associated with increased numbers of mDCs [242]. Statins also interfere with DC maturation by inhibition of NFκB activity; our data shows that blood mDCs in the ACS/CP population had an immature phenotype. Our CP cohort were not taking any of the above medications, however, mDCs increases might be related to the bacteremia induced by CP (explained below).

II. DCs activation signals: role of blood mDCs

DCs are equipped with pattern recognition receptors (PRR) for the recognition of danger signals in the form of pattern-associated molecular patterns (PAMPs) present in infectious agents. PRR-PAMPs interaction leads to DC activation and the production of co-stimulatory molecules which are essential for the induction of adaptive immune responses. The most common example of PRRs are the Toll-like receptors. For instance, TLR-4 plays a significant role in the initiation and progression of CAD and is expressed in blood mDCs, along with TLRs-2-10. Expression of TLRs in blood mDCs suggests that these cells have been exposed to activation signals, such as bacterial LPS, oxidized LDL, and heat shock proteins, typically recognized by TLR-4. In hyperlipidemic mice, TLR-2 and -4 are documented to contribute to the progression of atherosclerosis. Further, *P. gingivalis* infection was shown to promote atherogenesis in a ApoE (-/-) mouse model in a TLR-2 dependent fashion [9]. In the present study we did not investigate TLR expression of the isolated blood mDCs in the patient cohorts, but *ex-vivo* PBMCs, from the healthy controls, showed increased differentiation to MoDCs when pulsed with *P. gingivalis*; this effect was independent of the presence of growth factors GM-CSF and IL-4 and was possibly mediated via TLR-2 or 4 activation. Previous work has demonstrated that, in CAD patients, the concentration of GM-CSF did not correlate with the levels of blood mDCs or pDCs; the authors of this study suggested that other factors account for the altered frequency of blood mDCs [223]. For instance a previous study demonstrated that the intracellular pathogen, *M. tuberculosis*, can infect and differentiate monocytes into MoDCs [244].Thus *P. gingivalis*, along with other pathogens, provide activation signals to circulating monocytes that promote DC differentiation and explained the increase mDCs frequencies observed in the CP patients.

Our findings also shed light on the enigmatic functions of blood DCs. It has been speculated that blood DCs translocate antigenic material from its point of origin to remote target tissues [236]. The initial site of mDC infection by *P. gingivalis* is most likely the oral submucosa. Introduction of *P. gingivalis* into the oral mucosa in mice results in rapid (30 min) bacteremia (personal communication from C.A. Genco), but the role of mDCs in this bacteremia is not yet clear. Our results suggest that clearance of bacteremias may be a predominant function of blood DCs. Bacteremia is a well described phenomenon in patients with CP [215, 216], and is induced by tooth brushing, flossing, or undergoing S&RP [245]. Previous exposure to *Pg* was observed in our CP cohort by measuring serum antibody titers relative to the healthy controls. We demonstrate that both the chronic condition CP and acute bacteremia increases the *P. gingivalis* content of blood mDCs and results in an increase in blood mDC frequency. Other infectious conditions that alter blood DC frequency include tuberculosis [240], malaria [246] and filariasis [247]. To this end, we thus implemented an *in-vitro* infection assay to ascertain the mechanism. We show that indeed DC differentiation was enhanced by *P. gingivalis* infection but only at low MOIs. The underlying mechanism involved in infection induced mDC differentiation and the functionality of the resultant DCs was outside the purviews of this study, but is currently under investigation.

Consistent with this idea of pathogen clearance by mDCs we then examined the expression of co-stimulatory molecules CD40, CD80, CD83, CD86, and antigen presenting molecule MHC Class II on mDCs by flow cytometry. Our data indicates that *Pg* infected mDCs, in patients with CP, have an immature phenotype as determined by the low expression of co-stimulatory molecules, but moderate MHC-II expression (Fig. 6c). Expression of MHC-II is constitutive on mDCs, but is unlikely to be involved in antigen presentation. This is consistent

with inefficient maturation of mDCs by *P. gingivalis* due to the DC-SIGN ligand mfa-1 [127]. Similar findings have been found independently by *M. tuberculosis* generated MoDCs resulting in inefficient antigen presenting capabilities, also noted for the lack of T cell proliferation [244]. These findings suggest that mDCs may be involved in antigen capture and processing and thus may function in the clearance of transient *Pg* bacteremias. However, *Pg* blood mDCs infection might interfere with this process, leading to retention in the circulation and redistribution to peripheral distant sites such as coronary arteries.

III. Atypical migration of mDCs in CP: reverse transmigration

The proper function of immune surveillance requires well-coordinated mechanisms in order to guide the patrolling immune cells through peripheral tissues and into secondary organs [248]. Control over immune responses in the intestine and in lymph nodes is maintained by differential trafficking patterns of select lymphoid/myeloid subpopulations. Most notable among the molecular signals that regulate cell trafficking are the chemokines and their respective receptors [249]. For example, the expression patterns of the chemokines MIP3α and MIP3β, and their respective receptors CCR6 and CCR7 suggest that these molecules mediate mDC trafficking from infected tissues to T cell rich regions within human lymph nodes [250]. Immature mDCs *in-vitro* express high levels of CCR6, and low levels of CCR7, while mature mDCs express low levels of CCR6 and high levels of CCR7 [250]. Of the respective ligand(s), MIP3α is predominantly expressed at inflamed tonsillar crypts, while MIP3β is specifically expressed in T cell rich area, suggesting a role for MIP-3α /CCR6 in recruitment of immature mDC at sites of injury and for MIP-3β /CCR7 in accumulation of antigen-loaded mature mDCs in T cell rich areas [250]. The constitutive trafficking to and from skin of epidermal Langerhans cells is coupled to the differential expression of MIP-3α/CCR6 and MIP-3β /CCR7 [251]. MDCs

infection by *P. gingivalis*, and other pathogens, might disrupt DC homeostasis and promote atypical DC migration; that is to prevent DC movement to lymph nodes and promote relocation to other tissues, other than the lymph nodes.

Our preliminary unpublished observations supports this notion; in response to *P. gingivalis* stimulation, MoDCs increased the expression of C-X-C chemokine receptor type 4 (CXCR-4), but fail to induce CCR7. CXCR4 is a G-protein-coupled receptor expressed by a multiple cells, including mDCs. It is involved in hematopoiesis and development of the vasculature and central nervous system. CXCR4 expression is induced on monocyte-derived DCs after stimulation, and mDCs are chemoattracted to the CXCR4 ligand CXCL12 (stromal-cell derived factor-1). CXCL12 (stromal cell derived-factor-1/SDF-1a) is involved in haematopoiesis, stem cell mobilization, bone marrow engraftment, organ development, and angiogenesis. CXCL12 is also expressed in CP lesions and levels are induce by inflammatory cytokines, such as TNF- α [252]. This suggest the intriguing possibility that *Pg* may abrogate CCR7 in mDCs upon infection, disrupts lymph node trafficking and may recirculate back to the blood through CXCL12-CXCR4 mediated interactions. CXCL12 is also expressed by platelets at sites on arterial injury, further implying that CXCR4 positive leukocytes, such as blood *Pg*-infected mDCs might be recruited via interactions with CXCL12 to the vascular endothelium, and thus contributes to the pool of vascular DCs observed in CAD.

In addition to MIP-3α /CCR6 and MIP-3β /CCR7, the trafficking of mDCs through the gingival tissues is also aided, by matrix-metalloproteinase 9 (MMP-9) [183, 231]. MMP-9 is a biomarker of increased CAD risk and a mediator of plaque instability [253]. We show that human mDCs stimulated by *P. gingivalis* release high levels of MMP-9, and that this is dependent on expression by *P. gingivalis* of the DC-invasin mfa-1. Increased mobilization of

Langerhans cells, which express CD1c [254] and dermal DCs, which express DC-SIGN, is observed in CP gingival lesions and these DCs accumulate in close proximity to the vasculature [213, 255]. Moreover, we demonstrated that DC-SIGN+ mDCs associate with ICAM-2, an endothelial cell marker, on the gingival tissue vasculature, which possibly allows the redistribution of *Pg* infected mDCs back into the peripheral circulation. In addition, hypoxic conditions, as in the subgingival pockets in CP lesions where *P. gingvalis* resides, [256] promote the transmigratory activity of DCs through endothelium [254]. Overall, the hypoxic microenvironment and local infection with *P. gingivalis* may be a potent driving force for reverse transmigration of tissue mDCs into the blood to atherosclerotic plaques [236, 257].

IV. Recruitment to atherosclerotic plaques

DCs were first identified in the human atherosclerotic lesions in 1995 and were thought to play an important role in the development of atherosclerotic lesions [258]. Although lipid uptake and foam cell formation is mostly attributed to macrophages, recent evidence demonstrated that vascular mDCs accumulate lipids efficiently [59]. Moreover, oxLDL can potentially result in DC activation, possibly mediated by CD36-TLR-4/TLR-6 interactions; consequently leading to local T cell responses and vascular tissue inflammation. This is supported by evidence showing contact between DCs and T cells, resulting in the overexpression of heat shock protein-70 (HSP-70), an indicator of physiologic stress [196]. Hsp-70 is also expressed by intracellular bacterial species [259], including *P. gingivalis* [260], and as we show here, by mDCs infected with *P. gingivalis*.

DCs are normally present in low numbers in healthy arteries (~4 DCs per 10 endothelial cells) and are mostly localized to the sub-endothelial space, particularly at vascular areas

exposed to turbulent blood flow [261]. In atherosclerotic conditions, high numbers of mDCs infiltrate the vascular wall, particularly in rupture-prone plaque areas [57]. Moreover, symptomatic CAD patients have higher numbers of DCs relative to asymptomatic patients [262]. Evidence that blood mDCs infiltrate atherosclerotic plaques comes from studies showing BDCA-1, a blood mDC marker, in advanced CAD lesions [15]. Our results demonstrate the presence of BDCA-1-mDCs and their association to *P. gingivalis* in CAD tissues. Additional reports have identified mDCs in atherosclerosis by means of visualization of dendrite morphology, along with the identification of phenotypic expression markers, such as CD1a, S-100, CD83 and DC-SIGN [263]. Subsequent studies of atherosclerotic plaques showed an intense infiltrate of DC-SIGN+ immature mDCs [4], as we observed in CP lesions [255]. The immature state of these mDCs is consistent with a reported role for DC-SIGN ligation in inhibiting TLR-mediated DC maturation [127, 264] and in promoting differentiation of immature DCs from progenitors [229].

Regression of atherosclerosis is a desirable clinical goal. Impairment of mDC egression, from the vascular wall to secondary lymphoid organs, might be implicated in disease progression in CAD; leading to mDC accumulation and retention in the plaque area. Previous animal models of atherosclerosis have shown that mDCs deficient in CCR7 egress inefficiently from atherosclerotic plaques. Our preliminary results showed that CCR7 expression is abrogated in *Pg* infected MoDCs. Moreover, *Pg* infected mDCs were also observed in atherosclerotic tissue in the ACS/CP patients (Fig.8). This suggests that the presence of *Pg*, and perhaps other infectious agents, in atheromatous plaque may impede DC egression, and thus prevent resolution of inflammation within the arterial wall. Dyslipidemia, an imbalance of lipids such as, triglycerides and lipoproteins, in the blood has also been associated to defective mDCs egression from atheromas [265]. Moreover, previous reports have shown an association between CP and

previous exposure to systemic *Pg* LPS to abnormal triglyceride levels [266], pointing to systemic endotoxin (LPS) as the inducer of the hyperlipidemic state [267]. Our results validate this observation and demonstrate statistically significant higher levels of triglycerides, very low density lipoprotein (VLDL) and higher antibodies to *Pg* in the CP cohort relative to the healthy controls. It is unclear whether dyslipidemia in CP impairs mDCs egression from the vascular wall, but yet could be another mechanism that requires proof in animal models.

Collectively, these data indicate that mDCs in tissues and blood harbor pathogens of direct relevance to coronary artery disease and other human diseases. Infection with *P. gingivalis* promotes mDC differentiation and converts mDCs to an atherogenic phenotype. We have thus identified an important pathophysiological mechanism that links chronic low grade infections to an increased risk of cardiovascular disease and other diseases.

Future Directions

We have proposed that DCs may be directly involved in dissemination of pathogens from the oral cavity to distant sites within the body, such as to the coronary arteries. Moreover, the function of mDCs, clearance of pathogens in tissues and blood, may be exploited by certain pathogens such as *Porphyromonas gingivalis*, leading to immune evasion and bacterial persistence. The recovery of live *Pg* from isolated human mDCs was not possible, despite many attempts using various culture techniques. However, when cultured with mDCs, *Pg* appears to survive for a period of 24 hours. This has important implications because recent reports suggest the *Pg* is able to spread from infected epithelial, endothelial, and smooth muscle cells to new host cells where it multiples. Whether, *Pg* infected mDCs in CP lesions relocate to the circulation to atherosclerotic plaques and allows *Pg* to spread to the vascular endothelium leading to infection, remains an open question. Future studies should examine whether *Pg* is able to spread from mDCs to endothelial cells, which have a more direct relevance to CAD. In addition, our preliminary results suggest that *Pg*, not only survives, but decreases mDCs apoptosis, thus enhancing DC lifespan and chance of relocation to peripheral non-lymphoid tissues. We acknowledge that there are limitations when doing research with human subjects. Thus, animal studies are required to conclusively prove that mDCs traffic *P. gingivalis*, among many other unknown pathogens, from the oral mucosa to the periphery. This could be done by either tagging *Pg* or mDCs and follow their migration and trafficking through the system, or by ex-vivo labeling of bulk numbers of DCs, followed by adoptive transfer to recipient animals. Ongoing collaborations are underway to further prove that mDCs are a conduit for pathogen dissemination. The implications of this findings may not only be limited to CAD risk, but to other chronic systemic diseases as well.

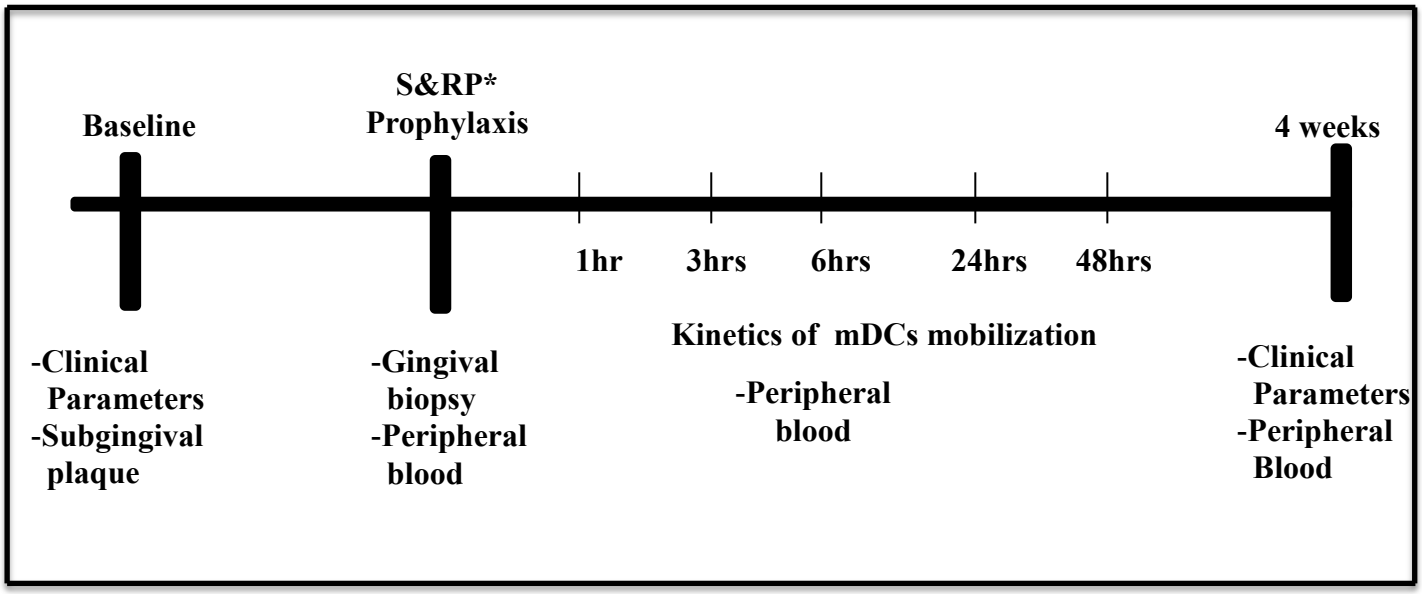

Figure 1: Study Design: Control (CTL), Chronic Periodontitis (CP) and Acute Coronary Syndromes and CP (ACS/CP) Groups. Sixty-six (N=66) subjects were enrolled in the study following IRB approval. This consisted of twenty-five (N=25) periodontally healthy (CTL), twenty-six (N=26) chronic periodontitis (CP) and fifteen (N=15) ACS/CP subjects. Clinical parameters collected included clinical attachment loss (CAL), bleeding on probing (BOP), probing depth (PD), tooth mobility and furcation involvement, plaque index (PI) and gingival index (GI). The blood mDCs response was examined before and after (24 hours) SRP in the CP individuals, relative to the healthy controls. In a small subset of CP patients (N=5) the kinetics of blood mDCs response, after SRP, was evaluated at the following intervals: 1, 3, 6, 24, 48 hours and 4 weeks. A similar approach was undertaken with a CTL subject (N=1) after supragingival prophylaxis at the same time points. Gingival biopsy and subgingival plaque was obtained before SRP or prophylaxis in the CTL, and CP patient cohorts. All samples were collected under non-fasting conditions.

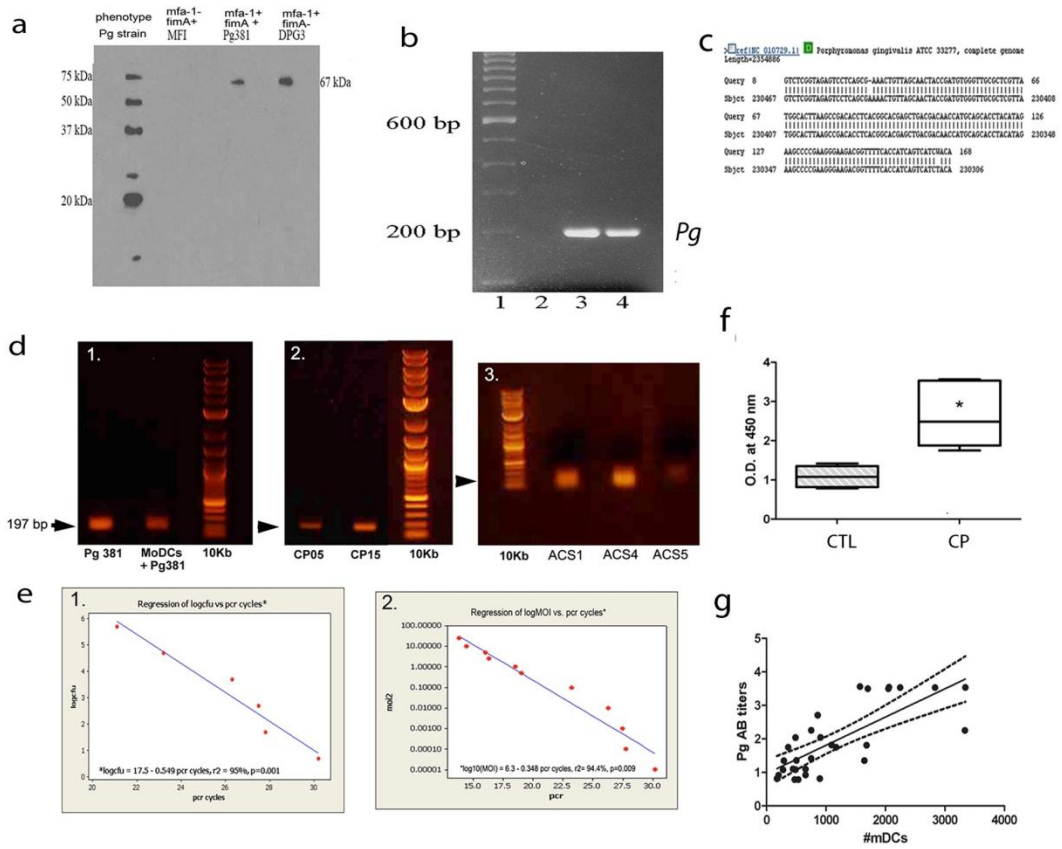

Figure 2. *P. gingivalis* infection and relative quantification in DCs. (a) Specificity of monoclonal antibody (AEZαMfa1) to 67 kDa mfa-1 (minor) fimbriae shown by Western blotting analysis, performed as described in material and methods. Lanes are as follows; Lane 1: MW standards; Lane 2: *P. gingivalis* MFI (minor mfa-1 fimbriae-/major fimA fimbriae+); Lane 3: wild type *Pg*381 (mfa-1+/fimA+) and Lane 4: DPG3) mfa-1+/fimA-). (b) Representative *Pg* 16srDNA in subgingival plaque sample from a patient with chronic periodontitis. DNA was purified as in material and methods and PCR amplified using 16s rDNA primers as previously described. Lanes: 1. 100bp DNA ladder; 2. No DNA; 3. Wild-type *Pg* DNA used as positive control; 4. *Pg* 16s rDNA (arrow) from subgingival plaque of a CP patient. (c) 200bp prducts were DNA sequenced, sequenced alignment performed with NCBI Genomic Blast online tools. (d) *Pg* 16s rDNA in: (1) WT *Pg*381 or MoDCs pulsed with WT *Pg*381 for 3 hours; (2) blood mDCs from representative CP patients and (3) ACS/CP patients. Genomic DNA and total RNA was purified as in methods. qRT-PCR condeucted with 1µl purified DNA using 16s rDNA primers as described above. (e) MoDCs were pulsed with WT *Pg*381 at a range of colony forming units (CFU) and multiplicities of infection (MOI). MoDCs analyzed for *Pg* by PCR of 16s rDNA. The number of PCR cycles was plotted with log 10CFUs (e.1) and MOIs (e.2), yielding a linear regression curves. Estimated CFUs (eCFUs) and MOIs of *Pg* in blood ml of CP and ACS/CP patients were derived from these models to quantitate relative levels of infection in blood mDCs. (f) Relative serum titers of anti-*P. gingivalis* DPG-3 IgG antibodies, determined by

ELISA, in healthy controls (CTL) and CP patients. *P< 0.05, Student t-test (g) Linear regression analysis of anti-*Pg* IgG titers (y-axis) vs. mean number of mDCs (#mDCs) in a subset of CTL and CP patients. Significant relationship is noted (r2=0.56, p=0.0001).

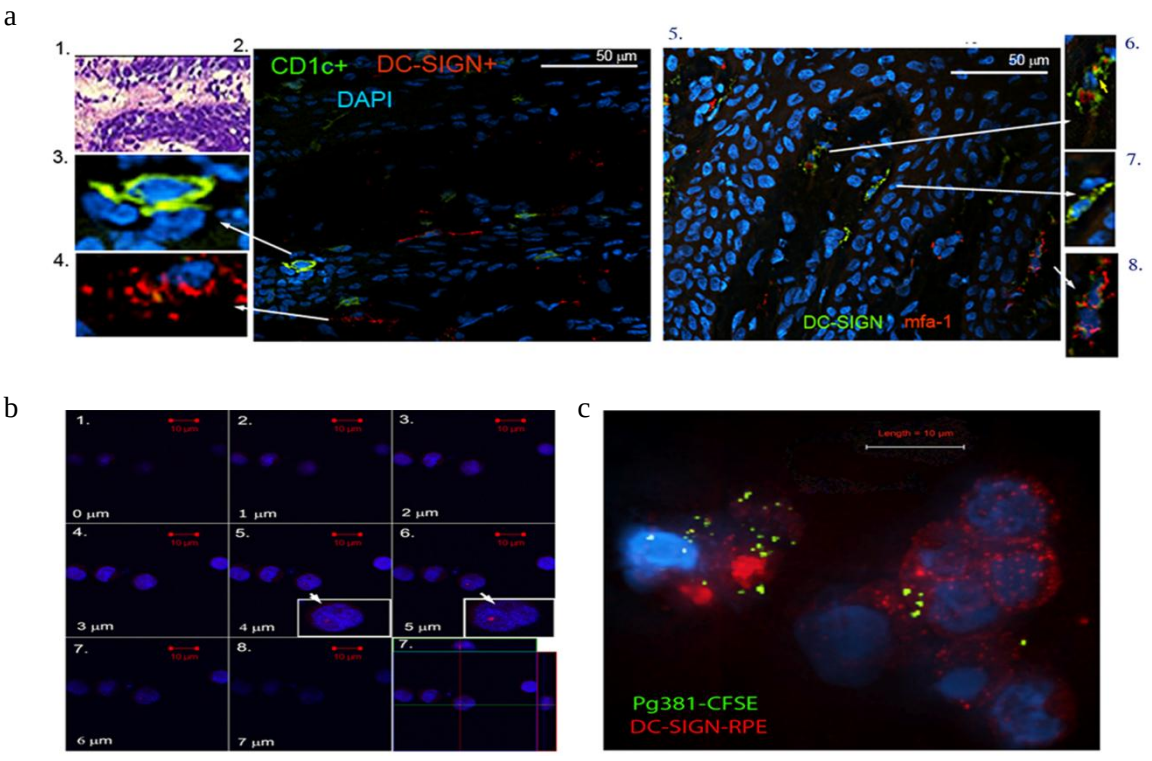

Figure 3. Infection of gingival tissues mDCs by *P. gingivalis* (a) Representative inflamed gingival tissue section (7µM thick) from a CP patient was stained with hematoxylin and eosin (H&E) (20x) (a.1). Section was stained with FITC-conjugated mouse anti-human CD1c (BDCA-1) and RPE-conjugated mouse anti-human CD209 (DC-SIGN) and image obtained at 100x(a.2). Shown at ~ 500x final enlargement (optical and digital) is detail view of individual of CD1c+mDC/LC in epithelium (a.3) and DC-SIGN+ mDCs in lamina propria (a.4) (200x). FITC-conjugated mouse anti-human CD209 and Alexa Fluor 594 conjugated to mfa-1 antibody AEZαMfa1 using commercial DyLightTM microscale antibody labeling kit in CP gingival tissues (100x). Shown in a.5-8 (200x) detail view of areas of colocalization. (b) Image series from scanning laser confocal microscopy (panels 1-7, z-stack, 1µm slices) of FACSorted CD19-CD1c+ (BDCA-1+) blood mDCs from oral carriage positive CP patient, permeablized and cytocentrifuged on slides and probed with AEZαMfa1-PE, followed by Vectashield TM mounting media. The presence of *P. gingivalis* (red) in mDC shown by arrow. (c) Epifluorescence deconvolution image analysis of blood mDCs from a healthy donor pulsed in vitro with CFSE-labeled *P. gingivalis* 381 (green) at an MOI of 1 for 3 hours. MDCs were cytocentrifuged and counterstained with DAPI (blue) and RPE-conjugated DC-SIGN (red).

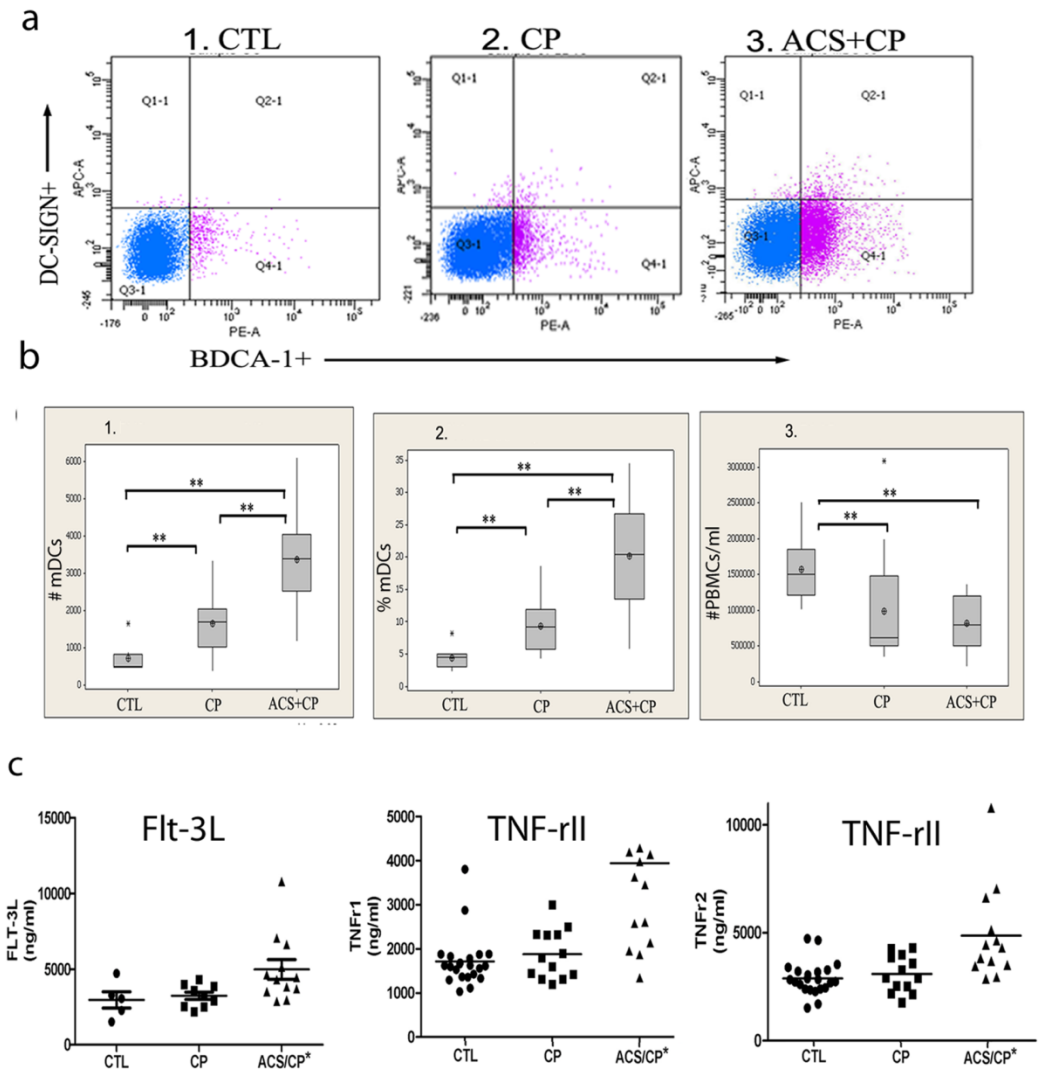

Figure 4. Increased frequency of blood mDCs in CP patients not related to DC poietin levels. (a) Representative scattergrams from flow cytometry analysis of blood mDCs at baseline in healthy control (CTL) subjects (n=25), CP subjects (n=26) and subjects with acute coronary syndrome and CP (ACS+CP) (n= 15). **(b)** Mean numbers (b.1) and percentages (b.2) of blood mDCs (mDCs/20,000 PBMCs) and total numbers of PBMCs/ml (b.3) of blood in CP, ACS/CP relative to CTL. ** $p<0.05$, Student t-test **(c)** Serum ELISA levels of FLT-3L, soluble TNFR-1, TNFR-2 (ng/ml) in all three patient groups performed in triplicate for each patient sample. *Significant difference in ACS+CP groups relative to CP and CTL ($p<0.05$, Student t-test).

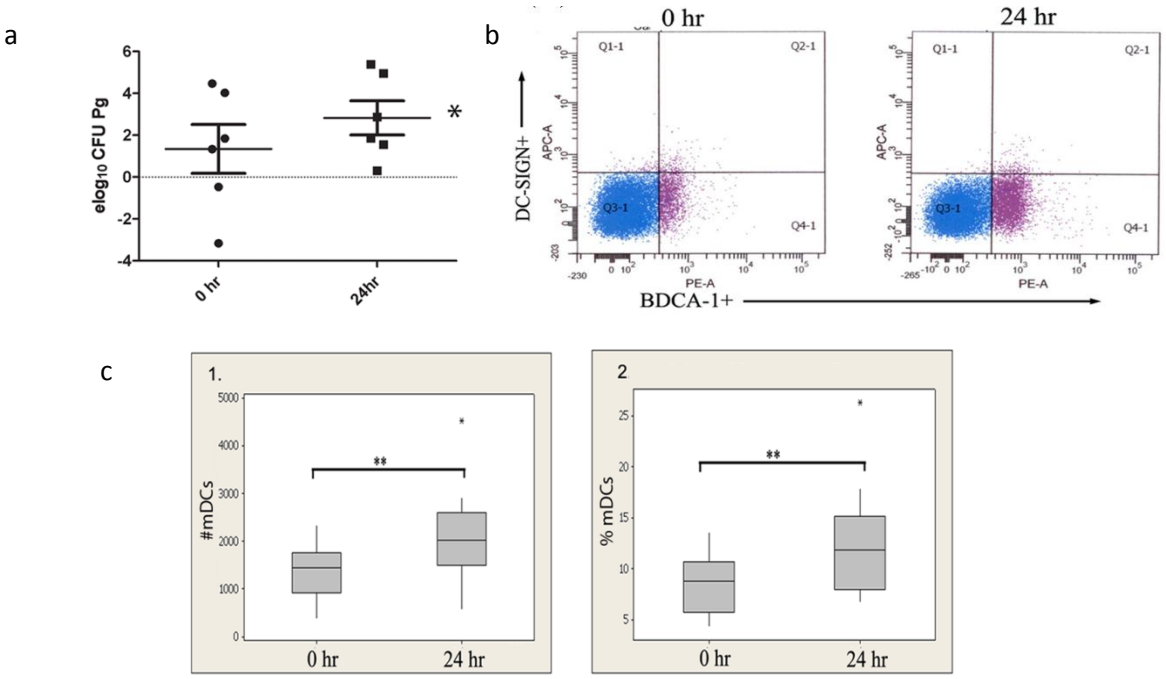

Figure 5. Induction of transitory bacteremia increases infection level and frequency of blood mDCs. **(a)** Significant increase in *P. gingivalis* 16s rDNA content of blood mDCs from CP subjects (n=6) 24 hours after local debridement (S&RP). MDCs *ex-vivo* isolated and analyzed for 16s rDNA of *P. gingivalis*. This was quantified by qRT-PCR, yielding estimated log10 CFU *Pg* (elog10 CFU) as in Fig. 2, Table 5. (*p=0.02, paired T-test). **(b)** Representative scattergrams from flow cytometry analysis of CD19- CD1c+ (BDCA-1+), CD209 (DC-SIGN+) blood mDCs before (0hr) and 24 hours after local debridement (S&RP) of CP patients as described in materials and methods. **(c)** Significant increase in mean number (c.1) and percentages (c.2) of blood mDCs 24 hours after S&RP (per 30,000 PBMCs) (**p<0.05, Paired t-test).

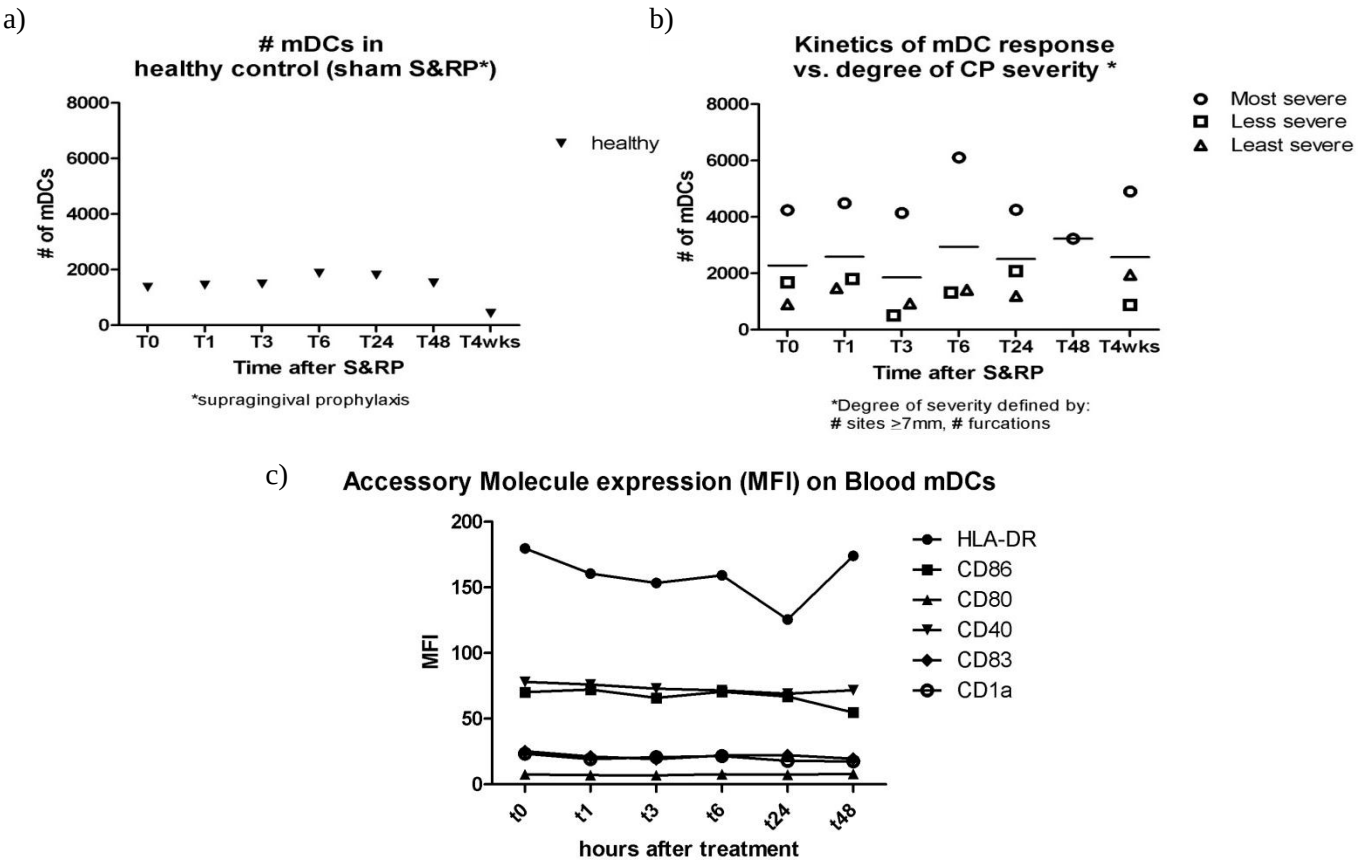

Figure 6. Kinetics of mDCs response after SRP and overall mDCs phenotype. CTL (N=1) and CP (N=5) patients were subjected to supragingival prophylaxis and SRP respectively. Peripheral blood was collected before and after treatment (1, 3, 6, 24, 48 and 4 weeks), and mDCs were isolated and purified. a) The mDCs from healthy controls showed minimal response after prophylaxis, while b) mDCs appears to have a more pronounced response to SRP, in particular at 6 hours. The highest frequency of blood mDCs, after SRP, was noted in the patient with most severe CP. Severity was determined by the number of sites with pocket depths of 7mm or greater and numbers of furcation involvement. Levels of blood DCs at 4 weeks were similar to baseline or before treatment. c) Relative expression of accessory molecules in the blood mDCs appears low, while constitutive MHC-II (HLA-DR) was significantly expressed, which is consistent with an immature DC phenotype.

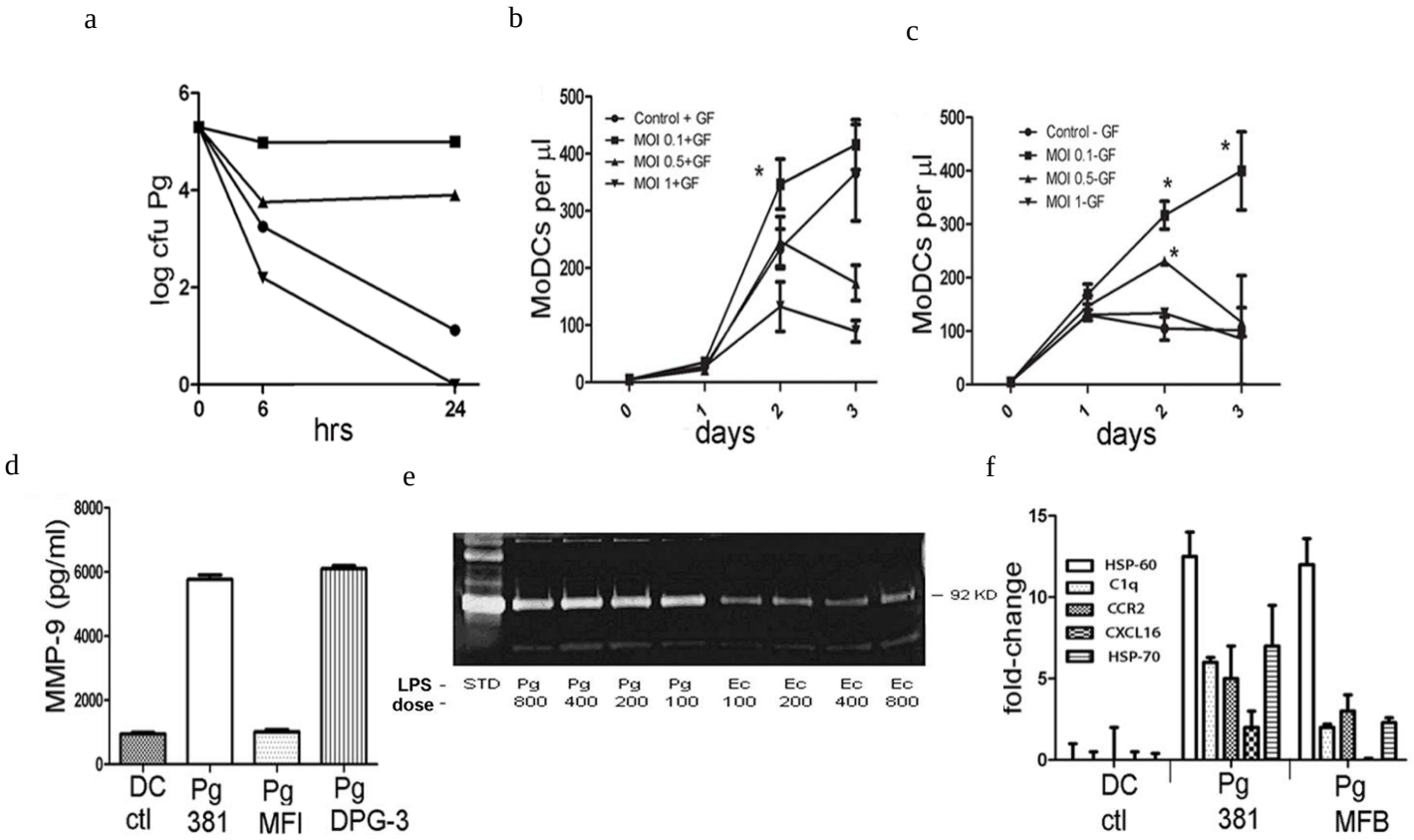

Figure 7. *P.gingivalis* survives in mDCs, induces differentiation and atherogenic phenotype in vitro. (a) Wild type Pg381 was incubated without monocyte-derived DCs (mDCs) (circle) or with mDCs (square), mDCs pre-treated with cytochalasin D (triangle), control PMNs (inverted triangle) at a multiplicity of infection of 100 for 0, 6, 24 hours. Internalization of CFSE-labeled *P. gingivalis* was confirmed by epifluorescence deconvolution microscopy. Cells were lysed and viable bacteria in lysates were streaked on enriched anaerobic 5% blood agar plates in triplicate under anaerobic conditions (10% H2, 5% CO2 in nitrogen) at a 1:10 dilution at 35o C for 14 days after which colonies were numerated and surviving cell forming units per mL were determined. Shown is Log10 cfu/ml. Experiment was repeated three separate times and data are representative of consistent results **(b)** Pre-DCs monocytes from healthy controls in triplicate were cultured with growth factors GM-CSF/IL-4 +/- *P. gingivalis* 381 at MOIs of 0.1, 0.5, and 1. The mean number of cells appearing in the MoDC gate (CD1c+DC-SIGN+) per µl + S.E. is shown. Phenotype of immature MoDCs was further confirmed by evidence of downregulation of CD14 and low to no expression of CD83 (not shown). *Significant difference between control+ GF (growth factors (GF) and MOI of 0.1 + GF (ANOVA, p< 0.05). Data are representative of results of assay repeated four separate times. **(c)** Performed as in (b), without growth factors. Significant differences were noted in mean # MoDCs per µl in MOI 0.1-GF vs MOI 0.5-GF at 2, 3 days, and in MOI 0.1-GF, MOI 0.5-GF vs control and MOI 1-GF at 2 days (ANOVA, p<

0.05). **(d)** MoDCs were pulsed with wild type *Pg* 381, its mfa-1 minor fimbriae deficient strain (MFI) and fimA major fimbriae deficient strain (DPG-3) or no Pg (DC ctl) for 18 h at a MOI of 1:25. Secretion of MMP-9 in pg/ml were assessed by ELISA. The data are the mean ± S.D. of triplicate assays. **(e)** MoDCs were stimulated with 100, 200, 400 and 800 ng/ml of *Pg* LPS and *E.coli* LPS for 24 hrs. MMP-9 in cell supernatants was analyzed by gelatin zymography. Equal amounts of protein were loaded and separated by electrophoresis as described in the Materials and Methods. Gelatin zymogram shows bands corresponding to 92 kDa (MMP-9). **(f)** MoDCs were pulsed in triplicate with wild type *Pg*381, its fimbriae-less mutant MFB or no *Pg* (CTL) at a 25:1 MOI for 3 hrs and uptake of CFSE-labeled Pg monitored by FACS analysis (not shown). After isolation of total RNA (RNeasy® Mini Kit-Qiagen) and synthesis of cDNA (Enhanced AviaN rtf IRST Strand Synthesis Kit- Sigma), qRT-PCR (BioRad) was used to determine expression levels, normalized to β-actin and expressed as fold-changes in mRNA. PCR primers were designed using PRIMER3 Sofware (76). For relative quantification of transcript expression, Ct values were obtained for each gene and data were analyzed using the Excel (Microsoft) macro GENEX v1.10 (Gene Expression analysis for iCycle iQ® Real-time PCR Detection System, v1.10, 2004, Bio-Rad Laboratories.

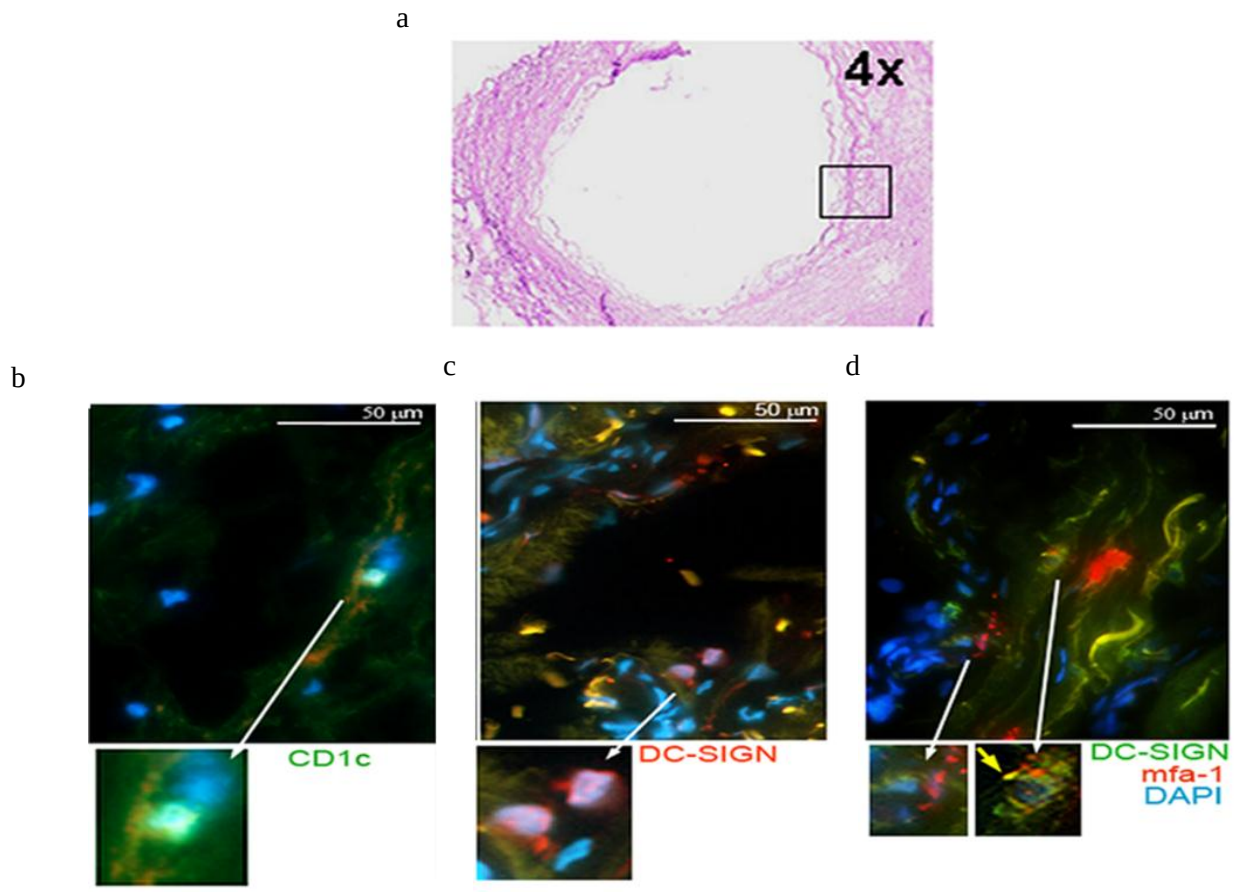

Figure 8. *P. gingivalis* infected mDCs invade atherosclerotic plaque of CP patient. **(a)** Representative post-mortem coronary artery 7µM tissue section from ACS/CP patient were stained with H&E. (4x) **(b)** CAD tissue sections were stained with FITC-conjugated CD1c+, **(c)** RPE-conjugated DC-SIGN+ and **(d)** DC-SIGN-mfa-1 double staining with FITC-conjugated DC-SIGN+ and Alexa Fluor 594 conjugated-mfa-1. Prior to staining, all tissue sections were blocked with 5% BSA in PBS, along with anti-human FcR block reagent. Appropriate controls included isotype matched antibodies and pre-immune antibodies. All sections were mounted with VectaShield mounting medium containing DAPI. Images were acquired with a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System. H & E images were obtained with enhanced light microscopy (Nikon E600).

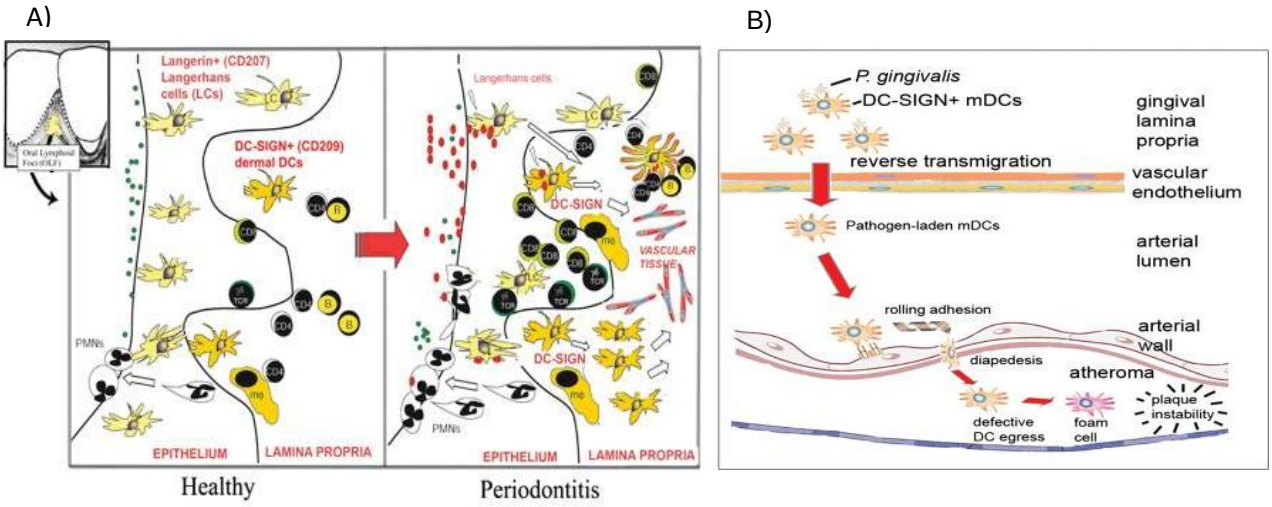

Figure 9. Pathogen trafficking blood DCs: links to CAD pathogenesis model. Schematic representation of how *P. gingivalis*-infected dendritic cells may contribute to the development and progression of CAD. (A) Representation of the CP lesion, its organized inflammatory infiltrate. The left panel depicts the healthy oral biofilm in the gingival crevice, comprised predominantly of Gram-positive bacteria (green dots). Healthy gingival tissue is infiltrated with numerous Langerhans cells in the epithelium, with sparse dermal dendritic cells in the lamina propria. In susceptible patients the disease progress and this is noted by changes in the oral biofilm to a predominantly Gram-negative subgingival flora (red dots). In response, a dramatic loss (efflux) of Langerhans cells occurs from the epithelium toward the lamina propria. Also observed is an influx of myeloid-derived DC-SIGN+ dermal dendritic cells (DCs) into the lamina propria. Present are neutrophils, macrophages (mϕ), B-cells, and CD4+ and CD8+ T-cells. In this study we have shown that *Pg* associates to mDCs via its surface receptor DC-SIGN, possibly a mechanism *Pg* uses to evade the host response. Unlike other cells, mDCs are highly migratory and we proposed that these Pg-infected mDCs redistribute from the initial site of infections to the circulation were they can travel to distant sites, such as the coronary arteries. The DCs form immune conjugates with CD4+ T-cells and also mobilize toward the vasculature. (B) Hypothetical model in periodontitis, showing mobilization of *P. gingivalis*-laden DC-SIGN+ myeloid DCs (mDCs) in the gingival lamina propria. These mDCs undergo reverse transmigration through the vascular endothelium. Once in the circulation, mDCs carrying *P. gingivalis* attach to endothelial integrins via DC-SIGN and, after rolling adhesion, undergo diapedesis between endothelial cells. As the atheroma continues to mature and DCs contribute to the foam cells and release MMP-9, the atheroma becomes highly unstable and, eventually, thrombus formation occurs. An animal model would be more suitable to prove this hypothesis, perhaps by tagging mDCs, and then follow their trafficking after infection. Other important questions that remain to be answered are whether *Pg* infection affects the homeostatic lymph node migration of mDCs upon infection. (Adapted from Zeituni et. al, J Oral Microbiol. 2010).

Table 1. Baseline Clinical and Radiographic Parameters of Periodontally Healthy (CTL) Subjects (n=25)

The dentition has ≥ 20 teeth, all of which exhibit:

- Probing depth < 4 mm
- Attachment loss not present
- No bleeding on probing
- Alveolar bone crest 1-2 mm from cemento-enamel junction (CEJ)

Table 2. Baseline Clinical and Radiographic Parameters of Chronic Periodontitis (CP) Subjects (n=26)

The dentition has ≥ 20 teeth with ≥ 8 teeth that exhibit:

- Probing depth > 4 mm
- Attachment loss > 3 mm
- Bleeding on probing
- Alveolar bone crest ≥ 3 mm from cemento-enamel junction (CEJ)

Table 3. Baseline Clinical and Radiographic Parameters of Acute Coronary Syndromes and Chronic Periodontitis (ACS/CP) Subjects (n=15)

The dentition has ≥ 20 teeth with ≥ 8 teeth that exhibit:

- Probing depth > 4 mm
- Attachment loss > 3 mm
- Bleeding on probing
- Alveolar bone crest ≥ 3 mm from cemento-enamel junction (CEJ)
- elevated hsCRP (>5 μg/ml)
- troponin+ unstable angina
- ECG evidence of ischemia
- 1, 2 or 3-vessel CAD, history of MI

Table 4. Clinical Description, Demographics, Serum Mediators			
	Patient Cohorts		
Clinical Description	**Control (n=25)**	**CP (n=25)**	**ACS/CP (n=15)**
	• non-smokers • without CP • no ACS, diabetes, cancer or other reported systemic disease	• moderate to severe CP • >20 teeth, • ≥ 8 with probing depth > 4mm • ALOSS > 3mm, BOP, alveolar bone crest > 3 mm from CEJ, Non-diabetic, non-smoker	• troponin+ unstable angina • ECG evidence of ischemia • 1, 2 or 3-vessel CAD, history of MI • moderate to severe CP • Excluded previous coronary bypass, untreated or incomplete treatment of coronary artery disease, life expectancy of <2 years.
	Age years		
Median (range)	51 (38-63)	52 (31-72)	65 (32-89)
	Gender		
Males	12	11	10
Females	13	14	5
	Self-reported race or ethnic group (%)		
White	12 (48)	16 (64)	11 (63
Black	9 (36)	2 (8)	0 (0)
Asian	0 (0)	2 (8)	0 (0)
Hispanic	4 (16)	4 (16)	4 (27)
Arab	0 (0)	1 (4)	0 (0)
	Serum Lipids (mg/dl)		
Triglycerides	102 ± 8.3	155 ± 16 *	n.d.
Total Cholesterol	209 ± 8	215 ± 9	n.d.
LDL	119 ± 7	134 ± 8	n.d.
HDL	70 ± 4	50 ± 3 *	n.d.
CHOL/HDL ratio	3.2 ± 0.2	4.6 ± 0.3*	n.d.
VLDL	20.3 ±1.6	31 ± 3 *	n.d.
	Serum Cytokines		
FLT-3L (pg/ml)	65 ± 24	66 ± 18	137 ± 27 †
TNFr-I (pg/ml)	1750 ± 564	1850 ± 867	3520 ± 2100
TNFr-II (pg/ml)	2969 ± 537	3239 ± 247	4982 ± 647 †
hsCRP (ng/ml)	2293 ± 1046	2797 ± 525	2851 ± 413
	Serum IgG titer against *P. gingivalis* DPG-3		
Anti-Pg IgG	1.06 ± 0.06	2.69 ± 0.19 *	n.d.

n.d. not determined; * significantly elevated vs. CTL ($p<0.05$, Students t-test)
† significantly elevated vs. CP and CTL ($p<0.05$, Students t-test)

Table 5. Oral Carriage with *Porphyromonas gingivalis* (Pg) and Infection of blood mDCs

Pt ID	*Pg* oral carriage State [1]	Sequence Match (%) [2]	*Pg* in mDCs [3]	Sequence match (%) [amplicons size; bp] in mDCs [4]	eCFUs of *Pg* in mDCs [5]
CP01	-	-	-	-	-
CP02	-	-	-	-	-
CP03	+	*Pg* (98%)	+	*Pg* (98%) [181], *Mc* (91%) [145], *Kp* (90%) [131]	0 [6]
CP04	-	-	+	*Pg* (91%) [161]	12
CP05	+	*Pg* (98%)	+	*Pg* (99%) [185], *Tp* (92%) [176], *La* (95%) [146]	3
CP06	+	No match	-	-	-
CP07	-	-	-	-	-
CP08	-	-	-	-	-
CP09	+	*Pg* (94%)	+	*Pg* (87%) [151]	12
CP10	-	-	-	-	-
CP11	-	-	-	-	-
CP12	+	*Pg* (96%)	+	*Pg* (99%) [187]	1.58×10^6
CP13	-	-	+	*Pg* (96%) [178]	5
CP14	-	-	-	-	-
CP15	+	*Pg* (96%)	+	*Pg* (99%) [198]	1.38×10^5
CP16	+	*Pg* (86%)	+	*Pg* (86%) [151], *Ssv* (100%) [121], *Bc* (90%) [118], *Lf* (92%) [135], *Cb* (95%) [148], *Bkc* (100%) [154], *Pa* (92%) [167], *Pv* (92%) [121]	0
CP17	+	*Pg* (96%)	-	*Sa* (92%) [156], *Bc* (100%) [176], Bo (100%) [154], Cp (95%) [133]	-

CP18	-	-	-	*Hp* (92%) [133], *Ssg* (92%) [154], *Sp* (86%) [121], *Lhr* (95%) [169]	-
CP19	+	*Pg* (96%)	+	*Pg* (98%) [165]	12
CP20	-	-	-	-	-
CP21	-	-	+	*Cd* (92%) [167], *Ey* (100%) [147]	-
CP22	+	*Pg* (96%)	+	*Pg* (95%) [168]	1.3×10^5
CP23	-	-	-	-	-
CP24	+	*Pg* (96%)	-	-	-
CP25	+	*Pg* (98%)	+	*Pg* (98%) [171]	1.1×10^3
CP26	+	*Pg* (98%)	-	*Pg* (98%) [167], *Cp* (92%) [178], *Hci* (90%) [155]	0
ACS/CP1(4)	nd	nd	+	*Pg* (95%) [135]	16
ACS/CP2 (6)	nd	nd	-	-	-
ACS/CP3 (7)	nd	nd	-	-	-
ACS/CP4 (8)	nd	nd	+	*Pg* (97%) [141]	3
ACS/CP5 (9)	nd	nd	-	-	-
ACS/CP6 (10)	nd	nd	-	-	-
ACS/CP7 (11)	nd	nd	+	*Pg* (92%) [138]	1
ACS/CP8 (13)	nd	nd	-	-	-
ACS/CP9 (15)	nd	nd	-	-	-

ACS/CP10 (16)	nd	nd	-	-	-
ACS/CP11 (17)	nd	nd	-	*Burkholderia cepacia* [7]	-
ACS/CP12 (20)	nd	nd	-	*Burkholderia cepacia* [7]	-

1. Positive (+) or negative (-) for 197 bp product (Pg-specific16s rDNA) in subgingival dental plaque by qRT-PCR
2. % concordance with *P. gingivalis* (*Pg*) type ATCC 33277 (Genomic BlastSequence [BLASTN 2.2.24+] [http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi])
3. Positive (+) or negative (-) for 197 bp PCR product in mDCs using *Pg*-specific 16s rRNA primer [219] in mDCs
4. % concordance of 197 bp PCR product with *P. gingivalis* (Pg) type ATCC 33277, or other PCR products using universal primer, including, *Moraxella catarrrhalis (Mc), Klebsiella pneumoniae (Kp), Streptococcus salivarious (Ssv), Bacillus cereus (Bc), Lactobacillus farciminis (Lf), Clostridium sporogenes (Cs), Clostridium botulinum (Cb), Burkholderia cepacia (Bkc), Prevotella sp. (Pv), Treponema pallidum (Tp), Lactobacillus acidophilus (La), Streptococcus anginosus (Sa), Streptococcus sanguinis (Ssg), Borrelia sp. (Bo), Clostridium perfringens (Cp), Clostridium difficile (Cd), Helicobacter cinaedi (Hci), Helicobacter pylori (Hp), Streptococcus pyogenes (Sp), Lactobacillus rhamnosus (Lrh), Eubacterium yurii (Ey),* (Genomic BlastSequence [BLASTN 2.2.24+] [http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi])
5. Estimated CFUs of *Pg* within mDCs, based on regression analysis of PCR cycles vs. $\log^{10}$ cfu of MoDCs pulsed with *Pg* 381 (Figure 2E)
6. Below the range of linear regression
7. Viable *Burkholderia cepacia* identified by AccuPro-ID™ (Accugenic, Newark, DE) was recovered from mDCs on blood agar in pure culture under anaerobic conditions.

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