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Microbial Carriage State of Peripheral Blood Dendritic Cells (DCs) in Chronic Periodontitis Influences DC Differentiation, Atherogenic Potential

Julio Carrion,* Elizabeth Scisci,† Brodie Miles,‡ Gregory J. Sabino,* Amir E. Zeituni,* Ying Gu,* Adam Bear,* Caroline A. Genco,§ David L. Brown,¶ and Christopher W. Cutler‖

The low-grade oral infection chronic periodontitis (CP) has been implicated in coronary artery disease risk, but the mechanisms are unclear. In this study, a pathophysiological role for blood dendritic cells (DCs) in systemic dissemination of oral mucosal pathogens to atherosclerotic plaques was investigated in humans. The frequency and microbiome of CD19−BDCa-1+DC-SIGN+ blood myeloid DCs (mDCs) were analyzed in CP subjects with or without existing acute coronary syndrome and in healthy controls. FACS analysis revealed a significant increase in blood mDCs in the following order: healthy controls < CP < acute coronary syndrome/CP. Analysis of the blood mDC microbiome by 16S rDNA sequencing showed Porphyromonas gingivalis and other species, including (cultivable) Burkholderia cepacia. The mDC carriage rate with P. gingivalis correlated with oral carriage rate and with serologic exposure to P. gingivalis in CP subjects. Administration of local debridement to elicit a bacteremia increased the mDC carriage rate and frequency in vivo. In vitro studies established that P. gingivalis enhanced by 28% the differentiation of monocytes into immature mDCs; moreover, mDCs secreted high levels of matrix metalloproteinase-9 and upregulated C1q, heat shock protein 60, heat shock protein 70, CCR2, and CXCL16 transcripts in response to P. gingivalis in a fibriae-dependent manner. Moreover, the survival of the anaerobe P. gingivalis under aerobic conditions was enhanced when within mDCs. Immunofluorescence analysis of oral mucosa and atherosclerotic plaques demonstrate infiltration with mDCs, colocalized with P. gingivalis. Our results suggest a role for blood mDCs in harboring and disseminating pathogens from oral mucosa to atherosclerotic plaques, which may provide key signals for mDC differentiation and atherogenic conversion. The Journal of Immunology, 2012, 189: 3178–3187.

Coronary artery disease (CAD) and its thrombotic complications are currently the most deadly and disabling cardiovascular diseases in affluent countries. CAD is the leading cause of mortality in the United States, and it is believed that CAD will continue to spread globally unless improved methods are developed to identify at-risk individuals (reviewed in Ref. 1) and institute preventive measures.

The most commonly recognized risk factors for CAD include diabetes, smoking, hypertension, and hyperlipidemia (2). Chronic low-grade infections with bacterial species Chlamydia pneumonia, Helicobacter pylori, Porphyromonas gingivalis, and others are also suspected of conferring increased CAD risk (3, 4); however, the mechanisms are not clear.

Chronic periodontitis (CP) is a low-grade infection identified as a risk factor for CAD (4–6) and other systemic diseases (7). The oral submucosa in CP is a niche for growth of oral Gram-negative anaerobes such as P. gingivalis. P. gingivalis is uniquely able to infect myeloid dendritic cells (mDCs) and reprogram them to induce an immunosuppressive T effector response (8–10). P. gingivalis has been identified in bacteremias (11, 12) and atherosclerotic plaques in humans (13); moreover, it accelerates atherosclerosis in ApoE−/− mice in a manner that is dependent on expression of fimbrial adhesins (4).

Invasion of the arterial vessel walls by inflammatory cells is indispensable to CAD development. Infiligrating cells include monocytes/macrophages (14, 15), lymphocytes, neutrophils, and mDCs (16, 17). An emerging body of literature supports a pivotal role for mDCs in CAD development in humans (18) and mice (19, 20), as reviewed previously (21). However, the predominant sources of mDCs in atherosclerotic plaques and the factors that trigger their activation, infiltration, and differentiation remain elusive.

Circulating dendritic cells (DCs) called blood DCs and their progenitors are likely sources of infiltrating DCs in CAD (22). In humans, blood DC subsets include CD123+CD303+ plasmacytoid DCs, CD19−CD1c− (BDCA-1) mDCs, and a minor subset of CD141+ mDCs (23). Blood DCs are derived from bone marrow...
progenitors, monocytes, and, ostensibly, DC-SIGN\textsuperscript{a} tissue DCs that have reverse transmigrated into circulation after capture of microbial Ags (24, 25). Previous work has documented mDCs actively infiltrating the oral submucosa in CP (26, 27) and rupture-prone atherosclerotic plaques (28). However, the role of blood mDCs in clearance of bacteremias and dissemination to distant sites such as atherosclerotic plaques is undocumented in humans.

In the current study, we show that blood mDCs of humans with CP harbor microbes identified in oral mucosa and atherosclerotic plaques. mDCs provide these microbes with a protective niche and mode of transport. The microbe in turn stimulates differentiation of mDCs from monocytes and converts mDCs into an atherogenic phenotype.

**Materials and Methods**

**Study population**

The Committee on Research Involving Human Subjects 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 CP consisted of 40 subjects with moderate to severe CP as determined by the presence of >20 teeth, of which at least 8 exhibited the following: probing depth >4 mm, attachment loss >3 mm, bleeding on probing, and alveolar bone crest >3 mm from cemento-enamel junction. Demographic data and clinical parameters of the study subjects are shown in Table I. Exclusion criteria included the following: steroidal anti-inflammatory agents, smoking, periodontal treatment within the past 6 mo, pregnancy, diabetes, heart disease, or cancer. After the initial examination, all CP patients were subjected to scaling and root planing (S&RP; local debridement of the root surfaces and pockets) under local anesthesia, and the blood mDC response was evaluated at 24 h. A subset of CP subjects included those with acute coronary syndrome (ACS) (n = 15), diagnosed as reported (29) and shown in Table I. ACS subjects without CP could not be identified. Healthy controls (CTL) consisted of 25 age- and gender-matched subjects and nonsmokers without CP, who had no history of ACS, diabetes, cancer, or other reported systemic disease. Healthy controls were not subjected to S&RP because there is no clinical need and it can be detrimental to clinical attachment levels.

**Blood mDC isolation**

PBMCs were isolated from 30 ml whole blood, and a nucleated cell suspension was prepared using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare). mDCs were isolated as described previously (30). Briefly, PBMCs labeled with BDCA-1 (CD1c) PE\textsuperscript{b} (BDCA-1; Miltenyi Biotec; catalog 130-090-508), CD209 allopheycocyanin\textsuperscript{a} (BD Biosciences; catalog 551545), and CD19 FITC\textsuperscript{a} (BD Biosciences; catalog 557697) were FACS sorted (FACSAria; BD Biosciences) and then separated again to remove CD19\textsuperscript{a} (B cells). These procedures routinely yielded mDC (CD1c\textsuperscript{a}CD209\textsuperscript{a}CD19\textsuperscript{c}) preparations of >95% purity.

### Table I. Clinical description, demographics, serum lipids, and cytokines

<table>
<thead>
<tr>
<th>Clinical description</th>
<th>Control (n = 25)</th>
<th>CP (n = 25)</th>
<th>ACS/CP (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical description</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers Without CP</td>
<td>Nonsmokers No ACS, diabetes, cancer, or other reported systemic disease</td>
<td>Moderate to severe CP &gt;20 teeth ≥8 with probing depth &gt; 4 mm ALOSS &gt; 3 mm, BOP, alveolar bone crest &gt; 3 mm from cemento-enamel junction, nondiabetic, nonsmoker</td>
<td>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 CAD, life expectancy of &lt;2 y</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>Median (range)</td>
<td>51 (38–63)</td>
<td>52 (31–72)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>12</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Females</td>
<td>13</td>
<td>14</td>
<td>5</td>
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<tr>
<td><strong>Self-Reported Race or Ethnic Group (%)</strong></td>
<td></td>
<td></td>
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<tr>
<td>White</td>
<td>12 (48)</td>
<td>16 (64)</td>
<td>11 (63)</td>
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<tr>
<td>Black</td>
<td>9 (36)</td>
<td>2 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0)</td>
<td>2 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>4 (16)</td>
<td>4 (16)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Arab</td>
<td>0 (0)</td>
<td>1 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Serum Lipids (mg/dl)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Triglycerides</td>
<td>102 ± 8.3</td>
<td>155 ± 16\textsuperscript{b}</td>
<td>146 ± 17</td>
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<tr>
<td>Total cholesterol</td>
<td>209 ± 8</td>
<td>215 ± 9</td>
<td>170 ± 11</td>
</tr>
<tr>
<td>LDL</td>
<td>119 ± 7</td>
<td>134 ± 8</td>
<td>96 ± 9.1</td>
</tr>
<tr>
<td>HDL</td>
<td>70 ± 4</td>
<td>50 ± 3\textsuperscript{a}</td>
<td>42 ± 11</td>
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<tr>
<td>CHOL/HDL ratio</td>
<td>3.2 ± 0.2</td>
<td>4.6 ± 0.3\textsuperscript{a}</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>VLDL</td>
<td>20.3 ± 1.6</td>
<td>31 ± 3\textsuperscript{a}</td>
<td>27 ± 4</td>
</tr>
<tr>
<td><strong>Serum Cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLT-3L (pg/ml)</td>
<td>65 ± 24</td>
<td>66 ± 18</td>
<td>137 ± 27\textsuperscript{b}</td>
</tr>
<tr>
<td>TNFr-1 (pg/ml)</td>
<td>1750 ± 564</td>
<td>1850 ± 867</td>
<td>3520 ± 2100</td>
</tr>
<tr>
<td>TNFr-II (pg/ml)</td>
<td>2969 ± 537</td>
<td>3239 ± 247</td>
<td>4982 ± 647\textsuperscript{b}</td>
</tr>
<tr>
<td>hsCRP (ng/ml)</td>
<td>2293 ± 1046</td>
<td>2797 ± 525</td>
<td>2851 ± 413</td>
</tr>
<tr>
<td><strong>Serum IgG Titer against P. gingivalis DPG-3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-PgIgG</td>
<td>1.06 ± 0.06</td>
<td>2.60 ± 0.19\textsuperscript{a}</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Significantly elevated versus CP (p < 0.05, Student t test).
\textsuperscript{b}Significantly elevated versus CP and CTL (p < 0.05, Student t test).

ALOSS, Attachment loss; BOP, bleeding on probing; CHOL, cholesterol; ECG, electrocardiogram; HDL, high density lipoprotein; LDL, low density lipoprotein; MI, myocardial infarction; n.d., not determined; Pg, P. gingivalis; VLDL, very low density lipoprotein.
Generation of monocyte-derived DCs

To serve as in vitro model of DC infectivity and *P. gingivalis* survival, monocyte-derived dendritic cells (MoDCs) were generated, as we have described previously (9, 27, 31). 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 catalog 300-03) and IL-4 (25 ng/ml; R&D Systems; catalog 204-IL-010) at a concentration of 1 x 10^6 cells/ml for 6–8 d, after which flow cytometry was performed to confirm the immature DC phenotype (CD14^+ CD83^+ CD1c^+ DC-SIGN^+; all Abs from BD Biosciences, except CD1c [Miltenyi Biotec]).

In vitro infection and MD differentiation model

After PBMC isolation and plastic adherence, monocytes were scraped off plates and collected. The total number of monocytes was counted using Accuri’s C6 Flow Cytometer System, and baseline levels of CD14, CD1c, DC-SIGN, CD86, and CD83 expression were obtained. Monocytes were then divided equally into 6-well plates and treated either with GM-CSF and IL-4 alone, wild-type Pg381 alone, GM-CSF and IL-4 plus wild-type Pg381, or RPMI 1640 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 MDs 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 DCs is represented as MoDCs per microliter of sample. Triplicates were averaged and assembled onto a line graph to show the differentiation of monocytes into DCs with and without growth factors. Intensities 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 (CD83^+CCR7^+ ) for each condition.

Flow cytometric phenotyping

MoDCs were labeled with combinations of PE, FITC, allophycocyanin, 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 FACS Calibur flow cytometer (BD Biosciences). 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 (MFI) and fluorescence index (FI) relative to the mean fluorescence intensity noted with the relevant negative control Ab for samples labeled in parallel and acquired using the same setting.

Blood mDC frequency change after treatment

Blood PBMCs were obtained from all study subjects at baseline and 24 h after a single intensive bout of S&R, as reported (32). This treatment has previously been shown to result in acute, short-term bacteremia (33, 34), as well as systemic inflammation (32). The MoDCs were labeled with fluorescent conjugated Abs (as described above), CD19, CD1c, DC-SIGN, CD80, CD83 flow cytometry (BD Biosciences). Markers 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 (MFI) and fluorescence index (FI) relative to the mean fluorescence intensity noted with the relevant negative control Ab for samples labeled in parallel and acquired using the same setting.

Quantitative RT-PCR and DNA sequencing

To detect and quantitate *P. gingivalis* genomic DNA (gDNA) and total RNA were isolated from subgingival plaque and MoDCs with the RNeasy Micro Kit (Qiagen), according to the manufacturer’s 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 MoDCs. cDNA was synthesized using STR1® Enhanced First Strand Synthesis Kit (Sigma-Aldrich, St. Louis, MO; catalog STR1-1KT). The cdNA template was standardized to a concentration of 0.1 μg/μl using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific; catalog ND3300) with Quant-iT dsDNA HS Assay Kit (Invitrogen; catalog Q32851). Quantitative real-time PCR (qRT-PCR) was used to detect the presence of *P. gingivalis* (16S rDNA) in MoDCs, MoDCs, and dental plaque samples. The primer set consisted of the non-*P. gingivalis*-specific primer 3180 MICROBIOME WITHIN HUMAN MYELOID BLOOD DENDRITIC CELLS

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; catalog 162-0102) along with a 0.1- to 10.0-kb DNA ladder (New England Biolabs; catalog N3200S). The resultant band was extracted and purified from the agarose gel using QIAEX II Gel Extraction Kit (Qiagen; catalog 20051), according to the manufacturer’s instructions. The DNA template (3.0 ng) was combined with 3.2 pmol 16S rDNA primer and sequenced using a 3730 DNA Analyzer (Applied Biosystems). The sequenced product was then aligned against all human, prokaryotic, and eukaryotic known database genomes using Genomic BlastSequence (BLASTN 2.2.24) (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

Intracellular survival

Wild-type Pg381 was used to infect MoDCs, or polymorphonuclear leucocytes (PMNs) at a multiplicity of infection of 100. Uptake by human cells was confirmed by observing complete internalization of CSFE-stained *P. gingivalis* via epifluorescence microscopy as soon as 60 min after inoculation. Cells were then washed twice in PBS and resuspended in culture medium for continued incubation. At each time point, cells were resuspended in sterile water on ice for 20 min to initiate cell lysis. Bacteria released from within the cells was resuspended in PBS and streaked on anaerobic 5% blood agar plates in triplicate under anaerobic conditions (10% H2, 5% CO2 in nitrogen) at a 1:10 dilution. Plates were incubated in anaerobic conditions at 35°C for 14 d, after which colonies were enumerated and surviving cell-forming U/ml were determined.

Derivation of mAb 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. Briefly, three female 6- to 8-wk-old BALB/c mice (Charles River) were immunized i.p. with three 50 μg doses of native minor fimbriae (Mfa-1) in Sigma adjuvant (Sigma-Aldrich) at 2-wk intervals, following which sera was drawn and tested by ELISA for the presence of Ag-specific Abs. The mouse selected for spleenectomy had a titer of >1:1000 to the protein. Prior to fusion, the mouse was boosted i.p. with 10 μg Mfa-1 in PBS (Life Technologies-Inviron, Carlsbad, CA). Four days following the booster, the mouse was sacrificed, and the spleen cells were isolated aseptically and fused with mouse myeloma cell line Sp2/0 (American Type Culture Collection), as described (36). Clones were screened by ELISA against native minor fimbriae. Clones were then further screened using a whole bacteria ELISA against mF1, which expresses only the major fimbriae (Fg min/ major fimbriae (Fg min/major fimbriae). Clone 89 was determined to be positive both by native minor fimbria ELISA and whole bacteria ELISA, and thus was selected for subcloning by limiting dilution. Subclone 89.15 was selected by ELISA for further study. mAb 89.15 was determined to be of the IgG1 isotype having a κ L chain, by use of the IsoStrip Mouse mAb Isotyping Kit (Roche Applied Science, Indianapolis, IN). Abs from this subclone are referred to as AEZmA1.

Immunofluorescence staining of oral mucosal tissues and coronary artery tissues

Oral mucosal tissues from the human gingiva were collected from untreated CP patients using a biopsy technique previously reported (37). Immediately after collection, the tissues were rinsed with sterile saline to remove traces of blood, embedded in Tissue Tek OCT compound (Sakura), snap frozen in
liquid nitrogen, and sectioned into 7-μM-thick sections using a cryostat (Leica CM1850). Coronal artery tissues were obtained from human postmortem tissues with atherosclerosis and CP (gift of E. Kozarov, Columbia University, New York) and cryosectioned, as described above. For immunofluorescent staining, sections were fixed in acetone for 5 min at −20°C, rehydrated in PBS lacking Ca2+ and Mg2+ (PBS+), blocked with 5% BSA (Sigma-Aldrich) in PBS+ along with anti-human FcR block agent (Miltenyi Biotec, Auburn, CA) for 1 h, and washed. Sections were incubated for 30 min at room temperature with conjugated primary Abs diluted in PBS+ and washed before mounting. All sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). To identify DC-SIGN within arterial plaques and gingival tissue sections, FITC-conjugated (BioLegend, San Diego, CA) or RPE-conjugated mouse anti-human CD209 (AbD Serotec, Raleigh, NC) was used. FITC-conjugated mouse anti-human CD1c was used (BD Biosciences) to identify CD1c. DyLight microscale Ab-labeling kit (Thermo Scientific, Rockford, IL) was used to conjugate Alexa Fluor 594 to mfa-1 Ab (EAEZaMf1a, according to manufacturer’s protocol. Controls included isotype-matched Abs and preimmune Abs. 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 H&E stain to examine cell and tissue morphology. The H&E images were analyzed by image-enhanced light microscopy (Nikon E600).

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

mDCs from blood of CP and ACS/CP patients were analyzed by immunofluorescence cytometry to determine infection with *P. gingivalis*. Briefly, FACS-sorted CD11c+ CD1c+DC-SIGN+ mDCs were permeabilized and fixed in Shandon Cytospin Collection Fluid (Thermo Scientific; catalog 6768315). The cytological specimen was deposited on Ultralast slides (Thermo Scientific; catalog 3039) and cyto centrifuged (Shandon Cytospin 4; Thermo Scientific). After blocking human FcR, as described above, mDCs were probed with PE-AEZaMf1a, and Vectashield mounting media with DAPI (VWR; catalog 101098-044) was added to the specimens; then slides were analyzed by conventional epifluorescence (Nikon E600) and by confocal microscopy, as above.

**Quantitation of serum DC poietins FLTs ligand, soluble TNF RI, soluble TNF RIi**

Serum analysis of DC poietin levels was conducted with colorimetric sandwich ELISA using respective Quantikine immunoassays, as follows: human Flt-3 ligand (Quantikine DFTK00), soluble TNF RI (Quantikine DRT100), and soluble TNF RIi (Quantikine DRT200) (R&D Systems). Briefly, standard dilutions and recommended serum sample dilutions were incubated in precoated microplates containing a specific mAb. Unbound debris was washed, and an enzyme-linked polyclonal Ab specific for the target molecule was added. Unbound Ab 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 the microplates were read immediately at 450 nm on a microplate reader with a correction wavelength of 540 nm (EMax; Molecular Devices).

**Determination of human anti-P. gingivalis IgG titers**

Serum levels of anti- *P. gingivalis* IgG Abs were determined by ELISA, as previously described (38). In brief, 96-well ELISA plates were coated with *P. gingivalis* strain DPG-3 (1 × 105 cells/well), followed by 1-h incubation at 37°C. The wells were blocked for 2 h at 37°C with 2% BSA (Sigma-Aldrich; catalog A3912) in PBS-Tween 20 (Sigma-Aldrich; catalog 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°C. HRP-conjugated goat anti-human IgG (H+L) Abs (Promega, Madison, WI; catalog W4031) were added and incubated for 1 h at 37°C. The 3.3',5.5'-tetramethylbenzidine liquid substrate for ELISA (Sigma-Aldrich; catalog T4444) was used as a substrate. The reaction was stopped by the addition of 3 N hydrochloric acid (LabChem, Pittsburgh, PA; catalog LC15360-2), and the OD was read using an E-max microplate reader (Molecular Devices, Palo Alto, CA) at 450 nm.

**Statistical analysis**

Summary statistics were computed for human peripheral blood mDC counts (numbers and percentages), number of PBMCs, serum IgG titers, serum DC poietins, and lipid profiles in humans, who were grouped according to disease status. D’Agostino-Pearson normality test at *p* < 0.05 was performed to confirm Gaussian distribution, after which differences in means ± SD of healthy versus CP or ACS/CP and CP versus ACS/CP subjects were analyzed by Student *t* test at *p* < 0.05 (GraphPad Prism 5; GraphPad Software, La Jolla, CA). The incidence of *P. gingivalis* in subgingival plaque and blood mDCs of CP patients was analyzed by χ2 and Fisher’s exact test, and Spearman rank test (data shown) with significance was assessed at *p* < 0.05. In vitro assays of DC differentiation, DC expression of matrix metalloproteinase (MMP)-9, and fold changes in ath- erosgenic marker mRNA were performed in triplicate and repeated a minimum of three times. Data were analyzed either by repeated measures ANOVA or Kruskal–Wallis test at *p* < 0.05.

**Results**

**Blood mDC frequency increases with chronic periodontitis and ACS**

The number and percentages (Fig. 1) of blood mDCs were analyzed in three groups, as follows: healthy controls, CP subjects at increased coronary artery disease risk due to lipid profile and serologic exposure to the atherosgenic pathogen *P. gingivalis* (Table 1) (39), and 100% coronary artery disease risk due to existing ACS, as well as CP. No ACS patients could be found without CP. The results show significant increases in blood mDCs in the following order: CTL < CP < ACS/CP. This was not attributable to an increase in total PBMCs, which were actually decreased in CP and ACS/CP (Fig. 1B). Due to previous correlation between blood DC frequency and serum levels of FLT-3L and soluble TNF receptors (40, 41), we analyzed these cytokines in the same patient sera. The results show no difference in FLT-3L, TNFr1, or TNFr2 in CP versus CTL subjects. However, a significant increase was noted in all three cytokines in ACS/CP, relative to CP and CTL (*p* < 0.05, Student *t* test) (Fig. 1C). As infectious seropositivity has been correlated with cardiovascular disease mortality (42), we analyzed the correlation between serum Ab titers against *P. gingivalis* DPG-3, a strain that solely expresses the DC adhesin, mfa-1 ligand (9), and blood mDC numbers. The results demonstrate a linear relationship between serum Ab titers to *P. gingivalis* DPG-3 and blood mDC frequency in CTL versus CP (*r*² = 0.56, *p* < 0.0001) (Supplemental Fig. 1A). Note that sera and subgingival plaque from the ACS/CP cohort were unavailable for analysis in this study, but previous studies have documented in ACS subjects the presence of serum IgG Abs to *P. gingivalis* and *P. gingivalis* DNA in saliva (43).

**Blood mDCs contain a microbiome, as well as viable pathogens and intact P. gingivalis**

To determine whether the blood mDCs contain microbes that could help explain their responsiveness in vivo, blood mDCs were isolated from CP and ACS/CP subjects and analyzed by 16s rDNA sequencing, immunofluorescence, and viable cultures. The 197-bp amplified product specific for *P. gingivalis* is shown in representative mDCs of CP and ACS/CP subjects, relative to in vitro controls (Supplemental Fig. 1B). Overall, 72% of CP subjects who were orally colonized by *P. gingivalis* (Supplemental Table I) were also positive for *P. gingivalis* 16s rDNA within blood mDCs, whereas no CTL mDCs yielded the amplified product. The correlation between oral colonization with *P. gingivalis* and mDC infection rate in CP subjects was positive and significant (Spearman *r* = 0.5192, *p* = 0.0078). Although dental plaque was unavailable from ACS/CP patients for analysis, 37.5% of their mDCs were positive for *P. gingivalis* 16s rDNA. To estimate the level of *P. gingivalis* infection of blood mDCs, MoDCs were pulsed with a range of known MOIs and CFUs of *P. gingivalis* and 16s rDNA used to generate linear regression models (Supplemental Fig. 1C, Supplemental Table I). Based on these models, we calculated estimated CFUs of *P. gingivalis* in blood mDCs from CP and
ACS/CP patients, which were equal to 132,623 estimated CFUs (±121,484 SE), whereas estimated MOIs were all <1. Other potential species identified and sequenced from 16S rDNA of mDCs included H. pylori, Pseudomonas spp., Moraxella catarrhalis, Klebsiella pneumonia, Salmonella enterica, and others.

Live bacteria recovered on blood agar from mDCs included Burkholderia cepacia from two ACS/CP patients (Supplemental Table I). Although viable P. gingivalis were not cultivable from blood mDCs, probably due to their dormant state (44), we did identify intact P. gingivalis at low MOI within mDCs of CP subject by immunofluorescence-confocal microscopy (Fig. 2A). The specificity of our mAb for P. gingivalis mfa-1 (AEZaMfa1) was first established, as shown (Supplemental Fig. 1D). To confirm the ability of P. gingivalis to infect CD1c+ DC-SIGN+ mDCs, we pulsed ex vivo isolated mDCs from a CTL subject with CFSE-labeled P. gingivalis in vitro, which were then counter-labeled with DC-SIGN. The results show intact P. gingivalis-CFSE within DC-SIGN+ blood mDCs (Fig. 2B).

Induction of short-term bacteremia increases blood mDC carriage state and frequency

To determine whether elevated peripheral blood DC counts correlated with the presence of blood-borne infection, CP patients were subjected to mechanical debridement (i.e., S&RP), the standard therapy for CP. This treatment is well documented to drive oral bacteria such as P. gingivalis and others into the bloodstream (11, 32, 33). mDCs were isolated 24 h later and analyzed for P. gingivalis 16S rDNA content and blood mDC frequency. The results (Fig. 2C) indicate a significant increase in P. gingivalis content of blood mDCs after S&RP. Accompanying this was a ∼25% increase in blood mDC frequency (Fig. 2D). This response was statistically significant when comparing pre- and posttreatment in all the CP subjects (p < 0.05, paired Student t test) (Fig. 2E). There was no increase in total PBMCs after therapy, nor were levels of DC poeins FLT-3L, TNFr1, or TNFr2 significantly altered by therapy (data not shown).

Increased mDC frequency involves de novo mDC differentiation in response to infection

A previous in vitro study indicated that differentiation of DCs from monocytes can be enhanced by Ab ligation of DC-SIGN (45). We reasoned that a similar ligation of DC-SIGN by P. gingivalis mfa-1, as reported (10), may enhance differentiation from monocytes. 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 monocytes into immature CD1c+ DC-SIGN+ mDCs, after an apparent 1-d delay. These were indeed immature mDCs, as they were negative for CD83 and CCR7 (data not shown). Moreover, mDC differentiation was enhanced ∼28% by P. gingivalis at a MOI of 0.1, but was retarded by MOI of 0.5 and 1 in a dose-dependent manner (Fig. 3A). Interestingly, P. gingivalis alone at 0.1 MOI induced differentiation of CD1c+ DC-SIGN+ mDCs in the absence of GM-CSF/IL-4 (Fig. 3B), and this continued for 3 d. When the MOI dose was increased to 0.5, the effect was diminished, whereas a MOI of 1 caused no enhancement of MoDC differentiation relative to controls.

mDCs provide a protective niche for P. gingivalis

Viable P. gingivalis (44) and C. pneumonia (46) have been recovered with some difficulty 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. 3C) show that *P. gingivalis* survives for 24 h within mDCs, whereas it dies rapidly in the absence of mDCs. Pretreatment of mDCs with cytochalasin D reduces the protective effect of mDCs 10-fold at 6 and 24 h. We further show professional phagocyte PMNs kill *P. gingivalis* rapidly.

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

MMPs are involved in extracellular matrix destruction, plaque rupture, and myocardial infarction (47). MMP-9 is particularly important in mDC migration (48). We therefore analyzed secretion of MMP-9 by mDCs pulsed with *P. gingivalis* strains that express the DC adhesin mfa-1 and gain access into mDCs (i.e., Pg381, PgDPG-3) or not (PgMFI). The results support mfa-1–dependent secretion of MMP-9 by mDCs (Fig. 3D). Other indicators of risk of plaque rupture, including C1q (49), heat shock protein (HSP) 60/HSP70 (reviewed in Ref. 50), CCR2 (51), and CXCL16 (52, 53), were analyzed at the mRNA level (Fig. 3E). Analysis of these transcripts indicates that C1q, HSP60, HSP70, CCR2, and CXCL16 are upregulated in response to fimbriated Pg381, relative to untreated control. Moreover, relative to the fimbriaeless mutant Pg MFB, Pg381 upregulates C1q, CXCL16, and HSP70 on mDCs.

**mDCs infected by *P. gingivalis* infiltrate oral submucosa and atherosclerotic plaques in situ**

Previous quantitative immunofluorescence histomorphometry established that the diseased oral mucosa (26, 54) and atherosclerotic plaques (16) are infiltrated with increasing numbers of DC-SIGN⁺ mDCs. In view of the current data and our previous in vitro work establishing mfa-1 fimbriae as a DC-SIGN (CD209) ligand (9, 10), we opted to ascertain whether mfa-1 could be identified within DC-SIGN⁺ and CD1c⁺ mDCs in oral mucosa. The results indicate that *P. gingivalis* mfa-1 colocalizes with DC-SIGN⁺ mDCs in oral submucosa (Fig. 4). 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 (data not shown). We then probed postmortem coronary artery biopsies from patients with CAD and CP using the same Abs (Fig. 5). A representative sample is shown. Note erosion and vascular inflammation of the intimal subendothelial layer (box) (Fig. 5A). Evident are CD1c⁺ (Fig. 5B) and DC-SIGN⁺ (Fig. 5C) mDCs, as well as colocalization of *P. gingivalis* mfa-1 with its receptor DC-SIGN (Fig. 5D).

**Discussion**

In this study, we provide evidence in humans for infection of mDCs as a significant route for pathogen dissemination to atherosclerotic plaques. The blood DCs respond to the low-grade infection CP, as well as the acute bacteremia elicited by treatment of CP. DCs were first identified in the human aortic intima in 1995 and were thought to be important in the development of atherosclerotic lesions (18). Soon after, DCs were shown to contact T cells and to overexpress, in early lesions, HSP70, an indicator of physiologic stress (55). HSP70 is also expressed by intracellular bacterial species (56), including *P. gingivalis* (57), and, as we show in this work, by mDCs infected with *P. gingivalis*. Subsequent studies of atherosclerotic plaques showed a prodigious infiltrate of DC-SIGN⁺ immature mDCs (58), as we observed in oral mucosa of CP subjects (54). The immature state of these mDCs is consistent with a reported role for DC-SIGN ligation in inhibiting TLR-mediated DC maturation (9, 59) and in promoting differentiation of immature DCs from progenitors (45). The present work isolated CD1c⁺/DC-SIGN⁺ mDCs from the blood of patients at increased risk for CAD and those with existing CAD and showed that they harbor DNA of *P. gingivalis* and other human pathogens. The infection of blood mDCs was linked to increased blood mDC frequency and to increased relative risk of CAD. The blood mDCs were immature despite containing whole intact *P. gingivalis*. This is consistent with inefficient maturation of mDCs by *P. gingivalis*.

**FIGURE 2.***P. gingivalis* carriage state and frequency of blood mDCs, before and after induced bacteremia.** (A) Image series from scanning laser confocal microscopy (1–7, z-stack series of 1-µm slices) of CD1c⁺ (BDCA-1⁺) blood mDCs from oral carriage-positive CP patient, permeabilized and cytocentrifuged on slides and probed with AEZaMfa-1-PE, followed by Vectashield mounting media. *P. gingivalis* (red) in mDC is shown by white arrow. (B) Epifluorescence deconvolution image analysis of blood mDCs from a healthy donor pulsed in vitro with CFSE-labeled *P. gingivalis* 381 (green) at a MOI of 1 for 3 h. MDCs were counterstained with DAPI (blue) and RPE-conjugated DC-SIGN (red). (C) Significant increase in *P. gingivalis* 16S rDNA content of blood mDCs from CP subjects (n = 6) 24 h after local debridement (S&R). MDCs were analyzed for 16S rDNA of *P. gingivalis*, quantified as in Supplemental Fig. 1C (*p* = 0.02, paired t test). (D) Representative scattergrams from flow cytometry analysis of CD1c⁺ (BDCA-1⁺) and CD209 (DC-SIGN⁺) blood mDCs before (0 h) and 24 h after local debridement (S&R) of CP patients, as described in Materials and Methods. (E) Significant increase in mean number (1) and percentages (2) of blood mDCs 24 h after S&R (per 30,000 PBMCs) (**p < 0.05, paired t test).
due to ligation of DC-SIGN by mfa-1 (9). The initial site of mDC infection by \textit{P. gingivalis} is most likely the oral submucosa. Introduction of \textit{P. gingivalis} into the oral mucosa in mice results in rapid (30-min) bacteremia (personal communication from C. Genco), but the role of DCs in this bacteremia is not yet clear. Trafficking of mDCs through tissues is aided, in particular, by MMP-9 (48, 60), a biomarker of increased CAD risk and a mediator of plaque instability (61). We show that human mDCs stimulated by \textit{P. gingivalis} release high levels of MMP-9, and that this is dependent on expression by \textit{P. gingivalis} of the DC-invasin mfa-1. Increased mobilization of Langerhans cells, which express CD1c (55), and dermal DCs, which express DC-SIGN, is observed in the oral submucosa in CP, and these DCs accumulate in close proximity to the vasculature (27, 54). Hypoxic conditions, as in the subgingival pockets where \textit{P. gingivalis} resides (62), promote the transmigratory activity of DCs through endothelium (63). Overall, the hypoxic microenvironment and local infection with \textit{P. gingivalis} may be a potent driving force for reverse transmigration.

\textbf{FIGURE 3.} \textit{P. gingivalis} induces mDC differentiation, survives within mDCs, and induces atherogenic mDC phenotype. (A) Pre-DC monocytes from healthy controls were cultured in triplicate with growth factors GM-CSF/IL-4+/+ \textit{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 \(\mu\)l ± SE is shown. Phenotype of immature MoDCs was further confirmed by evidence of down-regulation of CD14 and low to no expression of CD83 and CCR7 (data not shown). *Significant difference between control and growth factors (GF) and MOI of 0.1 + GF (ANOVA, \(p < 0.05\)). Data are representative of results of assay repeated three separate times. (B) Performed as in (A), but without growth factors. Significant differences were noted in mean number of MoDCs per \(\mu\)l in MOI 0.1-GF versus MOI 0.5-GF at 2 and 3 d, and in MOI 0.1-GF, MOI 0.5-GF versus control, and MOI 1-GF at 2 d (ANOVA, \(p < 0.05\)). (C) Wild-type Pg381 was incubated in mDC buffer alone (filled circle) or with mDCs (filled square), or with mDCs pretreated with cytochalasin D (filled triangle), or control PMNs (inverted filled triangle) at a multiplicity of infection of 100 for 0, 6, and 24 h. Internalization of CFSE-labeled \textit{P. gingivalis} was confirmed by epifluorescence microscopy. Cells were lysed, and viable bacteria was recovered on enriched anaerobic 5% blood agar plates in triplicate under anaerobic conditions (10% H\(_2\), 5% CO\(_2\) in nitrogen) at a 1:10 dilution at 35°C for 14 d, after which log\(_{10}\) CFU/ml was determined. Experiment was repeated three separate times, and data are representative of consistent results. (D) MoDCs were pulsed with wild-type Pg 381, its mfa-1 minor fimbiae-deficient strain (MFI) or fimA major fimbiae-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 was assessed by ELISA. The data are the mean ± SD of triplicate assays. (E) MoDCs were pulsed in triplicate with wild-type Pg381, its fimbiaeless mutant MFB, or no Pg (CTL) at a 25:1 MOI for 3 h, and uptake of CFSE-labeled Pg was monitored by FACS analysis (data not shown). qRT-PCR (Bio-Rad) was used to determine expression levels, normalized to β-actin, and expressed as fold changes in mRNA. PCR primers were designed using PRIMER3 Software (77). For relative quantification of transcript expression, cycle threshold 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).
of tissue mDCs into the blood to atherosclerotic plaques (8, 25, 64).

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 (25). Our results suggest that clearance of bacteremias may be a pathophysiological function of blood DCs. Bacteremia is a well-described phenomenon in patients with CP (33), and is induced by eating an apple (12), tooth brushing, flossing, or undergoing S&R (65). We demonstrate that both the chronic condition CP and acute bacteremia increase the P. gingivalis content of blood mDCs and result in an increase in blood mDC frequency. Other infectious conditions that alter DC frequency include tuberculosis (66), malaria (67), and filariasis (68). We initially suspected that DC poietin levels were altering blood mDC frequency in CP, as reported in a number of diseases, including CAD in humans (40, 41, 66, 69–74). Whereas elevated DC poietins were found in sera of our ACS/CP cohort, all had been on long-term cardiovascular care, including lipid-lowering drugs. These are known to raise mDC levels (73). Moreover, 100% of our ACS cohort had CP; none could be identified without it. It should be noted that our systemically healthy CP cohort were not on such drugs and DC poietin levels were unchanged in CP. We therefore surmised that the bacteremia itself was the impetus for increasing mDC frequency. We thus implemented an in vitro infection assay to ascertain the mechanism. We show that indeed DC differentiation was enhanced by P. gingivalis at low MOIs, but retarded at higher MOIs. We surmise that this differential response may be reflective of a balance between two driving forces. At low MOIs, the DC-SIGN ligand P. gingivalis mfa-1 (10) positively influences DC differentiation, as reported for anti–DC-SIGN Abs (45). This involves activation of a unique signaling pathway, the DC-SIGN signalosome, which is counterregulatory to TLR signaling (59). In contrast, infection by P. gingivalis at higher MOIs has a negative influence on DC differentiation due to enhanced apoptosis (75). Although we have no evidence yet that this effect is specific for P. gingivalis, previous studies using several other species suggest a predominantly negative influence of bacterial infection on DC differentiation (76).

Collectively, these data indicate that mDCs in tissues and blood harbor pathogens of direct relevance to coronary artery disease and other human diseases. MDCs protect the pathogen, whereas the infection promotes further 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.

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Disclosures
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References
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