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Anti-phosphorylcholine-opsonized low-density lipoprotein promotes rapid production of proinflammatory cytokines by dendritic cells and natural killer cells

T. Kikuchi*†, M. M. El Shikh†, R. M. El Sayed, D. B. Purkall, M. M. Elaasser, A. Sarraf, S. E. Barbour, H. A. Schenkein, J. G. Tew

Clinical Research Center for Periodontal Diseases, School of Dentistry, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA, USA

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Background and Objective: Epidemiological and animal studies suggest that periodontal infections increase atherosclerosis risk. Periodontitis patients have elevated levels of anti-phosphorylcholine (anti-PC) reactive not only with numerous periodontal organisms but also with minimally modified low-density lipoprotein (mmLDL). Dendritic cells (DCs) reside in arterial walls and accumulate in atherosclerotic lesions. The ability of anti-PC to bind mmLDL prompted the hypothesis that opsonized mmLDL would stimulate DCs and enhance the production of proinflammatory cytokines that promote atherogenic plaque development.

Material and Methods: Monocyte-derived DCs (mDCs) were generated using granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, then stimulated with mmLDL or with anti-PC-opsonized mmLDL. The anti-PC effect was determined using flow cytometry, cofocal microscopy and cytokine assays. The production of CD83, IL-12p35 mRNA, IL-12p40 mRNA, IL-12p70 and IL-10 by DCs was monitored.

Results: Dendritic cells stimulated with mmLDL expressed little CD83 and produced little IL-12p70. However, anti-PC-opsonized mmLDL enhanced DC maturation, as indicated by upregulated CD83 and rapid ($\leq$ 48 h) production of IL-12p70 if a source of interferon-γ (IFN-γ) was available. In leukocyte cultures, natural killer (NK) cells rapidly produced IFN-γ ($\leq$ 48 h) when interacting with IL-12-producing DCs activated by anti-PC-opsonized mmLDL. Moreover, IFN-γ promoted DC IL-12 responses that were further augmented when mmLDL was opsonized with anti-PC.

Conclusion: Minimally modified LDL-stimulated DCs and NK cells were mutually stimulatory, with DC IL-12p70 needed by NK cells and with NK cell IFN-γ

John G. Tew, PhD, Clinical Research Center for Periodontal Diseases, School of Dentistry, Medical College of Virginia Campus, Virginia Commonwealth University, 23298 Richmond, VA, USA
Tel: 804 828 9715
Fax: 804 828 9946
e-mail: tew@vcu.edu.

*Present address: Department of Periodontology, School of Dentistry, Aichi-Gakuin University, 2-11 Suemoridori, Chikusa-ku, Nagoya 464-8651, Aichi, Japan.

†Drs Kikuchi and El Shikh contributed equally to this work.

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needed by DCs. Moreover, production of these proinflammatory cytokines was markedly enhanced when LDL was opsonized by anti-PC. In short, the data suggest that the elevated anti-PC levels in periodontitis patients could promote a mechanism that facilitates atherosclerosis.

Phosphorylcholine (PC) is an immunogenic epitope in the capsular polysaccharide of *Streptococcus pneumoniae* and it has been suggested that the presence of serum anti-PC is partly a result of exposure to this organism (1,2). However, periodontitis patients exhibit elevated levels of anti-PC (3) and this antibody reacts with 30–40% of bacteria in dental plaque samples, including periodontitis-associated bacteria (3,4). Moreover, anti-PC levels in the gingival crevicular fluid from patients with aggressive periodontitis often exceed serum levels of anti-PC, suggesting that periodontal bacteria stimulate local anti-PC production (5). These relationships support the concept that periodontal bacteria are major inducers of anti-PC (3–5).

Atherosclerosis is a complex disease with a multifactorial etiology. Hypercholesterolaemia is clearly important, but the influence of inflammation and immune mechanisms in various stages of development are becoming increasingly apparent (6,7). Innate mechanisms involving leukocyte recruitment and the production of proinflammatory cytokines appear to be important in early atherosclerotic lesions, and adaptive immunity appears to contribute to disease activity and progression (6,7). Studies in murine models suggest that interferon-γ (IFN-γ) is a particularly important proinflammatory cytokine. Daily administration of IFN-γ promoted atherosclerosis in *Apoe* knockout mice (8) and athromatous lesions are decreased in mice deficient in the IFN-γ receptor (9). Dendritic cells (DCs) are known to interact with both natural killer (NK) cells and T cells and play a crucial role in the initiation of immune responses. Dendritic cells reside in the arterial walls and accumulate in atherosclerotic lesions where they may be important in producing type-1 mediators and promoting inflammation (10–12). Vascular DCs accumulate most densely in arterial regions that are subjected to haemodynamic stress by turbulent flow conditions and these areas are predisposed to immune complex (IC) deposition and development of atherosclerosis (11–13). In addition, many DCs in the atherogenic plaque bear C1q and are thought to be involved in the trapping of ICs (10). The distribution of DCs in the intima of athero-prone vs. athero-resistant areas of nondiseased aorta revealed DC clusters and possible activation of lymphocytes in the prone tissue (10,11). *In vitro*, some forms of oxidized low-density lipoprotein (oxLDL) induce DC maturation (14) and the same features can be seen in the vessel wall [the expression of CD83, major histocompatibility complex and CD40 is elevated (10,15)].

Anti-PC generated in response to periodontitis-associated bacteria interacts with oxLDL (16) and we reasoned that minimally modified low-density lipoprotein (mmLDL) might interact with anti-PC and form immune complexes (mmLDL-ICs) that might contribute to the increased risk for atherosclerosis in periodontitis patients. In previous studies we found that *Aggregatibacter actinomycetmcomitans*–DC and *Porphyromonas gingivalis*–DC interactions led to the production of interleukin (IL)-12p70 by DC and the rapid production of IFN-γ by NK cells (17,18). In the present study, we found that mmLDL could also promote the rapid production of IFN-γ by NK cells . Moreover, opsonization of mmLDL with anti-PC promoted IL-12 production by DCs and IFN-γ production by NK cells that, in turn, promoted the production of more IL-12 by DCs and ultimately more IFN-γ by NK cells. This ability of anti-PC-opsonized mmLDL to mature DCs and promote the rapid production of type-1 proinflammatory cytokines by DCs and NK cells, suggests that anti-PC may be involved in a mechanism that promotes the initiation of atherosclerotic lesions. Moreover, the elevated anti-PC levels in periodontitis patients may help to explain why their risk for atherosclerosis appears to be elevated (19,20).

Material and methods

Human subjects

Subjects for this study were obtained by the Clinical Research Center for Periodontal Disease, School of Dentistry, Virginia Commonwealth University, Richmond, Virginia. The Internal Review Board of Virginia Commonwealth University approved our use of human subjects and informed consent was obtained from all study subjects. Peripheral blood leukocytes (PBL) were isolated from heparinized whole blood from non-periodontitis subjects who had no evidence of attachment loss, except for recession on the buccal surface of anterior teeth at no more than one site and no pocket greater than 3 mm.

Lymphocyte separation

Peripheral blood leukocytes were obtained from heparinized blood by density centrifugation using lymphocyte separation medium (ICN, Aurora, OH, USA). Cells were centrifuged at 400 *g* for 20 min, and the cells were collected from the interface and washed three times in RPMI-1640 (Cellgro, Manassas, VA, USA). After washing, the cells were suspended in RPMI-1640 supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and antibiotics for cell culture.

Preparation of monocyte-derived DCs

For experiments where DCs were used for mRNA analysis, or where they were added to other cell types, they were purified by cell sorting using a MoFlo (Cytomation) cell sorter (Dako, Carpinteria, CA, USA) and cultured in medium enriched with 10% fetal calf serum containing 500 U/mL of recombinant human IL-4 and 800 U/mL of recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) for 5 d to generate monocyte-derived DCs (mDCs) (21). In some studies limited to analysis of the DC phenotype, the monocyte-rich adherent cell population was incubated with GM-CSF and IL-4 for 5 d, as indicated above. The presence of IL-4 interferes with the NK cell IFN-γ response. To ensure that the IL-4 used to induce the DCs was removed, the immature DCs were washed three times before they were added to PBL or NK cells. In each experiment monocytes were obtained from a single donor and were converted into mDCs, but different donors were used to reproduce experiments. Use of a single donor for comparison was important because the magnitude of the cytokine responses differed considerably from donor to donor. However, the relationships between cytokine responses within an experiment were reproducible in different donors.

Preparation of human LDL

Human LDL was isolated from the sera of nonperiodontitis subjects, as described previously (16), and chromatographed on a 1.5×90 cm Sepharose CL-6B (Sigma-Aldrich Corporation, St. Louis, MO, USA) column equilibrated in 0.01 M Hepes, 0.14 M NaCl and 0.05% NaN_3. The absorbance of the eluted fractions was monitored by A280 UV and protein content was assessed using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Samples of protein-containing fractions were diluted into phosphate-buffered saline (PBS)–Tween-20 and incubated on chromatographically purified goat anti-ApoB (Fitzgerald Industries International, Acton, MA, USA) coated ELISA wells for 30 min. All wells were washed three times in dilution buffer and then incubated with monoclonal anti-ApoB (Fitzgerald Industries International, Acton, MA, USA) for 30 min. The wash cycle was repeated and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Laboratories, West Grove, PA, USA) was added for another 30 min. Colour was developed using tetramethylbenzidine (TMB) in 0.1 M acetate buffer. Positive fractions were pooled and concentrated using Amicon Centriprep YM-30 (Millipore Corporation, Billerica, MA, USA). Concentrated preparations were passed over a Sephadex G-25 (Amersham Corporation, Burlington, MA, USA) column, equilibrated with Hanks' balanced salt solution (HBSS; pH 7.5), to remove NaN_3. The concentration of LDL was assessed using the ELISA strategy described earlier in this paragraph, utilizing LDL from Molecular Probes (Life Sciences Technologies, Carlsbad, CA, USA) as a standard. Our isolated preparation includes LDL that interacts with anti-PC (~60 ng/2 μg of LDL) and the preparation is referred to as mmLDL and was used at 2 μg/mL to stimulate DCs in culture. Although the LDL was not deliberately modified, ~60 ng of anti-PC-reactive LDL was present in the 2 μg/mL of LDL added to the cultures. The anti-PC activity indicates that some LDL had been modified sufficiently during isolation to permit anti-PC binding and we reasoned that modification would continue during culture with cells of the immune system.

Fluorescent labelling of LDL

Human LDL was isolated from the sera of nonperiodontitis subjects, as described in the preceding section. The freshly isolated LDL was labelled with NHS-Alexa 488 (Molecular Probes), following the manufacturers' instructions. DCs were stimulated using 2 μg/mL of labelled LDL.

ELISA for detection of anti-PC-reactive LDL

ELISA plates (Immulon 4 HBX; Thermo Fisher Scientific, Waltham, MA, USA) were coated with 4 μg/mL of affinity-purified human anti-PC. The anti-PC was diluted in phosphate buffer, pH 7.5, and the plates were shaken at room temperature for 30 min and then washed three times with PBS–Tween-20. The LDL samples were diluted in PBS–Tween-20 and added to anti-PC-coated wells. The plates were incubated for 30 min at room temperature with shaking, and then washed three times with PBS–Tween-20. Affinity-purified goat anti-human ApoB (Fitzgerald Laboratories) was diluted 1/1000 in PBS–Tween-20, then incubated for 30 min and washed as described above. Horseradish peroxidase-labelled rabbit anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA) was diluted 1/1500 in PBS–Tween-20 and incubated as described above, then washed once with PBS–Tween-20 and then three times with deionized water to remove residual Tween-20. The presence of HRP activity was detected using TMB diluted to 0.1 mg/mL in 0.1 M acetate buffer, pH 6.0, containing 0.006% H_2O_2, and incubating for 30 min with shaking, at room temperature. Development was stopped by adding 20 μL of 2.5 M H_2SO_4 and the plates were read at an absorbance of 450 nm using a Molecular Devices vMax ELISA reader (Sunnyvale, CA, USA). Volumes in the ELISA plates were 0.1 mL.

Levels of anti-PC-reactive LDL in LDL added to cultures

Low-density lipoprotein was incubated with supernatant fluid from stimulated polymorphonuclear leukocytes (PMNs), which we reasoned would mimic an acute inflammatory response and generate opsonizable LDL. Polymorphonuclear leukocytes were isolated as previously described (22), adjusted to 1×10^7 cells/mL in HBSS containing 0.008 M EDTA and 0.1% bovine serum albumin (Sigma; low endotoxin) and then incubated with 5 μg/mL of anti-CD32 (IV.3; ATCCGeorgetown, Washington, DC, USA) and anti-CD16 (a gift from Dr Shaun Ruddy) for 12 min at room temperature. The PMNs were washed

using HBSS and resuspended at 1×10^7 PMNs/0.2 mL in buffer. The PMNs were then activated by adding 5 μg/mL of $F(ab')_2$ goat anti-mouse IgG (Jackson Immunoresearch) and incubated at 37°C for 30 min. The PMNs were then pelleted and the active supernatant fluid was removed and filtered through a 0.2-micron filter to remove debris. Low-density lipoprotein was added to supernatant fluid at a concentration of 100 μg/10^7 PMNs used to make supernatant fluid. The LDL mixture was incubated at 37°C overnight to further oxidize the LDL and then passed through a Sepharose CL-6B (Sigma) column equilibrated in RPMI-1640, and the LDL peak was isolated as described above.

The concentration of LDL that had been modified sufficiently for anti-PC to bind was determined using anti-PC as the capture antibody to attach modified LDL to the ELISA plate. A standard curve was prepared using a murine monoclonal antibody for ApoB as the capture antibody. Goat anti-ApoB was used to measure captured LDL over a series of dilutions. Affinity-purified HRP-labelled mouse anti-goat IgG (H + L) was added for 30 min to label the goat antibody bound to the captured LDL (Jackson Immunoresearch). Colour was developed using TMB in 0.1 M acetate buffer. Sixty nanograms of anti-PC-reactive LDL was present in the 2 μg/mL of LDL used in our cultures and this increased to 150 ng of anti-PC-reactive LDL after encountering the PMN-supernatant fluid.

Reagents

Recombinant human IL-4, GM-CSF, monocyte colony-stimulating factor, monoclonal anti-human IL-12 and the mouse IgG1 isotype control were obtained from R&D Systems (Minneapolis, MN, USA). Anti-human CD83 was obtained from BD Biosciences (San Diego, CA, USA). Moderately oxidized LDL labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil-oxLDL) was purchased from Biomedical Technologies Inc (Stoughton, MA, USA).

Confocal microscopy

Dendritic cells were surface labelled with CD11c-fluorescein isothiocyanate (FITC) (11-0116; eBioscience, San Diego, CA, USA), and the LDL was labelled with Dil 1,1′-dioctadecyl – 3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate' (BT-920; Biomedical Technologies, Inc.). Dendritic cells were mounted with anti-fade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), coverslipped and examined using a Leica TCS-SP2 AOBS confocal laser scanning microscope fitted with an oil plan-apochromat × 40 and × 63 objectives. Two lasers were used: argon (488 nm) for FITC, and HeNe (543 nm) for Dil. Parameters were adjusted to scan at 512 × 512 pixel density and 8-bit pixel depth. Emissions were recorded in two separate channels, and digital images were captured and processed using Leica Confocal and LCS Lite software (Leica Microsystems Inc., Bannockburn, IL, USA) .

Purification of anti-PC and the nonspecific control antibody

Anti-PC was isolated by affinity chromatography on a *p*-Aminophenyl PC column (Pierce cat. # 20307; Thermo Fisher Scientific, Waltham, MA, USA), as previously described (16). Antibody that bound to lysine Sepharose (Amersham Biosciences, Burlington, MA, USA) was used as a nonspecific control. The bound antibodies were eluted from the columns using 0.1 M glycine, 0.2 M NaCl, pH 3.0, immediately neutralized with 0.1 M Tris–HCl, pH 8.0, and concentrated using a Centriprep 30 (Millipore Corp., Billerica, MA, USA). $F(ab')_2$ fragments of anti-PC were generated using Pierce Biotechnologies' ImmunoPure $F(ab')_2$ Preparation Kit, following the manufacturer's instructions. Reactivity of anti-PC was confirmed using ELISA (16).

Bacteria

A. actinomycetemcomitans strain Y4 and *P. gingivalis* strain W83 were employed in this study. *A. actinomycetemcomitans* and *P. gingivalis* were grown in brain–heart infusion medium (Difco Laboratories, Detroit, MI, USA). After culture the bacteria were washed three times with PBS and resuspended to the appropriate concentration in the same buffer. Any leukotoxin was inactivated by heating the organisms at 65°C for 30 min before use in cultures.

Flow cytometry

Cell-surface CD83 was analyzed using flow cytometry after gating on the monocyte/DC population. Cells (2×10^5) were incubated with 5 μL of FITC-labelled anti-CD83 (BD Biosciences) for 30 min on ice in the dark. The cells were washed once and resuspended in PBS containing 2% fetal bovine serum. The mean fluorescence intensity was then determined using a flow cytometer (FACScan; Becton Dickinson, Mansfield, MA, USA). Approximately 10,000 cells were included in each analysis.

Co-culture of mDC with PBL

Samples of 1×10^6 PBL suspended in 1 mL of RPMI-1640 supplemented with 10% fetal calf serum were cultured in 75-mm tubes (Falcon, Lincoln Park, NJ, USA) at 37°C in a humidified atmosphere containing 5% CO_2. In many cultures, 1×10^5 mDCs were used and cultures were stimulated with 2 μg/mL of mmLDL. Supernatant fluids were collected after 24 h of culture and the concentration of IFN-γ was measured using ELISA.

Magnetic cell separation separation of monocytes and NK cells

CD14 MicroBeads (130-050-201) and the NK Cell Isolation kit (130-092-657) from Miltenyi Biotec (Auburn, CA, USA) were used for isolation of monocytes and NK cells in studying the ability of anti-PC-opsonized mmLDL to enhance the rapid release of IFN-γ. In brief, monocytes were isolated from gradient-separated PBLs where the $CD14^+$ cells are magnetically labelled with CD14 MicroBeads, then loaded onto a magnetic cell separation (MACS) column placed in

the magnetic field of a MACS Separator (Miltenyi Biotec). The magnetically labelled $CD14^+$ cells are retained within the column, while the unlabelled cells run through. After removing the column from the magnetic field, the magnetically retained $CD14^+$ cells were eluted as the positively selected cell fraction. Binding of antibody to CD14 does not trigger signal transduction because CD14 lacks a cytoplasmatic domain. By contrast, NK cells were isolated from PBLs by depletion of non-NK cells (negative selection). Non-NK cells were magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies against lineage-specific antigens not expressed by NK cells and a cocktail of MicroBeads. Cells were layered over a MACS column placed in a magnetic field of a MACS Separator; the magnetically labelled non-NK cells were retained in the column, while the unlabeled NK cells were collected in the flow-through. Dendritic cells were generated from separated monocytes as detailed above.

Co-culture of mDC with NK cells, and assessment of cytokines

Samples of 1.5×10^5 MACS-separated NK cells suspended in 0.5 mL of RPMI-1640 supplemented with 10% fetal calf serum were cultured, at 37°C in an atmosphere of 5% CO_2, in a 48-well culture plate containing 1×10^5 mDCs, and cultures were stimulated with 4 μg/mL of mmLDL (Sigma; L8292), with or without 10 μg/mL of human anti-PC. In these studies, the concentrations of IL-10, IL-12 and IFN-γ were measured and the IL-10/IL-12 ratios were determined. To ensure that the responses were adequate for all cytokines, the LDL dose was increased for this experiment from 2 to 4 μg/mL. Supernatant fluids were collected after 48 h and the concentration of IFN-γ was measured using a Bio-Plex Human group 1 cytokine 7-plex panel (8800753; Bio-Rad) and a Bioplex reagent kit (8800547; Bio-Rad); data were collected on a Bio-Plex array reader (# LX10004042104) and analyzed using BIOPLEX MANAGER SOFTWARE 4.1. (Life Science Research, Hercules, CA, USA) IL-12p40 levels in the culture supernatant fluid were evaluated using an ELISA kit purchased from R&D Systems (Minneapolis, MN, USA).

Analysis of IL-12p35 and IL-12p40 mRNA levels by quantitative (real-time) PCR

At the end of cell culture, total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the one-step RT-PCR reaction was carried out using iCycler (Bio-Rad). The TaqMan™ one-step RT-PCR master mix reagent kit (ABI, Foster City, CA, USA) was used for RT-PCR. The amplification of IL-12p35, IL-12p40 and 18S RNA species was carried out in separate tubes using the same amount of total RNA retrieved from the same sample. The sequences for the various oligonucleotides starting with the forward primer, followed by the reverse primer, then the probe, and in the direction of 5′ to 3′, were as follows: IL-12p35: TCAAAACATGCTG GCAGTTAT, GAAGAAGTATGCA GAGCTTGAT, HEX-AGCTGATGC AGGCCCTGAATTTCA-BHQ-1; IL-12p40: CACAAAGGAGGCGAGGT T, TGGGTTCTTTCTGGTCCTTT, FAM-CCATTCGCTCCTGCTGCTT CACAA-BHQ-1; 18S ribosomal RNA: AAAATTAGAGTGTTCAAAGCAG GC, CCTCAGTTCCGAAAACCAA CAACY5-CGAGCCGCCTGGATAC CGCAGC-BHQ-2. The experimental conditions were extensively optimized for each target. Reactions were prepared in 96-well PCR plates (Bio-Rad) in a total volume of 25 μL containing the following components: 10 ng of total RNA, 12.5 μL of 2× Master Mix without UNG, 0.625 μL of 40× MultiScribe and RNase Inhibitor Mix, 250 nM of probe and 900 nM of forward and reverse primers. Thermal cycling conditions consisted of an initial reverse transcription step at 48°C for 30 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for IL-12p35 and IL-12p40. The cycling conditions for 18S were 20 cycles of 15 s at 95°C and 1 min at 60°C. Finally, fold differences in mRNA expression levels were calculated using the $\Delta\Delta C_T$ method (23). In short, the PCR efficiency was first ascertained to be close to 100% by performing multiple standard curves using serial mRNA dilutions. An amplification cycle threshold value (C_T value), defined as the PCR cycle number at which the fluorescence signal crosses an arbitrary threshold, was calculated for each reaction. The fold change between mRNA expression levels was determined as follows: fold change = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\ GoI} - C_{T\ Hk})$ Sample $- (C_{T\ GoI} - C_{T\ Hk})$ Control (C_T, cycle threshold; GoI, gene of interest; Hk, housekeeping gene).

Statistical analysis

Experiments were repeated a minimum of three times and the cultures were grown in triplicate. Group means were compared using analysis of variance (ANOVA) followed by Tukey's HSD test. Significance was accepted at alpha < 0.05.

Results

Anti-PC as an LDL opsonin

Dendritic cells express receptors for Ig-Fc (FcγR) and specific antibodies can serve as opsonins (24). This suggested that LDL-reactive antibody would enhance uptake by DCs. To test this, mDCs were incubated with labelled LDL in the presence or absence of anti-PC. Low-density lipoprotein was trapped by mDCs, as indicated by an increase in the mean cell fluorescence over the autofluorescence of the DCs alone, and anti-PC clearly enhanced the mmLDL–DC interaction (Fig. 1). By contrast, $F(ab')_2$ anti-PC failed to enhance mmLDL trapping, confirming the importance of Fc binding (data not shown).

Opsonins promote phagocytosis and, to determine if LDL was internalized, confocal microscopy was used to examine DCs with labelled LDL after 24 h of culture. Although the LDL used in Fig. 1 was not deliberately modified, the fact that anti-PC has activity indicates that some LDL had been oxidized during isolation and

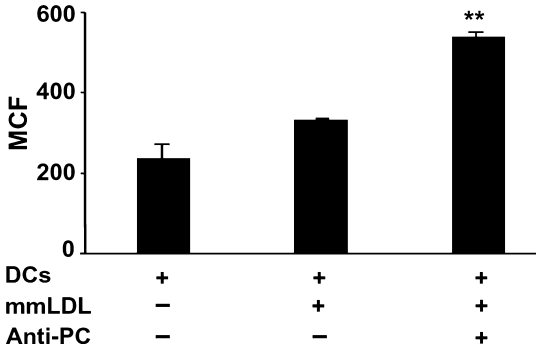

Fig. 1. Anti-phosphorylcholine (anti-PC)-mediated trapping of low-density lipoprotein (LDL) by dendritic cells (DCs). Purified DCs were incubated with Alexa 488-labelled LDL in the presence and absence of anti-PC (25 μg/mL). Data were collected using flow cytometry and are expressed as mean cell fluorescence (MCF). The data are typical of three experiments of this type. $^{**}p < 0.01$. mmLDL, minimally modified low-density lipoprotein.

culture. In this experiment, deliberately oxidized LDL was used to obtain sufficient LDL in DCs to be visualized in the absence of anti-PC. Anti-CD11c (green) was used to label the DC surface membrane and oxLDL was labelled red. Some DCs were LDL^+ in the absence of anti-PC but most were negative (Fig. 2B). In marked contrast, virtually all DCs became LDL^+ upon the addition of anti-PC opsonin and many DCs were strongly labelled (Fig. 2C). Note that the surface labelled CD11c typically appeared as a green ring around the DCs and this contrasted with LDL, which was largely inside the DCs. This was most apparent in cells incubated with opsonized LDL that are shown at higher power (Fig. 2D,E). A small amount of LDL was co-localized with the CD11c on the DC surface and appeared yellow, but the overwhelming majority of the LDL was internalized between the cytoplasmic and the nuclear membranes, and the cytoplasm appeared bright red, confirming internalization.

Anti-PC-opsonized LDL promoted DC maturation

We reasoned that opsonization of mm LDL would promote the maturation of

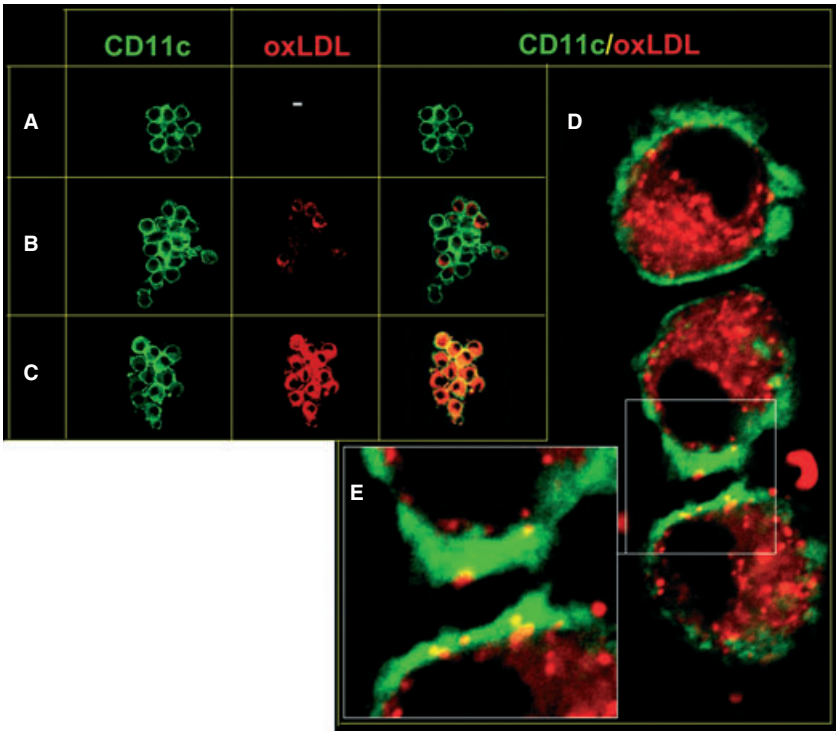

Fig. 2. Low-density lipoprotein (LDL) opsonization via anti-phosphorylcholine (anti-PC) confocal microsocopy was used to determine if anti-PC–LDL interactions result in dendritic cells (DCs) internalizing LDL. To achieve this, moderately oxidized LDL labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil- oxLDL) was incubated with anti-PC (25 μg/mL) and the DCs were examined using confocal microscopy after 24 h of culture. Anti-CD11c (green) was used to label the DC surface membrane and the Dil-OxLDL was red. (A) LDL was omitted so the overlay in the final box remained green. (B) Uptake of LDL by DCs in the absence of anti-PC. Note that surface-labelled CD11c typically appeared as a green ring around the DCs and this contrasted with the red LDL. (C) Anti-PC was included and the level of LDL trapped showed a clear increase over that in (B). (D) and (E) are shown at higher power and it is apparent that a small amount of LDL is co-localized with the CD11c on the DC surface and appears yellow. However, the overwhelming majority of the anti-PC-opsonized LDL had been internalized between the cytoplasmic and the nuclear membranes and the cytoplasm appeared bright red, confirming internalization.

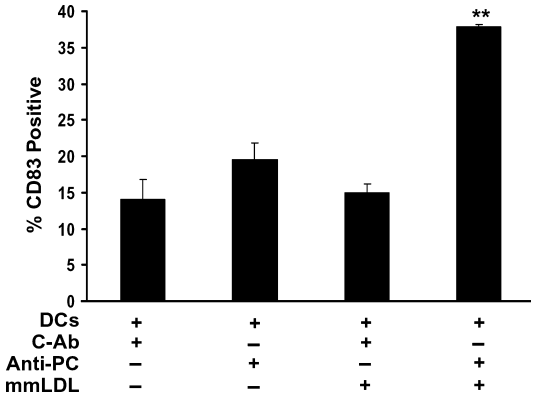

Fig. 3. Anti-phosphorylcholine (anti-PC)-opsonized low-density lipoprotein (LDL) promotes dendritic cell (DC) maturation, as determined by increased CD83 expression. Monocyte = derived DCs (2×10^6) were stimulated with anti-PC or LDL, or both, for 48 h and CD83 expression was monitored by flow cytometry. The negative control (C-Ab) is antibody that bound to lysine Sepharose and was eluted with the same reagents used to release anti-PC when performing affinity chromatography. These data represent the mean of three separate experiments of this type and are expressed as mean ± standard deviation. $^{**}p < 0.01$. mmLDL, minimally modified low-density lipoprotein.

DCs. The addition of anti-PC with the LDL resulted in a significant increase in CD83 expression, indicating DC maturation (Fig. 3). Similar results were obtained with major histocompatibiliity complex class II, further supporting maturation (data not shown).

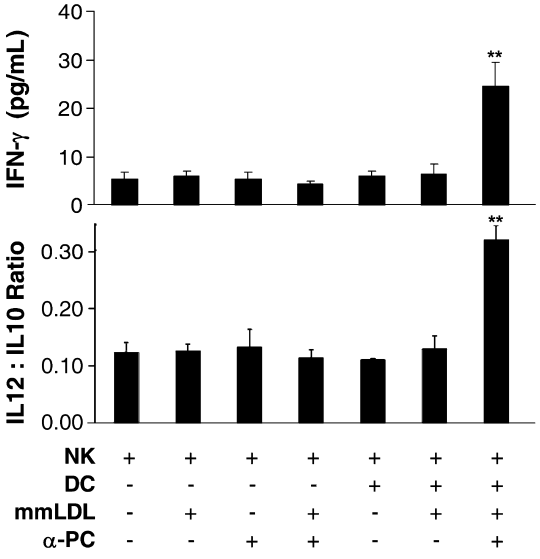

Fig. 4. Anti-phosphorylcholine (anti-PC) enhanced the ability of low-density lipoprotein (LDL)-stimulated dendritic cells (DCs) to produce interleukin (IL)-12 and promote interferon-γ (IFN-γ) production by natural killer (NK) cells. The upper panel shows the results of NK cells and DCs that were stimulated with LDL alone or with anti-PC-opsonized minimally modified low-density lipoprotein (mmLDL). To ensure that the responses were adequate for all cytokines being analyzed, the LDL dose was increased from 2 to 4 μg/mL. The supernatant fluids were collected 48 h later and IL-10, IL-12 and IFN-γ production were measured using Bio-Plex. In the lower panel the ratio of IL-12/IL-10 concentration is plotted. Opsonization of LDL increased IL-12 levels without significantly altering the level of IL-10, resulting in a higher IL-12/IL-10 ratio that corresponded with an elevated production of IFN-γ. $^{**}p < 0.01$

Anti-PC-opsonized LDL promoted DC-mediated enhancement of IFN-γ by NK cells

Our major hypothesis was that opsonized LDL would interact with DCs and promote the production of proinflammatory cytokines, including NK cell IFN-γ that is released 24–48 h after engaging mature IL-12-producing DCs (17). As hypothesized, the NK cell IFN-γ response was markedly enhanced when mmLDL was opsonized (Fig. 4; upper panel). While IL-12 from DCs is pro-inflammatory, IL-10 from DCs is anti-inflammatory and the IL-12/IL-10 ratio is important in determining whether a stimulus is proinflammatory or anti-inflammatory. Anti-PC opsonization did not significantly alter the IL-10 level, but increased the level of IL-12, and that increase in the IL-12/IL-10 ratio correlated with the production of NK cell IFN-γ. The importance of NK cells was confirmed in PBL cultures where the rapid, 24–48 h, response was eliminated by the removal of $CD56^+$ cells (data not shown).

Anti-PC enhanced IL-12 production by LDL-stimulated DCs

A. actinomycetemcomitans has a potent lipopolysaccharide that is capable of stimulating DC IL-12p70 (18). By contrast, *P. gingivalis* is unable to enhance IL-12p35 mRNA and the production of IL-12p70 (17). However, DCs and NK cells are mutually stimulatory, and the addition of IFN-γ, which may be obtained from NK cells, results in the induction of IL-12p35 mRNA and a substantial IL-12p70 response by *P. gingivalis*-stimulated DCs (18,25). The IL-12 stimulatory capacity of mmLDL was comparable with *P. gingivalis* when the production of IL-12p40 and IL-12p35 mRNA was studied using quantitative real-time PCR (Fig. 5). *A. actinomycetemcomitans* served as a positive control and stimulated both IL-12p40 and IL-12p35 mRNA. By contrast, both mmLDL and *P. gingivalis* stimulated modest levels of IL-12p40 mRNA, but no detectible increases in IL-12p35 mRNA. However, both mmLDL and

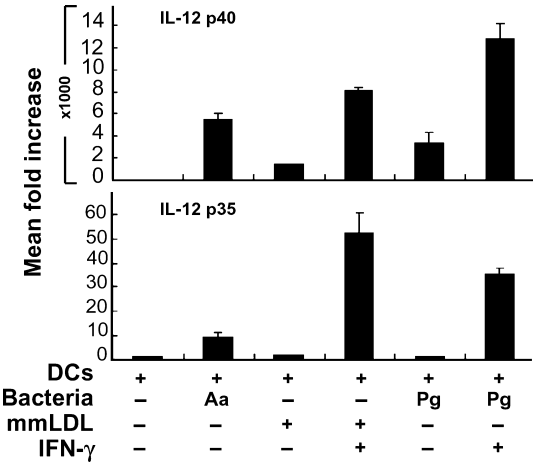

Fig. 5. Both low-density lipoprotein (LDL) and *Porphyromonas gingivalis* induced interferon-γ (IFN-γ)-stimulated monocyte-derived dendritic cells (DCs) to increase the production of interleukin (IL)-12p40 and IL-12p35 mRNA. IL-12p40 is illustrated in the upper panel and IL-12p35 is illustrated in the lower panel. DCs were stimulated with 2 μg/mL of LDL, *P. gingivalis* W83 (Pg) or *A. actinomycetemcomitans* (Aa), at 10^6 organisms, in the presence or absence of 1 ng of IFN-γ. Four hours later, total cellular RNA was extracted with Trizol from 10^6 DCs, IL-12p40 and IL-12p35 mRNA levels were measured using quantitative RT-PCR and the fold differences in mRNA expression levels were calculated using the $\Delta\Delta C_T$ method and expressed as mean fold increase. mmLDL, minimally modified low-density lipoprotein.

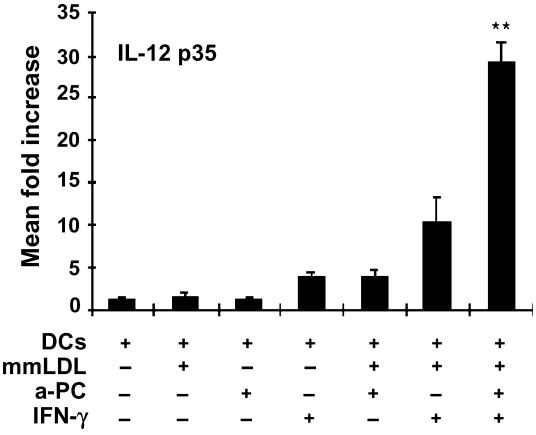

Fig. 6. Effect of anti-phosphorylcholine (anti-PC) and/or interferon-γ (IFN-γ) on low-density lipoprotein (LDL) induced the production of dendritic cell (DC) interleukin (IL)-12p35 mRNA. DCs were stimulated with 2 μg/mL of LDL in the presence or absence of 2 μg/mL of anti-PC and 1 ng/mL of IFN-γ. Three hours later, mRNA from 10^6 DCs was isolated and IL-12p35 mRNA levels were measured using quantitative real-time RT-PCR. The data are typical of three experiments of this type. $^{**}p < 0.01$. mmLDL, minimally modified low-density lipoprotein.

P. gingivalis stimulated substantial IL-12p35 and IL-12p40 mRNA responses upon addition of the NK-cell product IFN-γ (Fig. 5).

We postulated that opsonization would enhance the ability of LDL to stimulate DCs. To test this, mRNA for IL-12p35, which is the limiting component in the generation of bioactive IL-12p70, was studied using quantitative PCR. As illustrated in Fig. 6, the addition of IFN-γ, which may be produced by NK cells, resulted in a detectable enhancement of IL-12p35 mRNA. Similarly, the addition of opsonin with the LDL provoked an increase in IL-12p35 mRNA. However, the combination of IFN-γ plus opsonized mmLDL synergized to produce a substantial increase in IL-12p35 mRNA.

DC IL-12 responses increase with increased levels of anti-PC reactive LDL

We reasoned that the stimulatory activity of the LDL might relate to the level of anti-PC-reactive LDL. As illustrated in Fig. 7, anti-PC opsonization of mmLDL did not result in a detectable level of IL-12p70 in the absence of IFN-γ. This is consistent with the need for opsonization and IFN-γ for mmLDL to stimulate a substantial IL-12p35 response. However, deliberately modified LDL, with about threefold more anti-PC-reactive LDL, induced an IL-12p70 response when opsonized in the absence of IFN-γ. Nevertheless, the addition of IFN-γ still augmented this response, confirming the importance of this NK cell product for optimal DC IL-12 responses.

Discussion

Epidemiological studies indicate that periodontitis patients are at increased risk of atherosclerosis, and studies of periodontal pathogen-infected animals further support the concept that periodontitis may promote the initiation and progression of atherosclerosis (19,20,26–28). However, the mechanisms involved in promoting atherosclerosis by periodontal infections remain elusive. In recent years, the pathogenesis of cardiovascular disease has been linked to inflammation and a favoured hypothesis is that periodontitis represents an exposure to gram-negative pathogens that enhance systemic inflammation (6,29). Elevated levels of inflammatory cytokines might then exacerbate atherogenesis and thrombosis. Other inter-relationships include documentation of antigens and DNA from periodontal pathogens in atherosclerotic plaques, and experimentation with animal models indicates that *P. gingivalis* can invade endothelial cells and induce cytokine

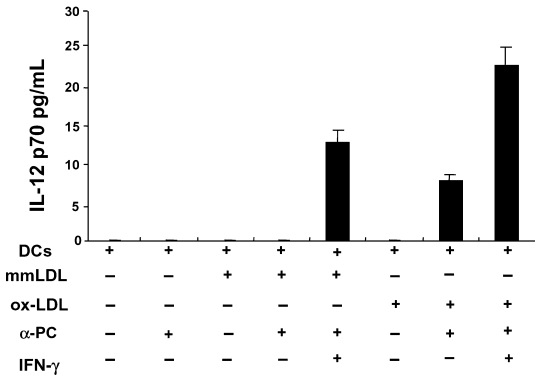

Fig. 7. Effect of low-density lipoprotein (LDL) modification on the production of interleukin (IL)-12p70. Dendritic cells (DCs) from a nonperiodontitis patient were stimulated with 2 μg/mL of LDL or polymorphnuclear leukocytes-modified LDL in the presence or absence of 2 μg/mL of anti-phosphorylcholine (anti-PC) and 1 ng/mL of interferon-γ (IFN-γ). The supernatant fluids were collected at 24 h, and IL-12p70 production was measured using ELISA. mmLDL, minimally modified low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein.

production and atheroma formation (30–32). Data in the present study suggest yet another pathway leading to increased proinflammatory cytokine production in periodontitis patients. Thirty to forty per cent of bacteria in dental plaque samples, including *A. actinomycetemcomitans, Fusobacterium nucleatum, Streptococcus oralis and Streptococcus sanguis*, express antigen recognized by anti-PC (3,4) and patients respond to this antigen as indicated by the local production of anti-PC in gingival tissues and increased levels of anti-PC in serum (3,5). Furthermore, anti-PC opsonizes *A. actinomycetemcomitans* (33) and, in the present study, we found that it opsonized mmLDL. Moreover, mmLDL opsonized by anti-PC stimulated DC maturation as well as promoting the rapid production of important proinflammatory cytokines, including IL-12p70, by the DCs and IFN-γ production from NK cells.

Anti-PC recognizes not only bacterial PC but also many PC containing self-antigens, including oxLDL, platelet-activating factor and apoptotic cells (34). Studies in mice have shown that anti-PC is largely IgM, and IgM remains within the vascular system where it appears to help clear oxLDL and to protect mice against atherosclerosis (35). Furthermore, murine anti-PC can be protective against infections with *S. pneumoniae* (35). Human anti-PC is largely IgG2 and studies of its ability to protect against *S. pneumoniae* have given variable results (35,36). The IgG molecule has the ability to pass through membranes and IgG-ICs may lodge in arterial regions that are subjected to haemodynamic stress as a result of turbulent flow conditions (11,12). In these areas, ICs, including LDL opsonized with anti-PC, would be in a position to interact with DCs in the subendothelial layer. This is consistent with observations indicating that LDL-ICs in the blood are risk factors for coronary artery disease (37,38). In addition, when LDL in developing atheromas is modified as a consequence of processes associated with inflammation, IgG anti-PC in the tissues may help to form LDL-ICs. Human anti-PC appears to promote the uptake of mmLDL, which may lead to foam-cell formation by macrophages and, as suggested by the present data, proinflammatory cytokine production by DCs present in atheromas (10,12,39,40).

It should be appreciated that DCs are located along the subendothelial layer of nondiseased arterial wall where they represent 2–5% of the cells and that DC numbers increase in atheroprone areas (11,12,39,40). Furthermore, analysis of the distribution of DCs in the intima of atheroprone vs. athero-resistant areas of nondiseased aorta revealed DC clusters and possible activation of lymphocytes in the prone tissue (11,12,39). Dendritic cells in the blood and intima of artery walls would be among the first cells to encounter blood-borne ICs, including ICs with oral bacteria or mmLDL-ICs. Some DCs in the lesions are resident in the normal arterial wall, while others probably originate from monocytes that infiltrate the arterial intima during atherogenesis (12). The vast majority of DCs in the atherosclerotic lesions do not accumulate substantial amounts of lipids, but some do and are transformed into foam cells (12). Dendritic cells in sites of chronic inflammation tend to accumulate, rather than migrate, and mature cells expressing CD83 may be found in contact with T cells, which are present in diseased arteries (12). Injury in the vessels may lead to DC maturation by mechanisms including pattern recognition from microbes, local release of chemokines and local release of reactive oxygen species, which may also cause LDL oxidation. Low-density lipoprotein and oxLDL have been shown to upregulate HLA-DR and CD86 (41), and oxLDL is able to engage pattern recognition receptors including Toll-like receptor 4 (TLR4) (42). High concentrations of TLR4 are located inside mDCs (43) and opsonization might promote DC maturation by bringing the mmLDL inside the DCs where it can engage TLR4.

Dendritic cells also have the remarkable ability to stimulate both NK cells of the innate immune system and naïve T cells of the acquired immune system and are uniquely able to initiate immune responses. The production of IL-12p70 by DCs is known to play a critical role in the induction of T helper 1 responses (7–10,17). T helper 1 cells are dominant in atherosclerotic lesions, especially early lesions, as the cytokines IFN-γ, IL-2 and tumor necrosis factor-α are highly expressed, whereas only small amounts of the T helper 2 cytokines IL-4, IL-5 and IL-10 can be detected. Direct evidence for a pro-athrogenic role for IFN-γ was obtained in *Apoe* knockout mice. Mice deficient in the IFN-γ receptor had a significant decrease in athromatous lesions (9). Furthermore, daily administration of IFN-γ promoted atherosclerosis in *Apoe* knock-

out mice (8). Lipoproteins have been shown to induce cytokine secretion by IL-2-primed NK cells (44), and the present data suggest that NK cells may participate in the initiation of atherosclerosis by rapidly producing IFN-γ in response to DC-derived IL-12. Studies of NK cells in atherosclerosis have been complicated by the lack of good animal models with NK cell deficiency and the inability to fully deplete *in vivo* with the antibodies currently available. However, depletion of NK cell function appears to decrease atherosclerosis in LDL receptor null mice (45) and reconstitution of LDL receptor knockout mice with Ly49A transgenic bone marrow suggests that NK cells are pro-atherogenic (46).

In conclusion, our data suggest that mmLDL opsonized with anti-PC promotes the production of proinflammatory cytokines by DCs and NK cells and thus may participate in the pathogenesis of atherosclerosis. Interaction with mmLDL is probably a frequent event that is well regulated. However, elevated levels of anti-PC may exacerbate the process and this could initiate arteritis and lead to chronic inflammation and atheroma formation. In short, opsonization of mmLDL by anti-PC could provide a mechanism to help explain epidemiological data, suggesting that periodontitis patients are more likely to develop atherosclerosis.

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