Phosphorylcholine-Primed Dendritic Cells Aggravate the Development of Atherosclerosis in ApoE−/− Mice

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Background: Atherosclerosis is an inflammatory disease involving activation of adaptive and innate immune responses to antigens, including oxidized low-density lipoprotein (oxLDL) and phosphorylcholine (PC). Dendritic cells (DCs), which are antigen-presenting cells that activate T cells, are present in atherosclerotic lesions and are activated in immune organs. However, the mechanism by which PC promotes atherosclerosis is unclear.

Methods and Results: To evaluate whether PC promotes atherosclerosis via DCs, 2×10^5 DCs activated by PC-keyhole limpet hemocyanin (DCs+PC-KLH) were injected into ApoE−/− mice and the features of the plaques and the effects of the DCs on cellular and humoral immunity against PC-KLH were determined. Mice injected with DCs+PC-KLH had significantly larger atherosclerotic lesions than controls, with increased inflammation in the lesions and plaque instability. Furthermore, DCs+PC-KLH were characterized using flow cytometry after coculture of bone marrow-derived DCs and naïve T cells. DCs+PC-KLH showed an inflammatory phenotype, with increased CD86, CD40, and major histocompatibility complex Class II molecules (MHC-II), which promoted PC-specific T helper (Th) 1 and Th17 cell differentiation in vivo and in vitro. Moreover, 2 weeks after the administration of DCs+PC-KLH to mice, these mice produced PC- and oxLDL-specific IgG2a, compared with no production in the controls.

Conclusions: These findings suggest that DCs presenting PC promote specific immunity to PC, increase lesion inflammation, and accelerate atherosclerosis, which may explain how PC promotes atherosclerosis.

Key Words: Atherosclerosis; Cellular immunity; Dendritic cells; Humoral immunity; Phosphorylcholine

In recent years, considerable research has focused on the immune inflammatory response associated with atherosclerosis and its clinical manifestations. It is now widely recognized that chronic inflammation caused by T cell-mediated pathogenic responses plays an important role in atherogenesis.[1] Recently, the pathogenic function of T helper (Th) 1 cells in atherosclerosis has been convincingly demonstrated in murine systems.[2,3] There is additional evidence supporting an important role for Th17 cells and interleukin (IL)-17 in atherogenesis.[4] In contrast, other studies have demonstrated an atheroprotective effect for naturally occurring CD4+CD25+Foxp3+ regulatory T cells (nTregs).[5] In addition, B1a lymphocytes are atheroprotective by secreting natural IgM, whereas conventional B2 cells

aggravate atherosclerosis by secreting IgG.[6,7] Meanwhile, dendritic cells (DCs), as antigen-presenting cells (APCs), have different roles in the progression of atherosclerosis through their regulation of T and B cell responses.[8]

Recently, oxidized low-density lipoprotein (oxLDL) was identified as a major autoantigen for the immune response associated with atherosclerosis.[1] oxLDL contains a variety of oxidation-specific neoepitopes on both its lipid and protein moieties, including phosphorylcholine (PC) and apolipoprotein (Apo) B100.[9] Hjerpe et al found that adoptive transfer of oxLDL-treated DCs significantly accelerated atherosclerosis in ApoE−/− mice.[10] In contrast, immunization with modified and native low-density lipoprotein (LDL) injected subcutaneously, as well as the injection of

antibodies against ApoB100 peptide sequences, reduced the progression of atherosclerosis.[11,12] These studies demonstrate that autoimmune responses against oxLDL and ApoB100 play an important role in atherosclerosis, and that the depletion of these molecules could protect against plaque development.

PC, another component of oxLDL, is also a component of multiple prokaryotic organisms, including *Streptococcus pneumoniae*. Indeed, Binder et al demonstrated that many autoantibodies to oxLDL share complete genetic and structural identity with antibodies from the classic anti-PC B cell clone T15, which protects against common infectious pathogens, including pneumonia.[13] Furthermore, pneumococcal immunization decreased the extent of atherosclerosis by producing oxLDL and PC antibodies, which indicates molecular mimicry between epitopes of PC and *S. pneumoniae*.[13] Caligiuri et al have verified that PC-targeting immunization directly reduces atherosclerosis by inducing PC-specific IgM.[14] However, how PC activates the immune response and induces an autoimmune reaction in atherosclerosis remains unclear.

To investigate the role of DCs in the PC-induced immune response, we transferred DCs pulsed with PC to ApoE$^{-/-}$ mice. This transfer induced specific humoral and cellular immune responses in vivo and significantly increased lesion development.

Methods

Reagents and Antibodies
The cell culture medium for DCs and splenocytes was RPMI 1640 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco-BRL) and 100 U/mL streptomycin-penicillin. Granulocyte-macrophage colony stimulating factor (GM-CSF; Lot no. 050755-1), IL-4 (Lot no.1106CY49), and IL-2 (Lot no.0608108) were obtained from Peprotech (Rocky Hill, NJ, USA). PC-keyhole limpet hemocyanin (PC-KLH) and keyhole limpet hemocyanin (KLH) were obtained from Biosearch Technologies (Novato, CA, USA). The purified anti-CD4 antibody (Ab; clone RM4-5) was from BD Systems (Franklin, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (Clone N418), phycoery-thrin (PE)-conjugated anti-CD86 (Clone PO3.1), anti-major histocompatibility complex Class II (MHC-II)-APC (Clone HIS19), anti-CD40-PE (Clone 5C3), anti-CD45-FITC (Clone HI30), anti-CD19-APC (Clone HIB19), anti-CD4-FITC (Clone GK1.5), anti-CD25-APC (Clone PC61.5), anti-Foxp3-PE (Clone FJK-165), anti-interferon (IFN)-γ-PE (Clone XMG1.2), anti-IL-17A-PE (Clone eBio17B7), anti-Foxp3-biotin (Clone FJK-16s), and anti-CD11c-biotin (Clone N418) Abs were obtained from eBioscience (San Diego, CA, USA). Anti-CD68 Ab was obtained from Millipore and anti-smooth muscle actin (SMA) Ab (Lot no. BM0002) was obtained from Boster (Wuhan, China). Control rat IgG was obtained from ThermoFisher Scientific (Rockford, IL, USA).

Animals
C57BL/6 mice and ApoE$^{-/-}$ mice from a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained in the Animal Center of Beijing University. The mice were kept in-house in a specific pathogen-free facility (Tongji Medical College, Wuhan, China) and were fed a normal chow diet or a Western-style diet containing 0.15% cholesterol and 21% fat. The mice were 6 weeks old at the start of the experiment. Diet and water were provided ad libitum. All experiments were performed in compliance with the guidelines of the Care and Use of Laboratory Animals (Science and Technology Department of Hubei Province, China, 2005. http://kjt.hubei.gov.cn/kjdt/ztzl/fzxczl/xfyf/dffg/202008/t20200826_2837541.shtml).

### Generation of Bone Marrow-Derived DCs (BM-DCs)
BM-DCs were generated with GM-CSF and IL-4 as described previously.[15] Briefly, bone marrow was isolated from C57BL/6 mice. Bone marrow cells were depleted of red blood cells and were cultured with RPMI 1640 for 6 days in tissue culture plates at 37°C under 5.0% CO_2. The RPMI 1640 culture medium was supplemented with 10% FCS, 100 U/mL penicillin, 100 U/mL streptomycin, 10 ng/mL GM-CSF, and 10 ng/mL IL-4. DCs were purified from the differentiated bone marrow cells using a CD11c magnetic cell-sorting kit (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. After purification, DCs were stimulated with KLH (DCs+KLH) or exposed to 50 μg/mL PC-KLH (DCs+PC-KLH) in RPMI 1640. After 48 h, the DCs and supernatants were collected for further experimentation.

T Cell and BM-DCs Coculture In Vitro
CD4$^+$ T cells were purified from splenocytes of ApoE$^{-/-}$ mice using the Naïve CD4$^+$ T Cell Isolation Kit, mouse (Miltenyi Biotec) according to the manufacturer's instructions. CD4$^+$CD25$^+$ regulatory T (Treg) and CD4$^+$CD25$^-$ T cells were isolated from all CD4$^+$ T cells using a CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Fluorescence-activated cell sorting (FACS) analysis indicated that the purity of each population was >93%. Using 24-well plates, 8×10^5 CD4$^+$CD25$^-$ T cells were cultured either alone or with 2×10^5 DCs+KLH or DCs+PC-KLH in RPMI 1640 in the presence of 2 μg/mL anti-CD3 Ab and 4 ng/mL IL-2 for 72 h. The cells were pulsed with 1 μCi [^{3}H]-thymidine/well during the last 18 h and the radioactivity in the cells was assayed at the end of the culture period. The supernatants were also collected at the end of the 72-h incubation period for cytokine quantification using ELISA (eBioscience).

Splenocyte Proliferation Assay
Spleens from ApoE$^{-/-}$ mice in the no DCs, DCs+KLH, and DCs+PC-KLH groups (n=3 per group) were dissected and passed through a Falcon 70-μm cell strainer. Erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 mol/L NH$_4$Cl, 10 mmol/L NaHCO$_3$, 0.1 mmol/L EDTA, pH 7.3). Splenocytes were cultured for 72 h in triplicate at a density of 2×10^5 cells per well in 96-well round-bottomed plates in the presence or absence of different concentrations of PC-KLH. RPMI 1640 (with L-Glutamine 2 mmoL, 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin; all from BioWhittaker Europe) was used as the culture medium. Cultures were pulsed for an additional 18 h with [^{3}H]-thymidine (1 μCi/well, specific activity 24 Ci/mmol; Amersham Biosciences). The amount of [^{3}H]-thymidine incorporated was measured using a liquid scintillation counter (TRI-CARB 2900R; Perkin-Elmer, Norwalk, CT, USA). At the end of the culture period, supernatants were collected for cytokine quantification using ELISA (eBioscience).
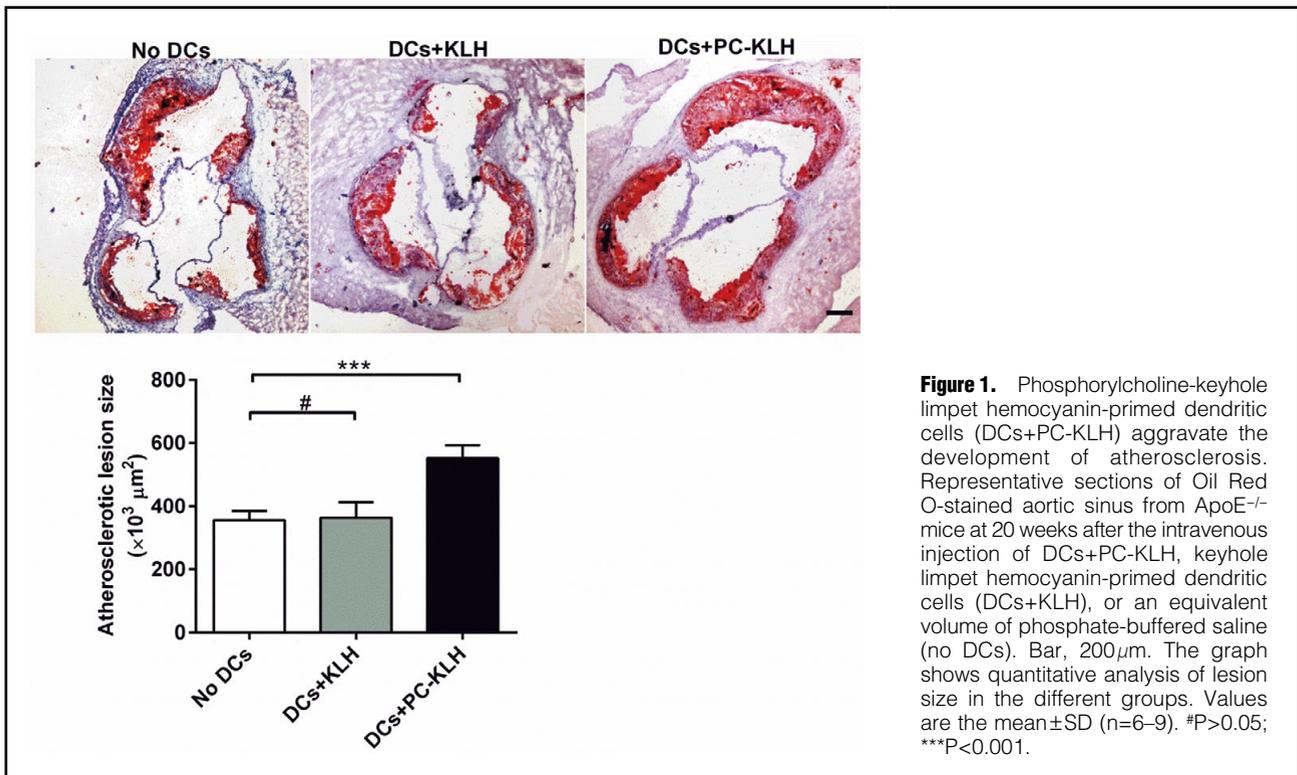
Figure 1. Phosphorylcholine-keyhole limpet hemocyanin-primed dendritic cells (DCs+PC-KLH) aggravate the development of atherosclerosis. Representative sections of Oil Red O-stained aortic sinus from ApoE$^{-/-}$ mice at 20 weeks after the intravenous injection of DCs+PC-KLH, keyhole limpet hemocyanin-primed dendritic cells (DCs+KLH), or an equivalent volume of phosphate-buffered saline (no DCs). Bar, 200μm. The graph shows quantitative analysis of lesion size in the different groups. Values are the mean±SD (n=6–9). #P>0.05; ***P<0.001.

Animal Groups

In the DC transfer experiments, 6-week-old male ApoE$^{-/-}$ mice were injected intravenously with $2×10^5$ DCs+KLH or DCs+PC-KLH (i.e., $2×10^5$ cells/mouse) or an equivalent volume of phosphate-buffered saline (PBS) alone (the no DCs group) once every 3 weeks until week 12. Five days after the first injection of DCs or PBS, mice were administered a Western-style diet for 12 weeks. Each group contained 6–9 mice.

Cytokine and Antibody ELISA

Supernatants of the cultured DCs and splenocytes and no DCs DCs+KLH DCs+PC-KLH after 12 weeks were collected for determination of IL-4, IFN-γ, and IL-17a using a commercially available ELISA (eBioscience) according to the manufacturer's instructions. To quantify oxLDL- or PC-specific antibodies, 96 well microplates were coated with 100μg/mL oxLDL or PC, washed wish wash buffer (0.05% tween20 in PBS, PH 7.2–7.4), and blocked wish Reagent diluent (1%BSA in PBS, PH 7.2–7.4, 0.2μm filtered). Then, plasma harvested from mice was added at an optimized dilution of 1:50 and specific antibodies to detect IgG1, IgG2a, and IgM (BD Biosciences) were added. Optical density (OD) was read at 450nm.

Flow Cytometry

Two weeks after the last administration of DCs, monocytes were isolated from the spleen using Ficoll-Paque Plus and erythrocyte lysis buffer to remove the erythrocytes. For the detection of CD45$^+$CD19$^+$ B cells, the cells were first stained with anti-CD45 Ab and anti-CD19 Ab. For detection of nTregs, cells were first stained with anti-CD4-FITC Ab (R&D Systems, Minneapolis, MN) and anti-CD25-PE Ab (R&D Systems), fixed and permeabilized according to the

manufacturer's instructions (eBioscience) and then stained with anti-Foxp3-APC Ab (eBioscience). For analysis of Th1 (CD4$^+$IFN-γ^+) and Th17 (CD4$^+$IL-17$^+$) cells, monocytes were suspended at a density of $2×10^6$ cells/mL in complete culture medium. Then, 1mL of the cell suspension was transferred to each well of a 24-well plate. Cultures were stimulated with phorbol myristate acetate (20ng/mL) plus ionomycin (1μg/mL; Alexis Biochemicals, San Diego, CA, USA) for 4h in the presence of 2μmol/mL monensin (Alexis Biochemicals) in an incubator at 37°C under 5% CO_2. After 4h culture, the monocytes were collected for staining refer to the R&D instruction manual. Fixation and permeabilization were necessary before staining with IFN-γ or IL-17 Ab. Isotype controls were used to enable correct compensation and to confirm antibody specificity.

To characterize the cultured DCs, purified CD11c cells were stained with CD86, CD40, or MHC-II for 30min. Flow cytometric acquisition was performed using a FACSCalibur (BD Immunocytometry Systems) and all analyses were performed using Flowjo software (Treestar Inc., Ashland, OR, USA).

Body Weight and Lipids

At the end of experiment, the weight of each mouse was recorded. Blood samples were collected, allowed to clot at room temperature, and the plasma was then isolated by centrifugation at 1,200g for 10min at 25°C. Plasma concentrations of total cholesterol, oxLDL, and triglycerides were measured by enzymatic assay and determined using an autoanalyzer (Hitachi 917).

Atherosclerotic Lesion Analysis

Atherosclerosis lesions were quantified in the aortic sinus and descending thoracic aorta, as described previously.[16]

Figure 2. Effects of phosphorylcholine-keyhole limpet hemocyanin-primed dendritic cells (DCs+PC-KLH) on plaque phenotype. Images show representative sections of the aortic sinus stained with antibodies specific for CD4 (CD4+ T lymphocytes), CD68 (macrophages), and α-smooth muscle actin (SMA), and Masson staining for collagen, from ApoE−/− mice at 20 weeks after the intravenous injection of DCs+PC-KLH, keyhole limpet hemocyanin-primed dendritic cells (DCs+KLH), or an equivalent volume of phosphate-buffered saline (no DCs) . Bar, 200 μm. The graphs show quantitative analysis of positively stained cells in each group. Values are the mean±SD (n=5–6). #P>0.05; *P<0.05.

Briefly, the hearts after 12 weeks including the aortic roots were prepared, which were remove from left auricle to right auricle and sections were fixed in 4% formaldehyde, processed, and embedded in oxytetracycline (OTC) compound. Sections (5–7 μm) were cut at 35-μm intervals starting from the 3-valve cusp of the aortic sinus. In addition, the descending thoracic aorta was dissected and fixed, opened longitudinally, and pinned onto black wax plates. All specimens were stained with Oil Red O (ORO) and hematoxylin. Plaque area was measured in each mouse using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

For immunohistochemical analysis, 5- to 7-μm serial cryostat sections of the aortic sinus adjacent to the ORO-stained sections and heart tissue were prepared. Sections were stained using the following molecule-specific antibodies: purified anti-CD68 Ab (1:200) for monocytes and macrophages, purified anti-α-SMA Ab (1:200) for smooth muscle cells (SMCs), and purified anti-CD4 Ab (1:50) for T cells.

For purified Ab, staining was visualized using biotinylated secondary antibodies. For biotinylated Ab, staining was visualized using streptavidinylated secondary antibodies and detected with the Avidin–Biotin Complex/Diamino-benzidine (ABC/DAB) system. Masson trichrome staining was used to detect collagen. Macrophages, SMCs, and collagen were quantified by determining the positively stained area as a percentage of the total plaque for each marker. CD4+ T cells were assessed by counting the number of stained positive cells per square millimeter in the plaque area.

Statistical Analysis

Unless indicated otherwise, results are expressed as the mean±SD. For normally distributed data with equal group variances, comparisons were made between 2 groups using Student's t-test. For data that were not normally distributed or if group variances were unequal, comparisons were made using the Mann-Whitney rank-sum test. One-way analysis

Table. BW and TC, oxLDL, and TG Plasma Concentrations in Mice in the Different Experimental Groups

	BW (g)	TC (mmol/L)	TG (mmol/L)	oxLDL (nmol/mL)
No DCs	29.78±1.61	17.04±1.88	1.60±0.17	1,050±139
DCs+KLH	29.75±1.50	16.65±1.94	1.64±0.14	1,054±148
DCs+PC-KLH	30.18±1.45	17.22±1.63	1.67±0.11	1,070±174

Data are presented as the mean±SD (n=6 in each group). Mice (6 weeks old) were injected intravenously with $2×10^5$ dendritic cells (DCs) cultured with keyhole limpet hemocyanin (DCs+KLH), $2×10^5$ DCs cultured with phosphorylcholine-keyhole limpet hemocyanin (DCs+PC-KLH), or an equivalent volume of phosphate-buffered saline (no DCs). All determinations were made after 12 weeks. BW, body weight; oxLDL, oxidized low-density lipoprotein; TC, total cholesterol; TG, triglyceride.
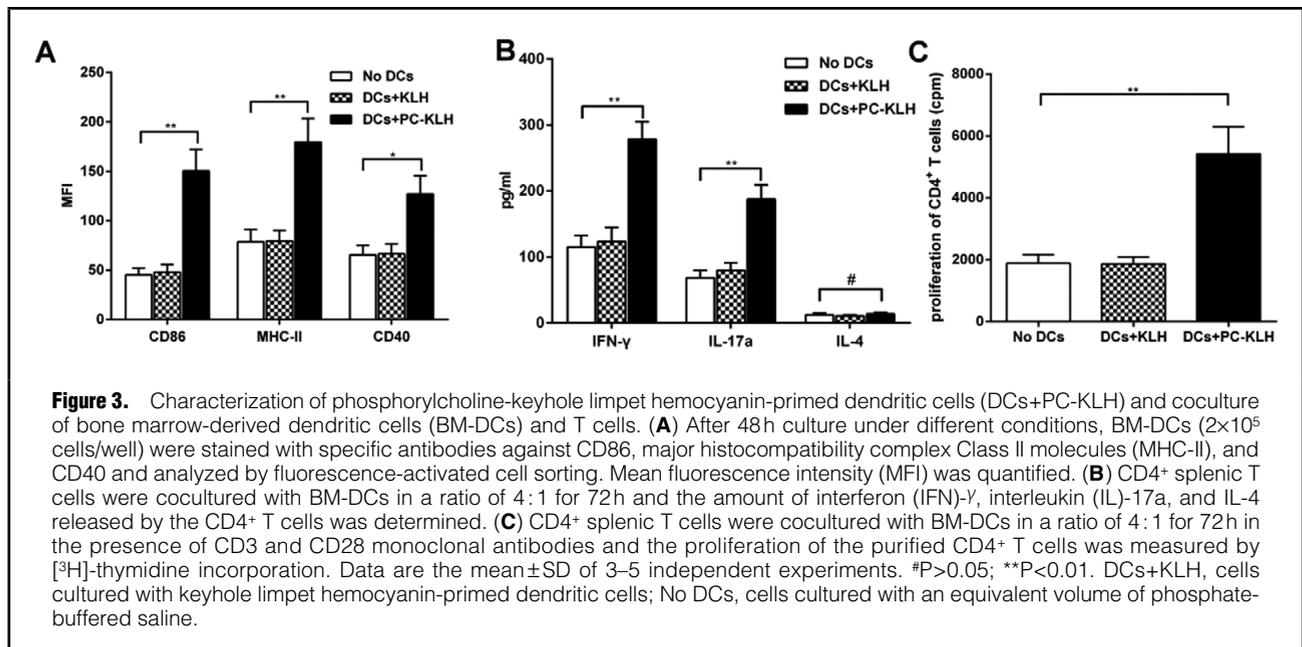
Figure 3. Characterization of phosphorylcholine-keyhole limpet hemocyanin-primed dendritic cells (DCs+PC-KLH) and coculture of bone marrow-derived dendritic cells (BM-DCs) and T cells. (**A**) After 48h culture under different conditions, BM-DCs ($2×10^5$ cells/well) were stained with specific antibodies against CD86, major histocompatibility complex Class II molecules (MHC-II), and CD40 and analyzed by fluorescence-activated cell sorting. Mean fluorescence intensity (MFI) was quantified. (**B**) CD4+ splenic T cells were cocultured with BM-DCs in a ratio of 4:1 for 72h and the amount of interferon (IFN)-γ, interleukin (IL)-17a, and IL-4 released by the CD4+ T cells was determined. (**C**) CD4+ splenic T cells were cocultured with BM-DCs in a ratio of 4:1 for 72h in the presence of CD3 and CD28 monoclonal antibodies and the proliferation of the purified CD4+ T cells was measured by [³H]-thymidine incorporation. Data are the mean±SD of 3–5 independent experiments. #$P > 0.05$; **$P < 0.01$. DCs+KLH, cells cultured with keyhole limpet hemocyanin-primed dendritic cells; No DCs, cells cultured with an equivalent volume of phosphate-buffered saline.

of variance (ANOVA) was used for multiple comparisons between ≥3 groups, followed by the Holm-Sidak test when data were normally distributed and group variances were equal. When data were not normally distributed or if group variances were unequal, the Kruskal-Wallis test followed by the Dunn test was used. All analyses were performed in GraphPad Prism 6.0. Two-sided $P < 0.05$ was considered significant.

Results

### Effects of PC-KLH-DCs on Atherosclerosis in ApoE⁻/⁻ Mice
To evaluate whether the observed promotion of atherosclerosis by PC was associated with DCs, mice were injected intravenously with different types of DCs (DCs+KLH or DCs+PC-KLH) or PBS as a control (no DCs). In mice injected with DCs+PC-KLH, there was a 55.2% increase in atherosclerotic lesion size compared with that in mice in the no DCs group ($5.52±0.42$ vs. $3.55±0.29×10^5 \mu m^2$, respectively; $P < 0.01$; **Figure 1**). In contrast, injection of DCs+KLH did not affect area of the atherosclerotic lesion compared with the no DCs group ($3.64±0.49$ vs. $3.55±0.29×10^5 \mu m^2$, respectively; $P > 0.05$; **Figure 1**).

Injection of PC-KLH-DCs significantly increased CD4+ T cell infiltration into the aortic sinus (**Figure 2**). Simulta-

neously, there was an increased percentage of CD68+ macrophages in the atherosclerotic lesions of mice in the PC-KLH-DCs group compared with the no DCs group ($P < 0.05$; **Figure 2**). Furthermore, there was a significant decrease in α-SMA and collagen after injection of PC-KLH-DCs (**Figure 2**). Interestingly, injection of PC-KLH-DCs did not affect the body weight or total plasma cholesterol, oxLDL, and triglyceride concentrations of ApoE⁻/⁻ mice (**Table**).

Characterization of Cultured DCs
In a previous study we showed that oxLDL promotes the maturation and differentiation of DCs and plays an important role in the progression of atherosclerosis.[15] However, there was no evidence regarding the effect of PC on DCs. In the present study, there was distinct upregulation of mean fluorescence intensity (MFI) for the costimulatory factors CD86, MHC-II, and CD40 in PC-KLH-treated DCs (**Figure 3A**). In order to investigate the effects of PC-KLH-DCs on T cells, T cells and BM-DCs were cocultured in vitro. In these studies, PC-KLH-DCs promoted the secretion of IFN-γ and IL-17a, but not IL-4 (**Figure 3B**), and the proliferation of CD4+ T cells from ApoE⁻/⁻ mice (**Figure 3C**), indicating differentiation of Th1 and Th17 cells.
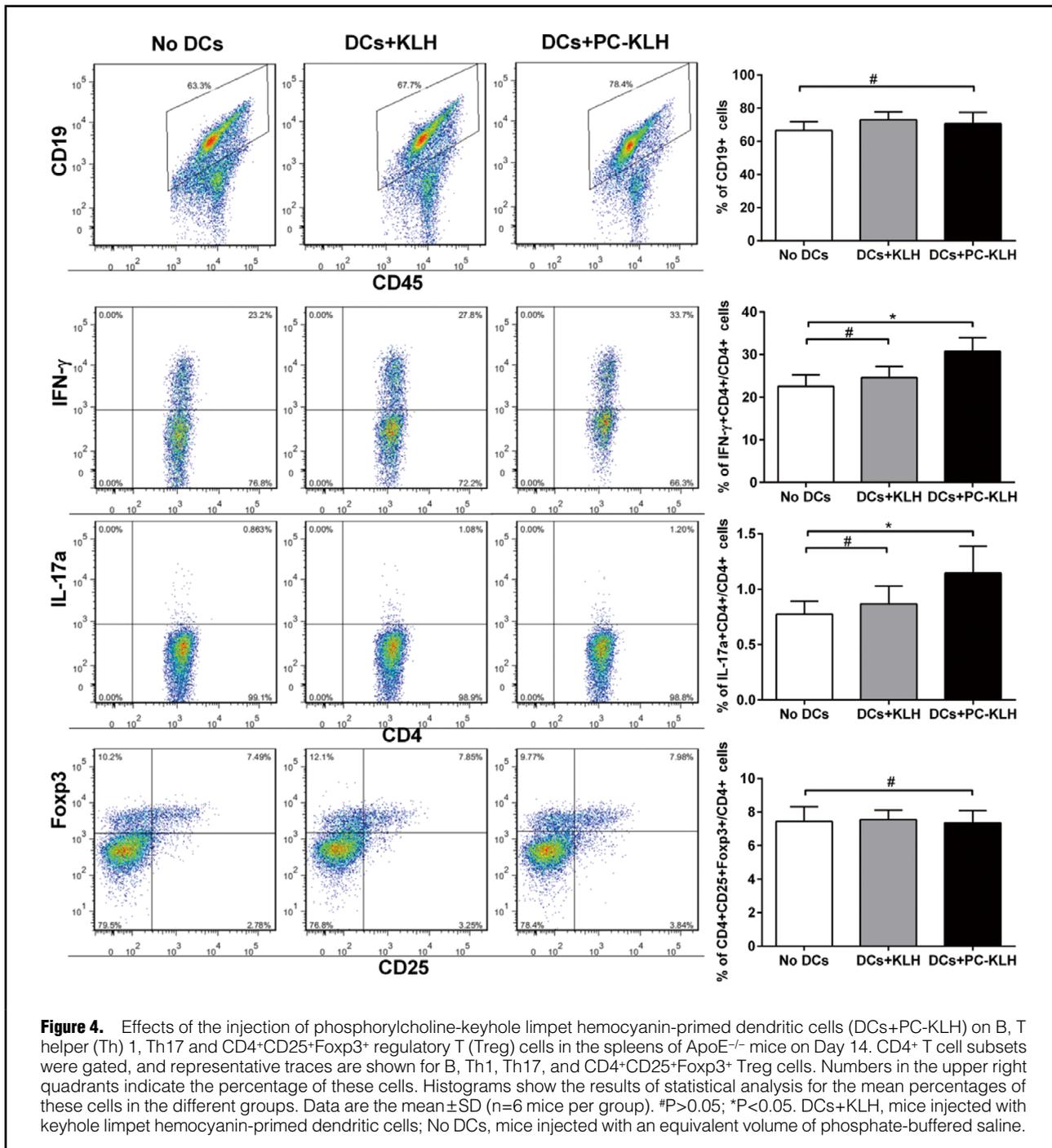
Figure 4. Effects of the injection of phosphorylcholine-keyhole limpet hemocyanin-primed dendritic cells (DCs+PC-KLH) on B, T helper (Th) 1, Th17 and CD4+CD25+Foxp3+ regulatory T (Treg) cells in the spleens of ApoE$^{-/-}$ mice on Day 14. CD4+ T cell subsets were gated, and representative traces are shown for B, Th1, Th17, and CD4+CD25+Foxp3+ Treg cells. Numbers in the upper right quadrants indicate the percentage of these cells. Histograms show the results of statistical analysis for the mean percentages of these cells in the different groups. Data are the mean±SD (n=6 mice per group). #P>0.05; *P<0.05. DCs+KLH, mice injected with keyhole limpet hemocyanin-primed dendritic cells; No DCs, mice injected with an equivalent volume of phosphate-buffered saline.

Effects of PC-KLH-DCs on the Induction of Th1, Th17, nTregs, and B Cells In Vivo

To assess the functional activity of PC-KLH-DCs on splenic immune cells, we examined CD4+ T cell subtypes and B cells in the spleen of mice 2 weeks after the end of the treatment. PC-DCs treatment significantly increased the percentage of Th1 and Th17 cells, but did not affect the total number of B and nTreg cells (**Figure 4**).

Role of Humoral Immunity in the Effects of PC-KLH-DCs on Atherosclerosis

Atherosclerosis is related to the formation of antibodies to oxidatively modified LDL, and the levels of this antibody are associated with the progression of atherosclerosis in mice.[17] The results of all the assays described above involved PC-KLH-DCs modulating cellular immunity, but we also investigated whether there was a humoral immune response to PC-KLH-DCs treatment by quantifying plasma antibody titers. Th1 cells induce IgG2a production, whereas Th2 cells facilitate IgG1 and IgE synthesis. In the present study we assessed the levels of oxLDL- and PC-specific antibodies. More PC-specific IgG2a were produced in PC-DCs-treated mice compared with controls 2 weeks after the last administration of PC-KLH-DCs (**Figure 5A**). Similar results
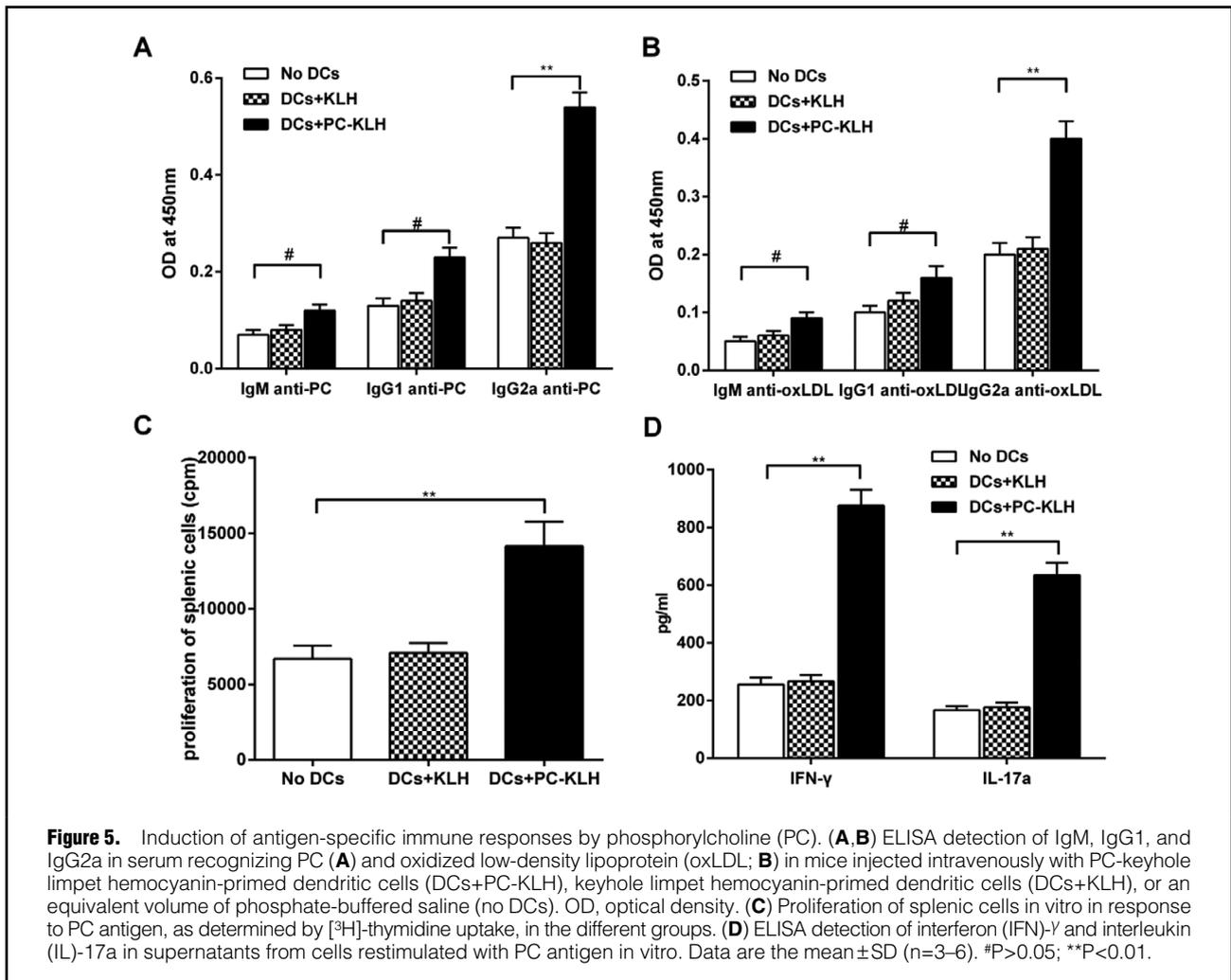
Figure 5. Induction of antigen-specific immune responses by phosphorylcholine (PC). (**A**,**B**) ELISA detection of IgM, IgG1, and IgG2a in serum recognizing PC (**A**) and oxidized low-density lipoprotein (oxLDL; **B**) in mice injected intravenously with PC-keyhole limpet hemocyanin-primed dendritic cells (DCs+PC-KLH), keyhole limpet hemocyanin-primed dendritic cells (DCs+KLH), or an equivalent volume of phosphate-buffered saline (no DCs). OD, optical density. (**C**) Proliferation of splenic cells in vitro in response to PC antigen, as determined by [³H]-thymidine uptake, in the different groups. (**D**) ELISA detection of interferon (IFN)-γ and interleukin (IL)-17a in supernatants from cells restimulated with PC antigen in vitro. Data are the mean ± SD (n=3–6). #P>0.05; **P<0.01.

were found for oxLDL-specific IgG2a (**Figure 5B**).

Antigen-specific T-cell activation was analyzed in vitro when cells from the spleen were restimulated with PC. Cells from mice injected with PC-KLH-DCs showed an approximate 2-fold increase in DNA synthesis (**Figure 5C**), but cells from KLH-DC-treated mice did not respond to PC antigen. In addition, PC induced the expression of IFN-γ and IL-17a in isolated splenocytes in vitro (**Figure 5D**).

Discussion

This report shows that myeloid DCs are important messengers between PC and the atherogenic effect, presenting PC to the immune system of hypercholesterolemic mice. We found that: (1) PC-KLH-DCs, but not KLH-DCs, accelerated atherosclerosis in recipient ApoE$^{-/-}$ mice; (2) atherosclerotic lesions of PC-KLH-DC-treated mice exhibited an unstable plaque phenotype; and (3) PC-KLH loaded onto DCs induced Th1 and Th17 cells, and a Th1-mediated humoral immune response in vitro and in vivo. Together, these data suggest that PC-KLH-DCs induce antigen-specific proatherogenic immunity that augments local and systemic inflammation and the growth of atherosclerotic lesions.

Recent evidence shows that increased DCs in atherosclerotic plaques and activated DCs in lymphoid organs in hypercholesterolemic mice contribute to local inflammation and regulate atherosclerotic development.[18,19] Indeed, Miyashita et al found that PC-KLH may be dose-dependently associated with increased IL-12p40 production from splenic DCs.[20] IL-12p40 is known to differentiate naïve T cells into Th1 cells and to inhibit Th2 immune responses.[21] Toll-like receptor (TLR) 4 and MyD88 (MyD88 is the canonical adaptor for inflammatory signaling pathways downstream of members of the toll-like receptor (TLR) and interleukin-1 (IL-1) receptor families) signaling pathways play an important role in lipopolysaccharide (LPS)-stimulated IL-12 production from DCs.[22] Similar to LPS, the binding of PC to TLR4 has been demonstrated using PC-containing glycoprotein ES-62 (ES-62, which is actively secreted by the rodent filarial nematode Acanthocheilonema viteae. ES-62 has been shown to exert anti-inflammatory actions through its phosphorylcholine (PC)-containing moiety on a variety of cells of the immune system, affecting intracellular signalling pathways associated with antigen receptor- and TLR-dependent response), a secretory product of parasitic nematodes.[23] These results demonstrated that PC can activate DCs to some extent.

In the present study we further investigated the effect of

PC-KLH on DCs and T cell activation. Increased expression of costimulatory factor CD86, MHC-II, and CD40 was found on the surface of PC-KLH-DCs, indicating DC maturation. These changes may contribute to the activation of T cells and the formation of larger atherosclerotic lesions in this study because a CD86 deficiency in atherosclerotic mice has been reported to result in reduced early and, to some extent, advanced atherosclerotic lesion formation.[24] Next, we found that PC-KLH-DCs induced the proliferation of T cells and Th1 and Th17 cell differentiation, th1 and th17 produce IFN-γ and IL-17. Thus, we infer that the increase in costimulatory factors on DCs induced by PC in this study led to Th17 polarization and IL-17a production. These results demonstrate that PC-KLH-DCs are inflammatory DCs. Previous research has convincingly demonstrated the pathogenic function of IFN-γ in atherosclerosis in murine systems, as well as a 60% reduction in lesion lipid accumulation, a decrease in lesion cellularity, and a marked increase in lesion collagen content in IFN-γ receptor-knockout mice.[2] Additional evidence supports an important role for Th17 cells and IL-17 in atherogenesis, in addition to promoting monocyte and macrophage recruitment into the aortic wall.[4] Therefore, the increases in IL-17a and IFN-γ may explain, in part, the aggravated and unstable plaques after the injection of PC-KLH-DCs into ApoE$^{-/-}$ mice.

T and B cells interact with each other: B cells are considered to have an important role in immunity by producing Abs with the help of Th cells, and antigen presentation to Th cells by conventional B cells can conceivably activate these T cells. Indeed, IgM-producing B1a lymphocytes are atheroprotective and conventional B2 cells aggravate atherosclerosis.[6,7] Although the relationship among T and B cells was not completely illuminated, PC-specific IgG2a and oxLDL-specific IgG2a but not IgM were found to be increased after PC-KLH-DCs treatment. These results indicate activation of B2 cells by PC-KLH-DCs. In contrast, Caligiuri et al found that PC immunization via intraperitoneal injection (IP) drives specific T15-like antibodies, PC-specific IgM, that block oxLDL uptake by macrophages, reduce foam cell formation in vitro, and are atheroprotective in vivo.[14] Together, this evidence demonstrates that different ways of immunization with PC activate different types of B cells, which determine a protective or pathogenic role in atherosclerosis.

Indeed, studies of autoantigen immunity in atherosclerosis have yielded complex results due to different forms of antigen presentation leading to qualitatively different immune responses. In general, subcutaneous injections of antigen with alum as the adjuvant or alum alone could protect against atherosclerosis.[25,26] In addition, intraoral or intranasal immunization with oxLDL, heat shock protein (HSP) 65, HSP60, or ApoB100 induces antigen-specific regulatory T cells and reduces atherosclerosis.[27–29] It may be speculated that mucosal treatment with atherosclerosis-associated antigen stimulates tolerogenic APCs, but not inflammatory APCs, and then antigen-specific CD4$^+$ T cells are activated within the plaque or immune organs by the development of adaptive immune cells that secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor-β1, which protect against plaque formation and promote plaque stability. These studies indicate that mucosal tolerance may be an important method for treating atherosclerosis. Similarly, the transfer of DCs loaded with oxLDL may either induce or protect against atherosclerosis

depending on the phenotype of the DCs transferred.[10,30] Treatment with immature IL-10-loaded DCs or thymic stromal lymphopoietin (TSLP) cultured DCs led to a protective effect against atherosclerosis[15,31] that was associated with the development of atheroprotective CD4$^+$CD25$^+$ Treg cells.[5] In contrast, in the present study, the administration of matured DC loaded with PC-KLH did not induce Treg cells, but promoted Th1 and Th17 cell differentiation. This may explain the opposite effects on atherogenesis between the 2 DC states.

Conclusions

In summary, PC-KLH-DCs aggravated the development of atherosclerotic lesions by promoting PC-specific Th1 and Th17 cell differentiation, and PC- and oxLDL-specific IgG2a production. Thus, the present findings explain the possible mechanism underlying the promotion of atherosclerosis by PC.

Disclosures

None of the authors have any conflicts of interest to declare.

IRB Information

This study was granted an exemption from requiring ethics approval by Huazhong University of Science and Technology because it did not involve human subjects.

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