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A combination of secondhand cigarette smoke and *Chlamydia pneumoniae* accelerates atherosclerosis

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ABSTRACT

Objective: Secondhand smoke (SS) induces chronic infection of endothelial cells by *Chlamydia pneumoniae* (Cpn) *in vitro*. We investigated the *in vivo* effect on atherosclerosis following exposure to SS and infection with Cpn both independently and in combination in ApoE−/− mice.
Methods and results: Plaques were largest in the combined SS + Cpn-exposed mice with 12–57% greater cross-sectional area compared with all other groups ($P < 0.03$). Quantitative RT-PCR (qRT-PCR) from aortic roots revealed a synergistic upregulation of both OX40L (CD134L) and MyD88 in SS + Cpn mice ($P < 0.05$). This upregulation occurred despite decreased numbers of macrophage, dendritic cell, CD4 T cell and smooth-muscle-cell infiltrates as determined by quantitative IHC and qRT-PCR. To elucidate whether enhanced apoptosis correlated with reduced plaque cellularity, area of Tdt-mediated dUTP nick labeling positive (TUNEL+) cells and expression of key bridging molecules necessary for efferocytosis (Mertk, Tgm2, FasL and C1qa) were examined. In SS + Cpn mice, there was an increase of the area of TUNEL+ cells in plaque cores ($P < 0.001$) and a downregulation of efferocytosis gene expression ($P < 0.05$). Systemic expression of cytokines in sera (Luminex) showed no differences between groups, suggesting that focal disease mechanisms within the plaque predominated.
Conclusions: The combination of SS exposure and Cpn infection enhanced atherosclerosis more than either variable did independently by activating inflammatory cells and by promoting growth and maturation of lesions *via* defective phagocytic clearance and accumulation of apoptotic cells.

1. Introduction

Secondhand smoke (SS), the most common indoor pollutant, is a recognized risk factor for atherosclerosis [1], while the contribution of *Chlamydia pneumoniae* (Cpn) to cardiovascular disease is still debated. However, Cpn is commonly detected in plaque but not in undamaged vessels [2]. The prevalence of SS exposure and the carriage of persistent Cpn are high in the general population, thereby increasing the likelihood that exposure to both may occur. The interaction of SS exposure and Cpn infection has been examined only *in vitro* [3], where SS induced chronic Cpn infection [3]. We hypothesized that the exposure to SS and infection with Cpn in combination would promote atherosclerosis. We investigated this possibility *in vivo* using an atherosclerosis-prone knockout mouse.

Innate immune and inflammatory signaling are key mechanisms in atherosclerosis progression. Data show that Cpn infection accelerates atherosclerosis in a myeloid differentiation marker 88 (MyD88)-dependent manner in ApoE−/− mice [4], that cigarette smoke induces MyD88 signaling-dependent pulmonary inflammation [5] and that nucleotide-binding oligomerization domain (NOD) protein expression is essential for the clearance of Cpn in the lung [6]. Furthermore, antigen-presenting cells (APCs) interact and potentiate atherosclerosis *via* T cell-stimulated pathogenic responses [7]. OX40L (on APC) and its receptor OX40 (on activated T cells) participate in a costimulatory signaling pathway [8] that has been strongly implicated in the onset and progression of atherosclerosis [9].

Apart from altered immune and inflammatory signaling, the accumulation of lipid-laden macrophages and apoptosis of these

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cells represent another factor in the progression of plaque. In early lesions, the effective clearance of apoptotic cells limits lesion maturation and suppresses necrosis. As lesions progress, phagocytic clearance (efferocytosis) becomes defective [10]. Efficient clearance requires the binding of apoptotic cells to phagocytes through key bridging molecules, such as Mertk [11], C1q, Transglutaminase 2 (Tgm2) and Fas ligand (FasL) [12]. Mice lacking these molecules [13–16] exhibit an accumulation of apoptotic cells, formation of necrotic plaques and more advanced atherosclerosis. This phenomenon also contributes to a persistent inflammatory state and is a hallmark of disease progression [12].

We report that atherosclerosis is exacerbated in mice exposed to the combination of secondhand smoke and *C. pneumoniae* infection and we elaborate upon inflammatory, immune and phagocytic mechanisms driving the disease progression.

2. Materials and methods

2.1. Mice, diet, infection, SS exposure

UCD Laboratory Animal Services approved the protocol. Six-week old female ApoE−/− mice (Jackson Laboratories) were housed in a P2 barrier facility. They were fed regular chow (4.5% fat) and water *ad libitum*. The Cpn CM-1 strain (ATCC), free of mycoplasma, was used for infection. As a control, Cpn was heat-inactivated (mock) at 65 °C for 30 min. Under light isoflurane anesthesia, the mice were inoculated weekly *via* the nares with 4×10^7 Cpn or mock for 3 inoculations (Fig. 1 A).

The mice were exposed to smoke for 8 weeks (Fig. 1A). The smoke was 85–90% sidestream smoke. The remainder was exhaled mainstream smoke. This surrogate for secondhand smoke [17] was generated by burning Kentucky 3R4F reference cigarettes in a smoking machine with standardized 35 ml puffs for 2 s durations once every minute for a total of 8 puffs per cigarette. Exposure was performed 6 h /day and 5 days/week for 8 weeks. Additional details are shown in online supplement. The mice were euthanized, as indicated (Fig. 1A).

2.2. Experimental design

A total of 52 mice received smoke; 26 of them received either mock (SS + Mock) or live Cpn infection (SS + Cpn). In each group, the mice were randomly assigned: 15 to assess lesion parameters *via* immunostaining and TUNEL staining, 8 to obtain mRNA for quantitative RT-PCR (qRT-PCR) analysis and 3 for DNA extraction for Cpn PCR. In parallel, 52 mice that received filtered room air (RA) were similarly analyzed and were assigned as RA + Mock or RA + Cpn. An additional 24 mice that were exposed to RA were divided such that half received mock treatment (RA + Mock) and half received live Cpn infection (RA + Cpn); 3 RA + Mock mice and 3 RA + Cpn mice were used for DNA extraction for Cpn PCR at 10, 12, 14 and 16 weeks of age. Sera from all 128 mice were collected and used for serology, lipid analysis and/or cytokine/chemokine detection.

2.3. Area of plaque

Five-micron cryosections that spanned the sinus were collected from OCT-embedded tissue beginning with the first visualization of the aortic valves and proceeded serially in a caudal to cranial direction, yielding 40 sections on average. Beginning most caudally, ten sections (every other in the series) were examined, spanning half of the sinus. Neutral fats within plaque stained with Oil Red-O (ORO) (American Master Tech) were imaged (Olympus BX61 microscope, 2× Planapo lens, 2× optivar). The cross-sectional area of plaque, which was reported in μm^2, was measured (NIH Image J).

2.4. Quantitative RT-PCR (qRT-PCR)

RNA was extracted from aortic roots (Qiagen Fiber tissue RNeasy; DNA digestion, RNA shredder), reverse transcribed and amplified in duplicate using TaqMan® analysis and low-density array (Lucy Whittier Molecular Diagnostic Core Facility, UC Davis). The assays (Table 1) were obtained from Applied Biosystems or designed and verified by the Core Facility. The samples were amplified by using standard amplification conditions for ABI (ABI PRISM 7900 HTA FAST). The most stable housekeeping gene, β-actin (standard deviation 0.6 and average Ct value 18.54) was used to normalize the Ct values of the target genes. The mRNA copy numbers were determined according to previous methods [18].

2.5. Detection of apoptotic cells

The Tdt-mediated dUTP nick labeling (TUNEL) assay was performed (Roche; IHC Core Facility Gladstone Institute, UC San Francisco). Apoptosis was quantified by circumscribing coalesced TUNEL+ cells that lay within plaque and measuring the cross-sectional area using NIH image J software.

2.6. Immunohistochemistry (IHC)

For qualitative assessment of OX40L protein, immunohistochemical staining of cryosections of the aortic root were examined (Fig. 2). The sections were incubated with rat IgG2a anti-mouse F4/80, (eBioScience), IgG2a DC marker MIDC-8 (Serotec), IgG2b OX40L (CD134L, BioLegend) or a rat anti-mouse IgG2a isotype control antibody (BioLegend). For quantitative IHC, cryosections (5 μm) of the aortic root were stained with rat anti-murine CD4 (BD Pharmingen™), F4/80 (BioLegend), dendritic cells (MCA978, Serotec) and smooth muscle cell α-actin (Sigma), as previously described [19,20]. The quantification of F4/80-, DCs- and SMC-positive staining was performed blindly using IMAGEPROPLUS software (Media Cybernetics) or Image J software (NIH, USA) image analysis and expressed as the percentage of intimal area in order to normalize for differences related to wall remodeling between the study groups. The quantification of $CD4^+$ T cells was performed by counting individual positively stained lesional cells but excluding foam cell-like positively stained cells. Additional details are shown in online supplement.

2.7. Systemic immune response

The systemic immune responses were examined by Luminex MILLIPLEX MAP Mouse Cytokine/Chemokine Panel using sera from fasting animals according to the manufacturer's instructions (Millipore Mpxmcyto-70k pmx22). Controls and standards were analyzed with the samples. The means from two runs using 19–20 mice per group were examined by Luminex 100™ IS V. 1.7.

2.8. Lipid analyses

The UC Davis Veterinary Hospital Laboratory performed cholesterol and triglyceride analyses on overnight fasting plasma from 14 to 16 animals/group (Cobas C501 System, Roche Diagnostic) using enzymatic colorimetric methods.

2.9. Detection of Cpn antibody and DNA

Cpn antibody titers were detected using a kit (*Cpn* MIF IgG, Ref IF1250G, Focus Technology) that provides for detection of mouse