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Genital *Chlamydia* infection in hyperlipidemic mouse models exacerbates atherosclerosis

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HIGHLIGHTS

- Genital *Chlamydia* infection exacerbated atherosclerosis in two independent hyperlipidemic mouse models of atherosclerosis.
- *Chlamydia* infection increased pro-inflammatory cytokines, chemokines, and VCAM-1 expression.
- Interestingly, *Chlamydia* infection showed uterine pathology only in apoE-deficient mice.

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ABSTRACT

Background and aims: Atherosclerosis is a chronic inflammatory disease, and recent studies have shown that infection at remote sites can contribute to the progression of atherosclerosis in hyperlipidemic mouse models. In this report, we tested the hypothesis that genital *Chlamydia* infection could accelerate the onset and progression of atherosclerosis.

Methods: Apolipoprotein E (*Apoe*$^{-/-}$) and LDL receptor knockout (*Ldlr*$^{-/-}$) mice on a high-fat diet were infected intra-vaginally with *Chlamydia muridarum.* Atherosclerotic lesions on the aortic sinuses and in the descending aorta were assessed at 8-weeks post-infection. Systemic, macrophage, and vascular site inflammatory responses were assessed and quantified.

Results: Compared to the uninfected groups, infected *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice developed significantly more atherosclerotic lesions in the aortic sinus and in the descending aorta. Increased lesions were associated with higher circulating levels of serum amyloid A-1, IL-1β, TNF-α, and increased VCAM-1 expression in the aortic sinus, suggesting an association with inflammatory responses observed during *C. muridarum* infection. Genital infection courses were similar in *Apoe*$^{-/-}$, *Ldlr*$^{-/-}$, and wild type mice. Further, *Apoe*$^{-/-}$ mice developed severe uterine pathology with increased dilatations. Apoe-deficiency also augmented cytokine/chemokine response in *C. muridarum* infected macrophages, suggesting that the difference in macrophage response could have contributed to the genital pathology in *Apoe*$^{-/-}$ mice.

Conclusions: Overall, these studies demonstrate that genital *Chlamydia* infection exacerbates atherosclerotic lesions in hyperlipidemic mouse and suggest a novel role for Apoe in full recovery of uterine anatomy after chlamydial infection.

1. Introduction

Atherosclerosis is a chronic inflammatory disease initiated by endothelial dysfunction, macrophage activation, and lipid accumulation in the vasculature [1]. Several studies provide evidence that infectious agents may accelerate atherosclerotic processes. For example,

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Porphyromonas gingivalis, Helicobacter pylori, HIV, and *Chlamydia pneumoniae* infection have been linked with atherosclerosis in humans [2–4]. Respiratory *C. pneumoniae* infection and *Chlamydia* heat shock protein have been associated with atherosclerosis in multiple human serologic studies, providing some of the first evidence of an association between infection and atherosclerosis [5–7]. *C. pneumoniae* has been detected in human atherosclerotic arteries in multiple studies [5–8]. Mouse models of *Chlamydia* lung infection support the clinical observations from *C. pneumoniae* association with atherosclerosis. *C. pneumoniae* respiratory infection accelerates hyperlipidemia-induced atherosclerotic lesion development in C57BL/6J (wild type, WT) [9,10], apolipoprotein-deficient ($Apoe^{-/-}$) [11], and low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mice [12,13].

Genital *Chlamydia trachomatis* infection is the most prevalent sexually transmitted bacterial infection and is a common cause of pelvic inflammatory disease (PID) [14]. *Chlamydia* infection followed by inflammation of the uterine lining (termed endometritis) and/or fallopian tubes (termed salpingitis), can lead to infertility in some women [15]. A link between genital chlamydial infection and atherosclerosis merits investigation because of the high prevalence of *Chlamydia* infection in youth, high prevalence of cardiovascular disease in adulthood, and the socioeconomic association of both infection and cardiovascular disease [16,17]. However, there have been no studies investigating whether or not genital *Chlamydia trachomatis* infection primes the host for accelerated atherosclerosis. We hypothesize that the inflammatory response to genital *C. trachomatis* infection could enhance the progression of atherosclerosis. This hypothesis was tested using the mouse model for genital *Chlamydia* infection, which has served as a powerful tool in the identification of key innate and adaptive inflammatory pathways [18,19]. Using two independent hyperlipidemic mouse models $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice, we show that *C. muridarum* genital infection exacerbated atherosclerotic lesions. Interestingly, $Apoe^{-/-}$ mice uteri also developed severe dilatation post infection, suggesting a Apoe-deficiency promotes uterine pathology after chlamydial infection.

2. Materials and methods

2.1. Chlamydial stocks, mice, and diet

Chlamydia muridarum, "Nigg" strain (UAMS, Little Rock, AR) was propagated as described in an earlier study [18]. Wild type mice (WT, C57BL/6J 000664), mice deficient in Apoe ($Apoe^{-/-}$, 002052) or Ldlr ($Ldlr^{-/-}$, 002207) in C57BL/6J background were purchased from the Jackson Laboratory. Institutional Animal Care and Use Committees at the University of Arkansas for Medical Sciences, University of Pittsburgh, and UNC at Chapel Hill approved all animal experiments. $Apoe^{-/-}$ mice were fed a high-fat diet (TD88137, 42% fat and 0.5% cholesterol), and $Ldlr^{-/-}$ mice were fed a high-fat (15.8% fat)/high-cholesterol (1.25%) diet (TD94059) [20,21].

2.2. Genital infections of mice

Seven days prior to infection, all mice (8-wks) received 2.5 mg of Depo-Provera subcutaneously in 100 µl of PBS. A week later, one set of mice were anesthetized with nembutal (240–250 µl of 5 mg/ml stock) and infected by administering 3×10^5 infectious forming units (IFU) of *C. muridarum* in 10 µl SPG buffer (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamate) into the vaginal vault. Uninfected mice did not receive SPG-buffer into the vaginal vault, since previous studies from our laboratory have observed no inflammation or no uterine pathology from SPG buffer. Beginning one week later, uninfected and infected mice were fed a high-fat ($Apoe^{-/-}$) or a high-fat/high cholesterol diet ($Ldlr^{-/-}$) for 7 weeks (Fig. 1A). Mice were euthanized at 8 weeks (56-days) post-infection. Chlamydial shedding was determined by swabbing the cervix and vaginal vault of infected mice with a calcium alginate swab (Spectrum Medical Instruments, Los Angeles, CA) at various times post-infection, and IFUs in the swabs were determined as described in an earlier study [19].

2.3. Genital histopathology

Genital tract tissues were extracted *en bloc* from mice and tissues were fixed in 10% buffered formalin and embedded in paraffin. Longitudinal sections (4 µm) were stained with hematoxylin and eosin (H&E) and evaluated by a pathologist to whom experimental design was not disclosed. Scoring was done as described in an earlier study [19]. Luminal distention of the uterine horns and dilatation of the oviducts was graded from 1 to 4, with a grade 4 representing peak severity or frequency of the parameter.

2.4. Atherosclerotic lesions

The heart and descending aorta were excised and fixed in PBS/4% formalin/30% sucrose overnight before mounted in optimal cutting temperature medium and frozen at −70 °C. Aortic sinus cryosections (8 µm) were stained with Oil Red O [20]. Atherosclerotic lesions were quantified as previously described [20], by determining the lesion area in each of the five sections for each mouse. *En face* analysis of the descending aorta was performed after staining descending aorta with Oil Red O [20].

2.5. Plasma lipids, SAA-1, and cytokines

Concentrations of plasma total cholesterol was determined using kits from Biovision (Milpitas, CA) as previously described [20]. Plasma TNF-α and serum amyloid A-1 (SAA-1) levels were determined by sandwich ELISA using kits specific for mouse TNF-α (RND Systems) and SAA-1 (Invitrogen). A HEK-IL-1 receptor reporter cell line (HEK-IL-1Ra, Invivogen) was used to estimate the functionally active mature form of mouse IL-1β in the plasma. Plasma samples from uninfected and infected $Apoe^{-/-}$ *and* $Ldlr^{-/-}$ mice were used in this bioassay along with recombinant mouse IL-1β as a standard. A HEK-IL-1Ra cell line expressing IL-1Ra linked to secretory alkaline phosphatase (SEAP) as a reporter was treated for 16 h with plasma from chlamydia infected $Apoe^{-/-}$ *and* $Ldlr^{-/-}$ mice. SEAP in the supernatant collected from the HEK-IL-1Ra cell line was quantified using Quantiblue substrate (Invivogen).

2.6. Immunohistochemical analysis

Serial aortic sinus cryosections (8 µm) were stained with goat anti-mouse vascular cell adhesion molecule-1 (VCAM-1) IgG (10 µg/ml, RND systems) using Vectastain ABC reagent (Vector Laboratories Inc.). The sections were developed with DAB (3,3′-diaminobenzidine) and counterstained with Mayer's hematoxylin. Images were captured using an Olympus microscope. Sections stained with goat IgG were used as a non-specific IgG control. The percentage of VCAM-1 + staining area was determined by measuring the total aortic sinus area. Macrophages at the lesion site were characterized by staining cryosections with the MOMA-2 antibody [22].

2.7. Chlamydia at the lesion site

DNA was prepared from the aorta, including the aortic arches, of Chlamydia-infected $Apoe^{-/-}$ mice. Detection of chlamydial DNA was determined by quantitative PCR using *C. muridarum*-specific primers for gene coding for 16s rRNA as previously described [23]. Purified chlamydial DNA was used as a positive control.

2.8. Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were generated from

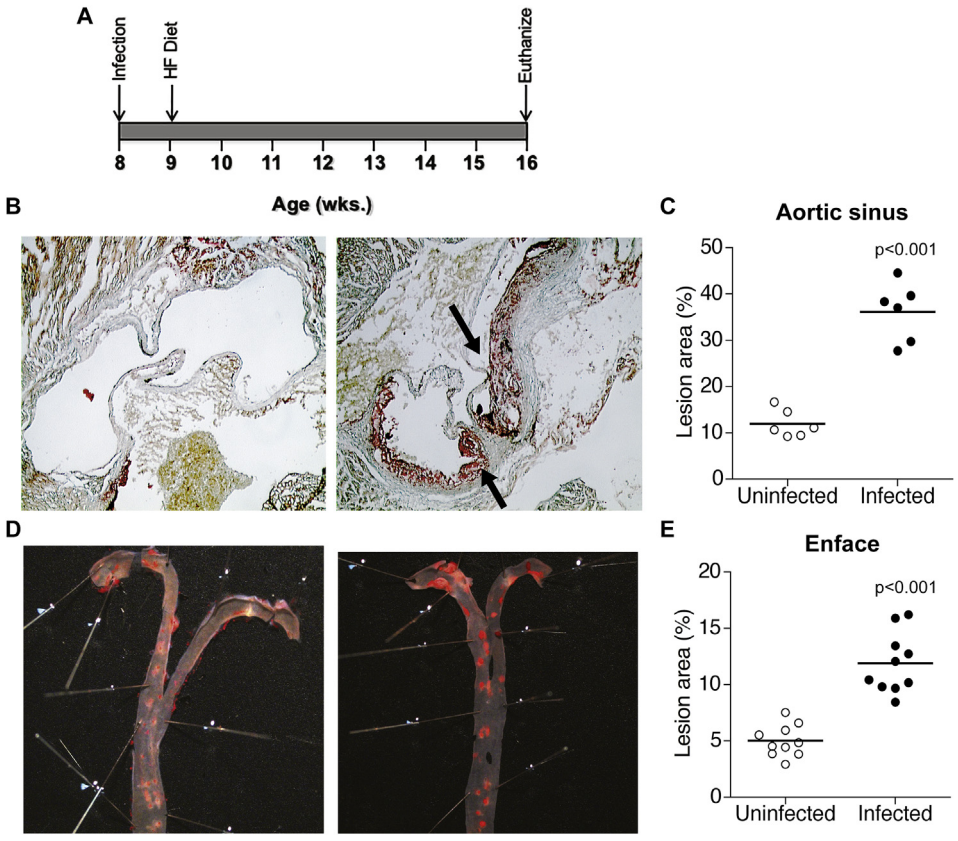

Fig. 1. Aortic lesions were increased in *Apoe*$^{-/-}$ mice with intra vaginal infection of *C. muridarum*. *Apoe*$^{-/-}$ mice (N = 10) were infected with 3 × 10^5 IFU vaginally and were fed high fat diet starting one week after infection.
Mice were sacrificed 8-weeks post infection (A). Aortic sinus (N = 6) and descending aorta (N = 10) were stained using oil red O to visualize plaques and average lesion area plotted. Aortic sinus from a representative mouse from each group is shown and (B) lesion area in aortic sinus (C) for all mice determined. Descending aorta from a representative mouse from each group is shown (D) and lesion area in descending aorta (E) for all mice determined. Significance determined by 't' test. Figure represents one of two independent experiments.

WT, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, as previously described [20]. After 6–8 days in culture, BMDM (0.8 × 10^5/well) were infected with *C. muridarum* (1 multiplicity of infection). Total RNA was isolated from uninfected and infected BMDM, and expression of cytokines and chemokines were determined by quantitative RT-PCR (qRT-PCR), using primers described in earlier studies [20,24,25], and in Supplemental Table 1. To confirm infection by observation of chlamydial inclusions, BMDM (0.8 × 10^5/well) were seeded onto coverslips, infected in parallel, and fixed with methanol for 10 min at room temperature at 24-h post infection. Coverslips were stained for chlamydial inclusion using mouse anti-chlamydial immune sera, followed by anti-mouse alexaFluor-488 secondary antibody (Invitrogen) and mounted with ProLong Antifade (Invitrogen) [19].

2.9. Statistical analyses

Columns and error bars represent mean ± SD. Differences between two groups were considered significant when $p < 0.05$ using the two-tailed Student *t*-test. Infection course was analyzed by 2-way ANOVA and significance of uterine pathological scores was determined using the Mann-Whitney *U* test. All data were analyzed using InStat version 3.1a for Macintosh (GraphPad, San Diego, CA).

3. Results

3.1. C. muridarum genital infection results in increased aortic lesions in both Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice

Apoe$^{-/-}$ and *Ldlr*$^{-/-}$ mice, fed a hyperlipidemic diet, were used to determine whether genital chlamydial infection accelerates the progression of atherosclerosis. Infection course was assessed by chlamydial IFU measurements in genital swabs from *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice and found to be comparable to WT mice (Supplemental Figs. 1A and B). Both knockout mouse strains cleared infection by day 25 and IFUs were not detected in the swabs, thereafter.

To determine whether atherosclerosis was exacerbated in mice infected with *C. muridarum*, atherosclerotic lesions were compared between infected and uninfected mice at 8 weeks post-infection that were given hyperlipidemic diet starting a week after infection (Fig. 1A). Larger lesion areas were observed in the aortic sinuses of infected *Apoe*$^{-/-}$ mice (Fig. 1B), which was significantly different between uninfected and infected mice (Fig. 1C). *En face* analysis of the descending aorta, including aortic arch, also showed significantly increased lesions in mice that were infected with *C. muridarum* (Fig. 1D and E). *Ldlr*$^{-/-}$ mice were used as another model to independently test and confirm the finding from *Apoe*$^{-/-}$ mice. The aortic sinus atherosclerotic lesion area in infected *Ldlr*$^{-/-}$ mice was significantly larger (50% increase) than in the uninfected *Ldlr*$^{-/-}$ mice (Fig. 2AB). *En face* analysis showed a similar increased in descending aorta of infected mice (Fig. 2C). At this early time point (7 weeks on a high fat diet), Chlamydia-infected wild type mice (fed a high fat diet) did not show detectable atherosclerotic lesions (data not shown). These observations demonstrate that genital chlamydial infection exacerbates atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, two well-characterized hyperlipidemic mouse models.

3.2. Chlamydia infection induces systemic and arterial inflammation

Cholesterol levels were similar in uninfected and infected *Apoe*$^{-/-}$ mice, and similar results were observed in *Ldlr*$^{-/-}$ mice (Supplemental Figs. 2A and 2B). Therefore, increased atherosclerotic lesions in infected mice could not be attributed to plasma cholesterol levels. To evaluate if systemic inflammatory responses [26] were different in uninfected and infected mice, SAA-1, TNF-α, and IL-1β levels were measured in the plasma at 8-weeks post infection. SAA-1, TNF-α, and IL-1β levels were elevated in the plasma of *Chlamydia*-infected *Apoe*$^{-/-}$ mice (Fig. 3A–C). Plasma levels of bioactive IL-1β were also significantly higher in *Chlamydia*-infected *Ldlr*$^{-/-}$ mice compared to the

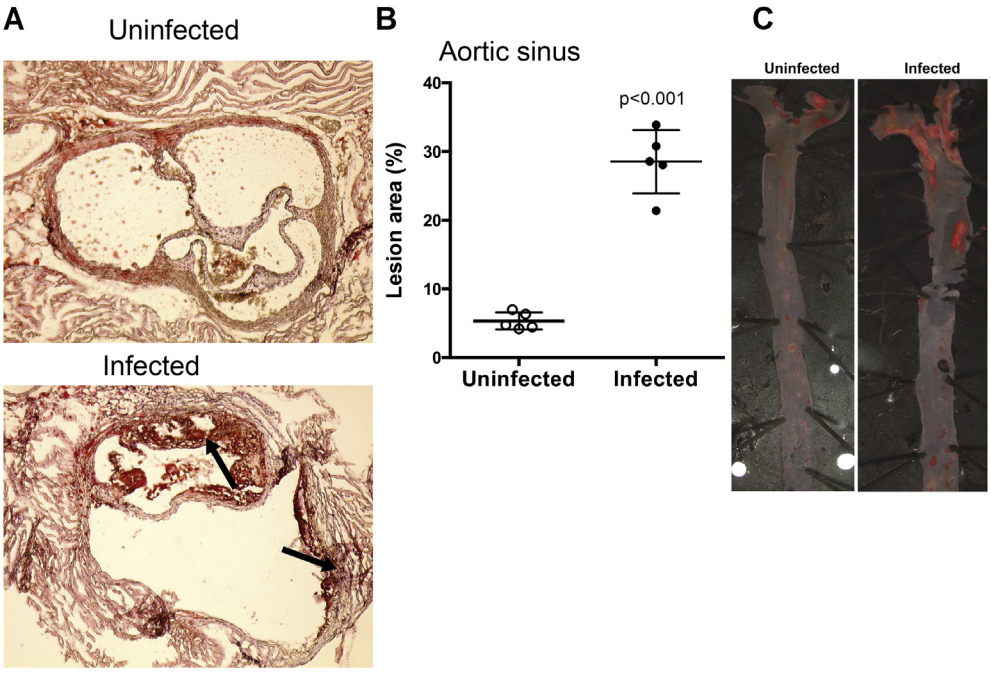

Fig. 2. Aortic lesions were increased in *Ldlr*$^{-/-}$ mice infected with *C. muridarum*. *Ldlr*$^{-/-}$ mice (N = 10) were infected with 3×10^5 IFU vaginally and were fed high fat/high cholesterol diet starting one week after infection (as shown in Fig. 1A). Mice were sacrificed 8-weeks post infection. Aortic sinus (N = 5) was stained using oil red O to visualize plaques. Aortic sinus (A) from a representative mouse from each group is shown and average lesion area (B) plotted. *En face* analysis of descending aorta from a representative mouse from each group is shown (C). Uninfected *Ldlr*$^{-/-}$ mice fed a high-fat/high cholesterol diet was used as control. Significance determined by 't' test.

corresponding uninfected mice at same time post infection (Fig. 3D). To determine if inflammatory responses were different in the vascular endothelium between uninfected and infected mice, we analyzed the expression of an inflammation marker; VCAM-1 at the lesion site in the aortic sections. VCAM-1 served as a candidate inflammatory marker as VCAM-1-dependent monocyte adhesion and transmigration promote formation of fatty streak lesions [27]. Significantly more VCAM-1+ staining was observed in the aortic lesions of infected *Apoe*$^{-/-}$ mice compared with uninfected *Apoe*$^{-/-}$ mice (Fig. 4A and B). A similar trend was also observed in infected *Ldlr*$^{-/-}$ mice, compared with uninfected *Ldlr*$^{-/-}$ mice (Fig. 4C and D). Immunohistochemical analyses of the aortic sinus using MOMA2 antibody showed more macrophages in aortic lesions of infected *Apoe*$^{-/-}$ mice, compared to uninfected mice (Fig. 4E). These findings suggest that chlamydial infection-induced systemic inflammatory response could induce endothelial cell activation, resulting in increased adherence, and transmigration of monocytes at the lesion site in infected mice.

Clinical studies have shown that *C. pneumoniae* lung infections result

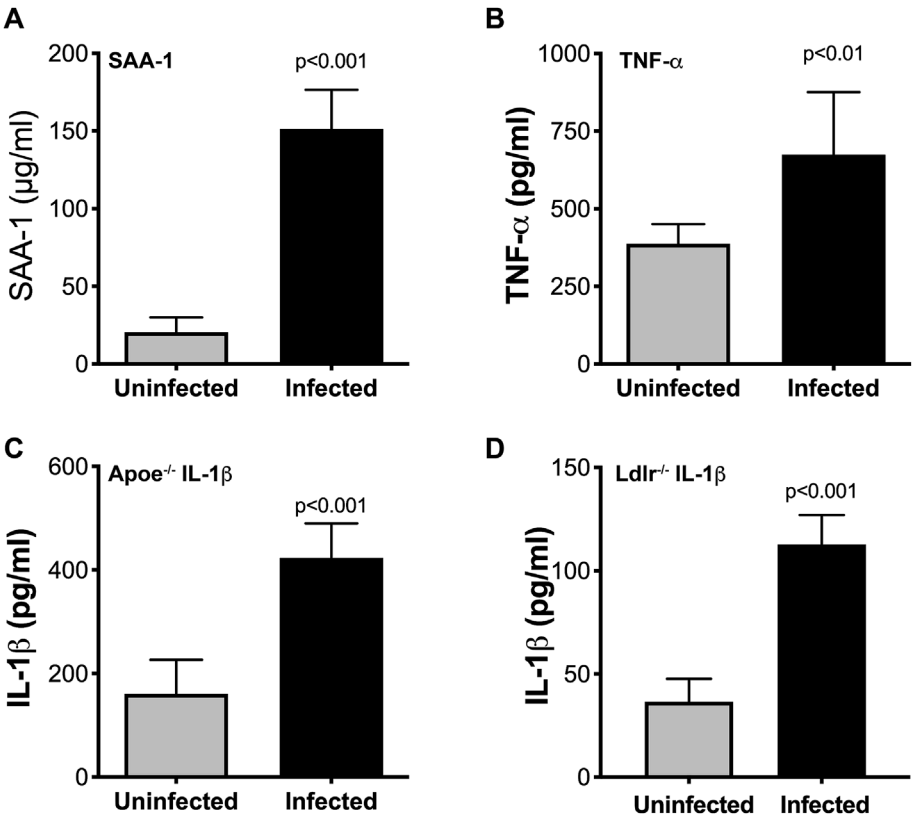

Fig. 3. Increased levels of SAA-1, TNF-α, and IL-1β in the plasma of infected *hyperlipidemic* mice. *Apoe*$^{-/-}$ mice (N = 10) or *Ldlr*$^{-/-}$ were infected with 3×10^5 IFU genitally, and one week after infection mice were fed hyperlipidemic diet. Mice were sacrificed 8-weeks post infection (7 weeks on a high fat diet). Plasma from uninfected mice were used as controls. Plasma levels of SAA-1 (A), TNF-α (B) in *Apoe*$^{-/-}$ mice, and IL-1β in *Apoe*$^{-/-}$ (C), and *Ldlr*$^{-/-}$ (D) were determined as described in methods. Significance determined by 't' test.

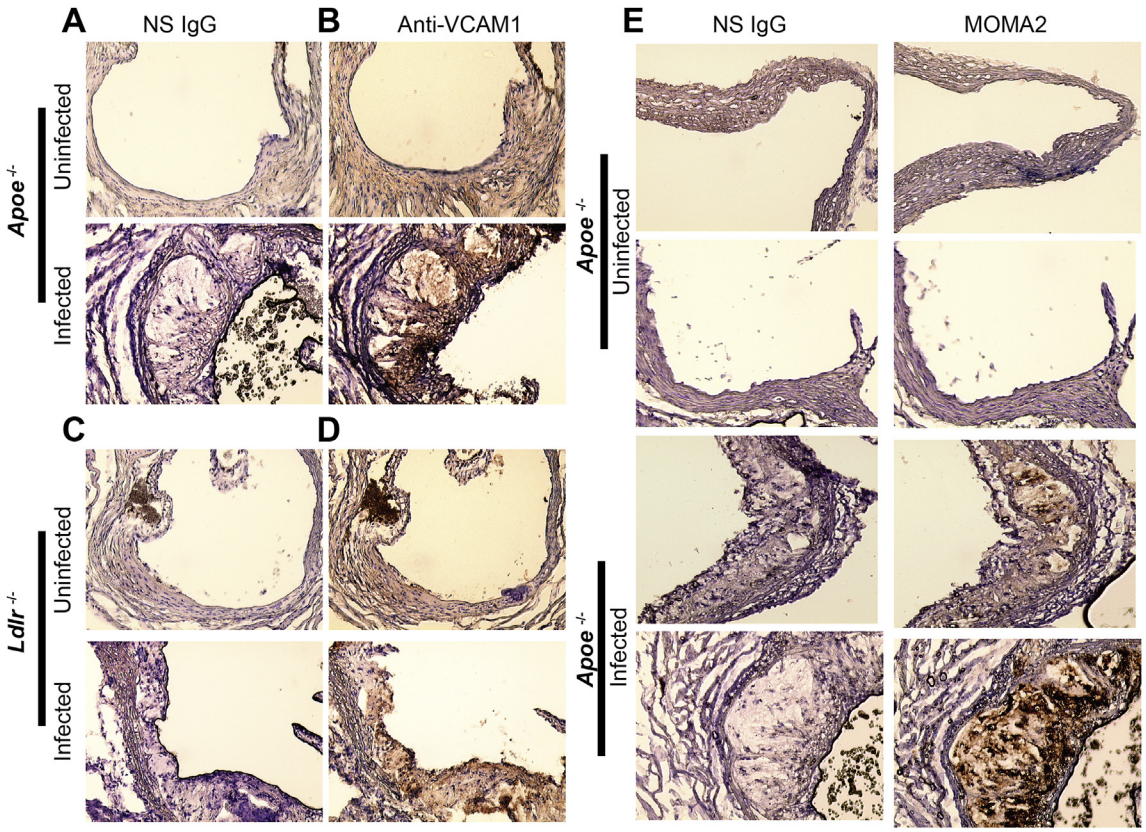

Fig. 4. Increased VCAM-1 expression and macrophages in *Chlamydia*-infected mice.
Representative aortic sinus sections from uninfected or *Chlamydia*-infected *Apoe*$^{-/-}$ (A and B) or *Ldlr*$^{-/-}$ (C and D) were stained with anti-mouse VCAM-1 IgG to detect VCAM-1 protein expression at the lesion site. Arrows indicate VCAM-1 positive staining. (E), Macrophages in aortic sections from uninfected and *Chlamydia*-infected or *Apoe*$^{-/-}$ mice was determined as described the Methods section. Under similar conditions, aortic sections incubated with an isotype-matched rat IgG control were minimal. Representative of five aortic sinus sections from uninfected and chlamydia-infected *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice are presented (100X magnification).

in dissemination of *C. pneumoniae* at the vascular site [28,29]. Therefore, we investigated whether increased incidence of lesions observed in hyperlipidemic mice is caused by dissemination of *C. muridarum* to the vascular site. Quantitative PCR analyses of DNA from the aortic arch at 8 weeks post-infection did not show chlamydial DNA (Supplemental Figs. 3A and B). These findings suggest that there is no persistent chlamydial infection in the lesion-prone vascular site. However, this does not preclude dissemination of *Chlamydia* to the vascular sites in early stages of infection.

3.3. Increased uterine pathology was observed in *C. muridarum*-infected *Apoe*$^{-/-}$ mice

Chlamydia infection leads to significant pathology of the oviduct, characterized by fluid filled hydrosalpinx. Additionally, some mice also develop uterine pathology with dilatation. Excision of the genital tract at 8 weeks post-infection showed no significant differences in hydrosalpinx incidence between WT and *Ldlr*$^{-/-}$ mice and between WT and *Apoe*$^{-/-}$ mice, that were fed high fat diet (data not shown). However, infected *Apoe*$^{-/-}$ mice developed severe uterine dilatation relative to infected WT mice (Fig. 5A). Histopathological observation of H&E stained section showed dilated uteruses in infected *Apoe*$^{-/-}$ mice with severe thinning of uterine wall (Fig. 5B). To determine if feeding high fat diet exacerbated uterine pathology, in an independent study, *Apoe*$^{-/-}$ mice were infected and fed a high fat or regular chow diets (non-hyperlipidemic diet). Histopathological scoring of genital tract tissues showed more uterine horns with a score of 4 in high fat diet fed *Apoe*$^{-/-}$ mice. However, the differences in uterine dilatation between mice fed high fat or regular chow diet were not statistically different, although both were significantly increased compared to uninfected mice (Fig. 5C). Histological scoring of inflammatory cells at this time revealed no significant differences in acute (neutrophils), chronic (mononuclear), or plasma cells in mice fed high fat (Fig. 5D) or regular diets (data not shown). Further, the severe uterine dilatation observed in infected *Apoe*$^{-/-}$ mice, was not observed in infected *Ldlr*$^{-/-}$ mice (data not shown). These data suggest that uterine pathology observed in infected *Apoe*$^{-/-}$ mice is an outcome of absence of Apoe and not due to hyperlipidemia.

3.4. Inflammatory cytokine response to Chlamydia infection is increased in *Apoe*$^{-/-}$ macrophages

To determine if increased genital pathology in *Apoe*$^{-/-}$ mice is a result of increased inflammatory response of immune cells to genital Chlamydia infection, we generated BMDM from WT and *Apoe*$^{-/-}$ mice and their inflammatory gene expression in response to Chlamydia infection determined. Chlamydia-infected WT macrophages showed increased *Ifnb, Il1b, Il6,* and *Tnfa* expression (Supplemental Fig. 4A) compared to uninfected macrophages. Moreover, Apoe-deficient macrophages infected with Chlamydia showed higher pro-inflammatory cytokine response (Supplemental Fig. 4A) compared to infected WT macrophages. We then determined whether macrophages from two hyperlipidemic mouse models differ in cytokine response following Chlamydial infection. BMDM from *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were infected with Chlamydia, and their cytokine response to infection determined by qRT-PCR. Interestingly, some cytokine and chemokine expression of *Apoe*$^{-/-}$ BMDM were significantly higher than those of *Ldlr*$^{-/-}$ BMDM (Fig. 6). To rule out if the increased cytokine response

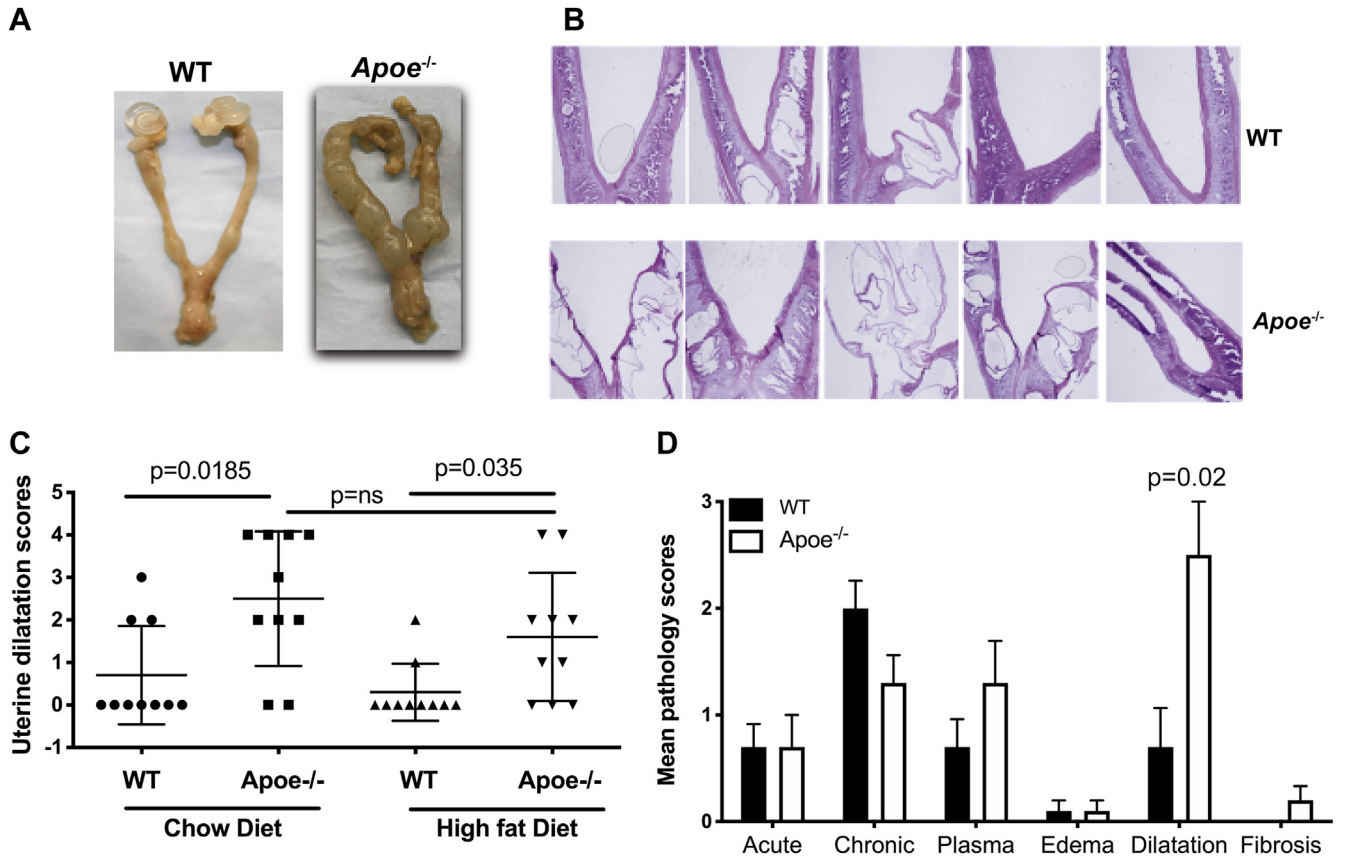

Fig. 5. Severe uterine dilatation in *Apoe*$^{-/-}$ mice in comparison with WT mice.
(A) Representative photograph of genital tracts from infected WT and *Apoe*$^{-/-}$ mice (N = 10). (B) H&E staining of longitudinal sections of genital tracts from WT and *Apoe*$^{-/-}$ mice (N = 5), and (C) histopathological scoring for immune cells and dilatation (N = 5). (D) Dilatation scores from an independent experiment where 5 mice from each group were fed high fat diet or fed a regular chow diet. Statistics were determined by non-parametric two-tailed Mann Whitney test.

was a result of increased infection, infected macrophages were stained for chlamydial inclusion. No differences in inclusion size or numbers were observed between *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice BMDM macrophages (Supplemental Fig. 4B). These findings suggest that Apoe-deficiency augments chlamydial infection-induced pro-inflammatory cytokine response, which could contribute to genital pathology.

4. Discussion

In this study we addressed the hypothesis that genital chlamydial infection can accelerate atherosclerosis under hyperlipidemic conditions. Using two hyperlipidemic mouse models (*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$), we observed increased atherosclerotic lesions following *Chlamydia* genital infection. The infected mice had enhanced systemic and local inflammation, indicated by increased SAA, TNF-α, and IL-1β in plasma, and upregulation of VCAM-1 at the lesion site. In addition, we also

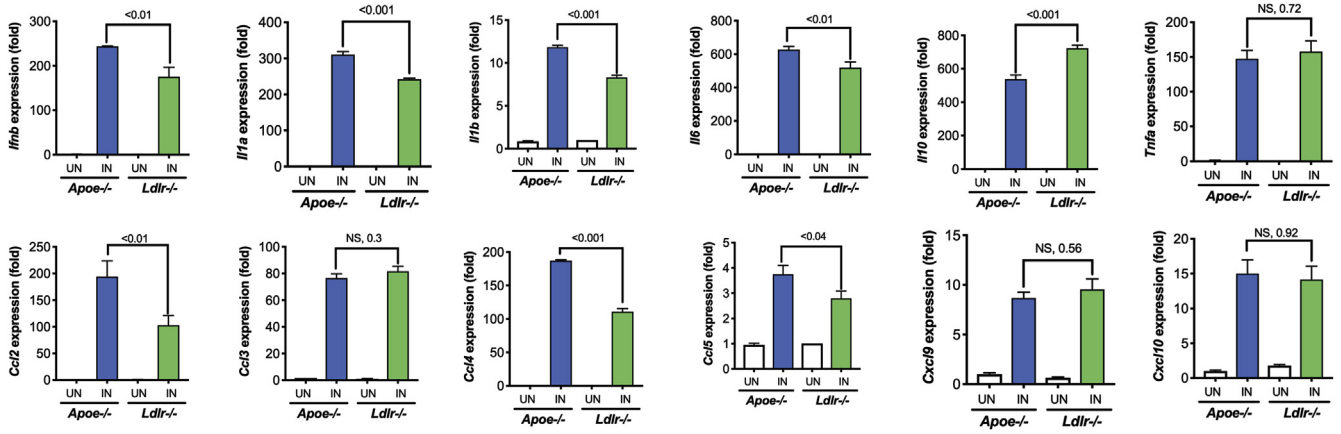

Fig. 6. Augmented inflammatory responses in Chlamydia infected *Apoe*$^{-/-}$ *macrophages*.
BMDM from *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ were infected with *C. muridarum* (1 multiplicity of infection) for 8 h, and expression of inflammatory cytokine and chemokine responses was determined by qRT-PCR. Uninfected macrophages were used as controls. Values are mean ± SD of triplicates. Representative two independent experiments are presented. Cytokine expression in Chlamydia infected *Apoe*$^{-/-}$ BMDM was compared to infected *Ldlr*$^{-/-}$ BMDM. Significance determined by 't' test.

discovered a novel role for Apoe in the maintenance of uterine architecture after chlamydial infection is resolved in the genital tract.

There is significant but conflicting literature regarding *C. pneumoniae* lung infection promoting atherosclerosis in the mouse model. Earlier studies have reported increased atherosclerotic lesions in *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice inoculated intra-nasally at multiple times with *C. pneumonia* [11,12,30]. However, other studies showed no differences in total lesion area [31,32]. Different *C. pneumoniae* strains, age of the mice, mouse strains used; the frequency and route of infections could be contributing to the conflicting reports. Unlike previous studies [33,34], we have used genital *C. muridarum* infection model which requires a single inoculation to establish genital infection to examine genital *C. muridarum* infection contributing to atherosclerotic lesions in mice. Atherosclerotic lesions were analyzed as early as 8 weeks post-infection, including 7 weeks on a hyperlipidemic diet. Unlike other studies where lesions are measured at later times (> 12 weeks on a high-fat diet) [31,35], we chose to collect samples at this early time when lesions are not fully developed in *Apoe*$^{-/-}$ mice, to detect increases in lesion area post infection, before excessive lesions appeared in uninfected hyperlipidemic mice.

Bacterial infection or bacterial ligands regulate cholesterol homeostasis by regulating reverse cholesterol transporter expression [36]. However, we found no differences in plasma cholesterol levels between infected and uninfected mice, indicating that the increased lesions in the infected groups are independent of plasma lipid levels. Infectious microbial agent-induced atherosclerosis could occur by direct effect through infection of vascular cells and/or indirectly by induction of inflammatory cytokines by infection at other sites, such as the aortic vascular endothelium. *C. pneumoniae* was detected within atherosclerotic plaques, suggesting an association between atherosclerosis and *C. pneumoniae* infection [37]. A recent report has reported that intranasal administration of *C. muridarum* resulted in the infection of blood monocytes, which subsequently could transport *C. muridarum* to the vascular site [38]. In our study, PCR analyses did not show chlamydial 16s ribosomal RNA in the aortic arch at 8 weeks post-infection. However, this does not rule out the possibility of early dissemination of *C. muridarum* to the mouse aorta, as we have observed early dissemination followed by rapid clearance of *C. muridarum* in the lungs and spleens of WT mice [39]. Further, *C. muridarum* infection has been shown to persist in the gut well after it has been cleared from the genital tract [40,41]. Whether or not this persistence in the gut could have affected atherosclerotic lesions needs further investigation. Dissemination of *C. muridarum* is likely due to its ability to grow in macrophages [42]. However, with the exception of the less prevalent human Chlamydia strain like Lymphogranuloma venereum, genital strains of *C. trachomatis* grow poorly in human macrophages. Nevertheless, immunodeficiency or co-infection with other sexually transmitted infectious pathogens can compromise host immunity and allow *C. trachomatis* to survive in macrophages and disseminate to remote sites.

In mice, systemic inflammation induced by either intraperitoneal LPS injection or by cecal ligation puncture accelerated atherosclerosis by increased VCAM-1 expression and monocyte adhesion to the aorta [43,44]. Further, we have shown that *C. muridarum* infection in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice increased expression of VCAM-1 and macrophage accumulation at the lesion site. We have also shown that chlamydial genital infection results in augmented systemic inflammatory response as evidenced by elevated levels of TNF-α and IL-1β. Mechanistic studies have indicated that TNF-α severely accelerates lesion development by upregulating vascular cell adhesion molecules and CD36, one of the scavenger receptors implicated in foam cell formation and atherosclerosis [45,46]. These findings suggest that *C. muridarum*-induced production of TNF-α and IL1β could increase endothelial cell VCAM-1 expression resulting in increased monocyte adhesion, and subsequent accelerated atherosclerosis in infected mice [27,47].

Genital *Chlamydia* infection in the mouse model leads to the development of fluid-filled oviduct pathology, described as hydrosalpinx. Occasionally, C57BL/6 mice also develop uterine cysts or swelling post-infection. However, in most animals, the uterine horns recover fully and resume an intact epithelial layer. A surprise finding in this study was that infected *Apoe*$^{-/-}$ mice developed severe uterine pathology in addition to oviduct pathology. The uterine swelling led to complete loss of the epithelial architecture. We have reported similar uterine horn pathology *in Irf3*$^{-/-}$ mice and suggested increased cellular proliferation as an associated phenotype [48]. However, the exact mechanism for this phenotype is not clear. The uterine pathology observed in *Apoe*$^{-/-}$ mice was not associated with a high fat diet, and not observed in *Ldlr*$^{-/-}$ mice, suggesting an alternate function specifically for Apoe in uterus. Apoe is expressed in macrophages, and is essential for efficient cholesterol efflux [49]. However, several new functions of Apoe have emerged. An anti-inflammatory role has been attributed to Apoe as it aids in clearance of apoptotic bodies by macrophages [50]. In this report, we showed that *Chlamydia* infection augmented cytokine expression in Apoe-deficient macrophages compared to WT and Ldlr-deficient macrophages. These findings suggest that lack of anti-inflammatory function of Apoe may contribute to the augmented inflammatory cytokine response following *Chlamydia* infection. *Apoe*$^{-/-}$ mice display impaired immunity to *Listeria monocytogene* [51] and *Klebsiella pneumoniae* [52] infection. Apoe has also been shown to inhibit cell proliferation and migration [53], suggesting that in the absence of Apoe, excessive proliferation of uterine stromal cells could lead to the observed dilatation.

In conclusion, our findings show that inflammatory response during genital Chlamydia infection may be sufficient to promote the formation of atherosclerotic lesions under hyperlipidemic conditions in mouse models. To our knowledge, this is the first study to suggest a link between genital Chlamydia infection-induced PID and subclinical atherosclerosis, suggesting a causal link between genital infection and atherosclerosis. Further clinical studies are required to establish this association in humans. Additionally, the contribution of Apoe in uterine health during infection warrants further investigation.

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Author contributions

UMN and SN conceived and designed the experiments. JDS, RLB, RJ, BP, PF, UMN, and SN performed experiments. JDS, RLB, BP, UMN, and SN analyzed the data. LH performed pathology score of the samples, UMN, CLH, and SN wrote the manuscript with contribution from other authors. UMN and SN contributed equally to this work.

Declaration of Competing Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.09.021.

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