Chlamydia and Lipids Engage a Common Signaling Pathway That Promotes Atherogenesis

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ABSTRACT

BACKGROUND Recent studies indicate that Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) signaling promote the development of high fat diet-induced atherosclerosis in hypercholesterolemic mice.

OBJECTIVES The authors investigated the role of TLR4/MyD88 signaling in hematopoietic and stromal cells in the development and infection-mediated acceleration of atherosclerosis.

METHODS The authors generated bone marrow chimeras between wild-type and Tlr4−/− mice, as well as wild-type and Myd88−/− mice. All mice were on the Apoe−/− background and fed high fat diet. The authors infected the chimeric mice with C. pneumoniae (CP) and fed them high fat diet.

RESULTS Aortic sinus plaques and lipid content were significantly reduced in Apoe−/− mice that received Tlr4−/− or Myd88−/− bone marrow compared with control animals despite similar cholesterol levels. Similarly, Tlr4 or Myd88 deficiency in stromal cells also led to a reduction in the lesion area and lipid in aortic sinus plaques. Myd88 expression only in CD11c+ dendritic cells (myeloid cells) in cells was sufficient in otherwise Myd88-deficient mice to induce CP infection-mediated acceleration of atherosclerosis, underlining the key role of Myd88 in CD11c+ dendritic cells (myeloid cells). Whereas CP infection markedly accelerated atherosclerosis in TLR4- or Myd88-positive chimeras, CP infection had a minimal effect on atherosclerosis in TLR4- or Myd88-deficient mice (either in the hematopoietic or stromal cell compartments).

CONCLUSIONS The authors show that both CP infection and metabolic stress associated with dyslipidemia use the same innate immune response pathway, utilizing TLR4/Myd88 signaling, with similar relative contributions in bone marrow-derived hematopoietic cells and in stromal cells. Further studies are required to understand this intricate and complex cross talk among innate and adaptive immune systems in various conditions to more effectively design dendritic cell-mediated atheroprotective vaccines and other therapeutic strategies. (J Am Coll Cardiol 2018;71:1553–70) © 2018 by the American College of Cardiology Foundation.

Immune cells and their mediators drive the chronic arterial inflammation that is a hallmark of atherosclerosis (1). Virtually every major immune cell lineage used in host defense has been identified in human and/or animal plaques (1–3). Evidence from numerous genetic loss- or gain-of-function studies in animal models shows that immune cell types are generally neither bystanders nor a consequence of plaque development, but instead directly participate in atherogenesis. The array of cytokines that are implicated in atherosclerosis are strikingly similar to those used by immune effector cells to kill
foreign pathogens and defective or diseased host cells (1). Although it is evident that host defense mechanisms play a key role in the development and progression of atherosclerotic disease, it is far less clear how microbial pathogens influence this process. Toll-like receptors (TLR) are a major mechanism by which the host detects the presence of foreign pathogens and initiates a defensive response (4–6). We have identified that TLR4 is a major player in atherosclerosis, which has led to a paradigm shift in the field (7). This was followed by studies that detected the activation of several other TLR in human atherosclerotic plaques as well (8). Furthermore, previous studies from our lab showed that atherosclerosis-prone mice (ApoE−/−) that are genetically deficient in TLR2, TLR4, or the common downstream TLR adaptor protein myeloid differentiation factor 88 (MyD88) develop significantly less atherosclerotic lesion in their aortas and aortic roots when compared with control ApoE−/− mice (9–12).

Dendritic cells (DC) are professional antigen-presenting cells that centrally direct innate immune responses and initiation of acquired immunity (13–16). Only mature DC have high levels of surface expression of major histocompatibility complex and costimulatory molecules that are essential for efficient T-cell stimulation. DC are present in normal and atherosclerotic intima (17) and adventitia (18,19) and play an important role in inflammatory vascular disorders such as arteritis (18–21). When activated, DC become trapped in the adventitia, local tolerance is broken, and the mature DC can then activate local CD4+ T cells and promote local inflammation (22). We have previously shown that DC become trapped at sites of plaque development and can no longer maintain tolerization to self, leading to autoimmune mechanisms that trigger inflammation at plaque sites (23). Besides inducing adaptive immune responses, mature DC also secrete cytokines that recruit immune cells to the site of infection and this requires sufficient TLR stimulation. TLR signaling in DC can also drive regulatory T cells (Treg) (24). Importantly, Subramanian et al. (25) showed that MyD88 plays a key role in CD11c+ DC (or myeloid cells) by promoting atheroprotective Treg generation and the absence of MyD88 in CD11c+ DC leads to loss of these atheroprotective Treg. This results in a concomitant increase in proatherogenic effector T cells and increase in monocyte chemotactic protein 1 (MCP-1)-mediated myeloid-derived inflammatory cells with a net effect of increase in lesion size. However, to fully understand the role of MyD88 signaling in CD11c+ DC in atherosclerosis, we have used mice that transgenically express MyD88 only in the CD11c+ DC (or myeloid cells) in a MyD88 knockout background.

In our previous studies, we showed that MyD88-dependent signaling from TLR2 and TLR4 pathways play a key role in high fat diet (HFD)-induced as well as CP infection-induced acceleration of atherosclerosis (33). These seminal studies described how some infectious agents can exacerbate atherosclerosis in hypercholesterolemic mouse models. Furthermore, we reported that this effect was associated with the presence of activated myeloid DC and by the presence of plasmacytoid DCs (41). Immunohistochemical staining revealed the presence of CP in DC in the atherosclerotic plaques, thus supporting a likely role for DC in bridging the pathogen-induced proinflammatory responses to the acceleration of atherosclerosis (42). To better understand the molecular mechanism by which CP infection leads to accelerated atherosclerosis, we wished to determine the relative contribution of the MyD88 or TLR4 signaling in bone marrow (BM)-derived cells (particularly macrophages or DC) versus the non-hematopoietic stromal cells and compare it with that seen in HFD-induced atherosclerosis.

In this study, we show that TLR4 and MyD88 signaling in both stromal and hematopoietic cells contribute to atherogenesis, and we found a key role of MyD88-dependent signaling in CD11c+ myeloid cells, in both HFD-induced and CP infection-mediated
atherosclerosis in mice. Our results show CP infection-induced acceleration of atherosclerosis is significantly blocked in TLR4 and MyD88 deficiencies in either BM-derived or stromal compartments. However, to our surprise the transgenic expression of MyD88 only in CD11c+ DC (myeloid cells) in otherwise MyD88-deficient background was enough to restore CP infection-induced acceleration of atherosclerosis. These findings thus reveal critical information regarding the contribution of MyD88-dependent signaling in the CD11c+ DCs and myeloid cells to atherogenesis during infection as well as lipid-mediated atherosclerosis.

**METHODS**

**ANIMAL STUDIES.** All animal experiments were performed according to the guidelines and approved protocol (IACUC Protocol #2096) of the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee. Cedars-Sinai Medical Center is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and abides by all applicable laws governing the use of laboratory animals. Laboratory animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the U.S. Department of Health and Human Services publication, *Guide for the Care and Use of Laboratory Animals.*

**MICE.** All mice were on the C57BL/6 background for these studies. Male and female *Apoe*−/− and *Tlr4*−/−/*Apoe*−/−, *Apoe*−/−/*Myd88*−/−, CD11c−Myd88-Tg/*Myd88*−/− mice were used, but the majority of the animals in each group were male (80% in each group investigated).

**ATHEROSCLEROSIS.** Mice were fed HFD containing 0.15% cholesterol (Harlan Teklad, Madison, Wisconsin) starting at 8 weeks of age before infection and continuing until sacrifice unless otherwise noted. Atherosclerosis data were compared between the sexes, male and female mice were pooled for these studies.

**BM CHIMERIC MICE.** All mice (both donor and recipients) were on the *Apoe*−/− background. Eight-week-old recipient mice were irradiated 9.5 Gy (Gammacell 40 Cs γ-irradiation; GammaCell Bio-Technologies, Ramat Hasharon, Israel) to eliminate endogenous BM stem cells and most of the BM-derived cells. A group of control mice received no transplantation. BM from donor mice was harvested by flushing the femurs and tibias with phosphate-buffered saline (pH 7.4). Single-cell suspensions were prepared by passing the cells through a 70-μm cell strainer (BD, Breda, the Netherlands). Next, 2 × 10⁷ donor BM cells were injected into the tail vein of the irradiated, recipient mice. The mice were kept in micro isolator cages for 6 to 8 weeks to allow reconstitution of the BM. After recovery, chimeric mice were placed on HFD for an additional 16 weeks. At the termination of each experiment, BM was harvested, and mice were genotyped for the corresponding genes (*Tlr4* or *Myd88*) by polymerase chain reaction from the deoxyribonucleic acid obtained from BM-derived macrophages, peritoneal macrophages, peripheral blood mononuclear cells (donor), and endothelial cells (EC) (recipient) to confirm chimerism. As previously shown, we routinely obtain over 95% chimerism with our BM transplant protocols (43).

**GENERATION OF THE MyD88 TRANSGENIC MICE IN CD11C+ MYELOID CELLS IN Apoe-DEFICIENCY BACKGROUND.** CD11c−Myd88-Tg/*Myd88*−/− (CD11c−Myd88-transgenic mice on *Myd88*−/− background) mice (44) were kindly provided by Ruslan Medzhitov (Yale University, New Haven, Connecticut) and were crossed with *Apoe*−/−/*Myd88*−/− double knockout mice that we have previously generated (9) to generate *Apoe*−/−/CD11c−Myd88-Tg mice.

**CP LUNG INFECTION.** We used the same protocol of CP infection that we previously described to investigate infection-mediated acceleration of atherosclerosis (31,33). Briefly, CP strain CM-1 (American Type Culture Collection, Manassas, Virginia) was propagated in Hep-2 cells as in Naiki et al. (33). Mice were anesthetized with isoflurane before intranasal application of 5 × 10⁴ inclusion-forming units of CP suspended in sucrose-phosphate-glutamate buffer (40 μl per nostril) per mouse. The intranasal administration of the buffer alone was performed as a negative control. Mice were inoculated a total of 3 × 1 week apart, HFD was continued for 4 months, at which point, mice were sacrificed and dependent variables were measured.

**SERUM LIPID PROFILES.** Mice were sacrificed and sera from mice were obtained at the end of experiments and after an overnight fast. Total cholesterol, high-density and low-density lipoprotein, and triglyceride in the plasma were measured by colorimetric assay (cholesterol E, L-type triglyceride M, L-type low-density lipoprotein cholesterol, high-density lipoprotein cholesterol E [Wako Diagnostics, Richmond, Virginia]) per the manufacturer’s instructions.

**MORPHOMETRIC ASSESSMENT OF ATHEROSCLEROTIC LESIONS IN THE AORTA AND AORTIC SINUS.** Mice were anesthetized with isoflurane before the aorta and the heart were excised. Aortas were dissected from the
aortic arch to the iliac bifurcation. Adherent (adventitial) connective fat was removed and specimens were fixed in Histo-CHOICE (Amresco, Solon, Ohio). Whole aortas were opened longitudinally and mounted en face, then stained for lipids with oil red O. Hearts were embedded in O.C.T. Compound (Tissue Tek, Sakura, Torrance, California) and cross sections of the aortic sinus were stained with oil red O. Lesion areas were quantified with Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland). Image analysis was performed by a trained observer, who was blinded to the genotypes of mice as previously described (9,31,45). The lesion area size and composition of the lesions by lipid-stained areas in the aortic sinus were measured as described in Tumurkhuu et al. (46). Lipid content in localization was measured by fluorescence microscopy with BZ-II analyzer (Keyence America, Itasca, Illinois).

**IMMUNOHISTOCHEMICAL STAINING OF MACROPHAGES AND T CELLS IN THE AORTIC SINUS.** To investigate the type of cells infiltrating, we performed immunofluorescence staining with rat anti-F4/80 (1:100; eBioscience, Thermo Fisher Scientific, Waltham, Massachusetts) or rat anti-CD3 (1:50; eBioscience) for 1 h at room temperature. After washing, the sections were incubated with Alexa Fluor 594-conjugated anti-Rat mAb (1:500; Life Technologies, Thermo Fisher Scientific) for 2 h. Serum-free protein block reagent (Dako, Agilent, Santa Clara, California) was used for blocking and ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (Invitrogen, Thermo Fisher Scientific) was used to counter stain. The data were obtained by fluorescent microscope (BZ-9000E, Keyence).

**SERUM LEVELS OF CHEMOKINES AND CYTOKINES.** Serum concentrations of MCP-1, IL-6, and IL-12p40 (eBioscience) were detected by enzyme-linked immunosorbent assay according to the manufacturer’s instructions. The chemokine expression profiles of the serum were assessed using a mouse Chemokine array kit (ARY020, R&D Systems, Minneapolis, Minnesota) according to the manufacturer’s instructions. Briefly, the arrays were incubated in the supernatants and detection antibody cocktail overnight at 4°C. After washing, the arrays were incubated with streptavidin-hors eradish peroxidase and then exposed to the Chemi Reagent Mix. The reaction intensity was analyzed using BioSpectrum UVP Imaging system (Bio-Rad, Hercules, California).

**STATISTICAL ANALYSIS.** Results are reported as mean ± SEM. All data were analyzed with the Prism statistical program version 4.03 (GraphPad, San Diego, California). Statistical differences were assessed by Student’s t-test between 2 groups and 1-way analysis of variance for 3 or more groups with Tukey post hoc test, and values of p <0.05 were considered significant. For data with 2 independent variables 2-way analysis of variance was used with Bonferroni post hoc test.

**RESULTS**

**TLR4 AND MYD88 EXPRESSION IN BOTH HEMATOPOIETIC AND STROMAL CELLS PLAY A ROLE IN HFD-INDUCED ATEROGENESIS IN ApoE−/− MICE.** We have previously reported that both MyD88 and TLR4 deficiency lead to significantly reduced atherogenesis in ApoE−/− mice fed HFD despite similar levels of hypercholesterolemia (9). Additionally, we showed that these animals have diminished numbers of macrophages and reduced cyclo-oxygenase 2 expression in the aortic sinus at the aortic root lesions (9). Here, we investigated the specific role that TLR4 or MyD88 plays in hematopoietic cells versus stromal cells in atherosclerosis. We first investigated the relative contribution of TLR4 in hematopoietic cells versus nonhematopoietic or stromal cells during the development of atherosclerosis by generating BM chimeras between Tlr4−/− mice and wild-type (WT) mice (Tlr4−/+), all mice are on the ApoE−/− background. The experimental schematic is shown in Online Figure 1.

After 16 weeks of HFD, we found a significant reduction in atherosclerotic lesion size (39%) and lipid content (38%) in the aortic root of irradiated WT (Tlr4−/−/ApoE−/−) mice that were reconstituted with Tlr4−/−BM mice compared with control BM chimeric mice (Tlr4−/+/ApoE−/− BM → Tlr4−/+/ApoE−/−) (Figures 1A to 1C, despite similar blood cholesterol levels (Table 1). Tlr4−/−/ApoE−/− mice that received WT (Tlr4−/+/ApoE−/−) BM also demonstrated a significant reduction in the atherosclerotic lesion size (25%) and lipid content (30%) in the aortic sinus plaques, compared with control chimeric mice (WT: Tlr4−/+/ApoE−/−) into WT: Tlr4−/+/ApoE−/− (Figures 1A to 1C, Tlr4−/−/ApoE−/− BM into Tlr4−/+/ApoE−/−) recipients also led to a significant reduction in lesion size and lipid content that was not significantly different from either deficiency alone (in the hematopoietic or stromal cell compartments). Finally, we also assessed the lesion coverage in the en face aorta preparations and observed a similar reduction (Figures 1D and 1E).

Downstream of TLR4 signaling lies the adaptor protein MyD88. MyD88 is also required for most other TLR signaling, as well as IL-1/IL-18/IL-33 signaling. Similar to the TLR4 chimeras, we generated Myd88−/− chimeras (all on ApoE−/− background) (Online Figure 1) and observed the effect of its deficiency in
hematopoietic or stromal cells on HFD-mediated atherogenesis. We found that hematopoietic deficiency of MyD88 significantly reduced the atherosclerotic lesion size in the aortic root (44%) and sinus as well as the lipid content of these lesions (42%) (Figures 2A to 2C), despite similar blood cholesterol levels (Table 1). When compared with control mice, Myd88−/−/Apoe−/− mice that received WT Myd88+/−/Apoe−/− BM also demonstrated a reduction in the lesion size of the cross-sectional aortic root area (31%) as well as significantly less lipid content (36%) in aortic sinus plaques (Figures 2A to 2C). As before, we found a
TABLE 1  TC Level, Lipoprotein Profile, and Triglyceride Concentrations in Serum (mg/dl) Chimeric Mice

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<th>Donor (BM) to Recipient</th>
<th>Donor (BM) to Recipient</th>
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<tr>
<td>Tc, mg/dl</td>
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<tr>
<td>WT to Apoe−/−</td>
<td>913 ± 67</td>
<td>WT to Apoe−/−</td>
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<td>Tlr4−/− to Apoe−/−</td>
<td>892 ± 53</td>
<td>Myd88−/− to Apoe−/−</td>
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<td>947 ± 61</td>
<td>WT to Apoe−/−; Myd88−/−</td>
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<td>879 ± 84</td>
<td>Myd88−/− to Apoe−/−; Myd88−/−</td>
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<td>HDL, mg/dl</td>
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<td>WT to Apoe−/−</td>
<td>61.0 ± 15.6</td>
<td>WT to Apoe−/−</td>
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<td>Tlr4−/− to Apoe−/−</td>
<td>57.0 ± 13.7</td>
<td>Myd88−/− to Apoe−/−</td>
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<td>51.0 ± 17.2</td>
<td>WT to Apoe−/−; Myd88−/−</td>
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<td>64.0 ± 18.6</td>
<td>Myd88−/− to Apoe−/−; Myd88−/−</td>
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<td>Ldl, mg/dl</td>
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<td>98.0 ± 18.1</td>
<td>WT to Apoe−/−</td>
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<td>105.0 ± 20.0</td>
<td>Myd88−/− to Apoe−/−</td>
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<td>WT to Apoe−/−; Tlr4−/−</td>
<td>116.0 ± 17.4</td>
<td>WT to Apoe−/−; Myd88−/−</td>
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<td>109.0 ± 16.7</td>
<td>Myd88−/− to Apoe−/−; Myd88−/−</td>
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<tr>
<td>Triglyceride, mg/dl</td>
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<td>WT to Apoe−/−</td>
<td>128.0 ± 26.0</td>
<td>WT to Apoe−/−</td>
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<td>Tlr4−/− to Apoe−/−</td>
<td>132.0 ± 19.1</td>
<td>Myd88−/− to Apoe−/−</td>
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<td>WT to Apoe−/−; Tlr4−/−</td>
<td>141.0 ± 16.9</td>
<td>WT to Apoe−/−; Myd88−/−</td>
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<td>Tlr4−/− to Apoe−/−; Tlr4−/−</td>
<td>123.0 ± 15.6</td>
<td>Myd88−/− to Apoe−/−; Myd88−/−</td>
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Values are mean ± SD. BM = bone marrow; HDL = high-density lipoprotein; LDL = low-density lipoprotein; TC = total cholesterol; WT = wild type.

reduction in the lesion coverage in en face aorta preparations from both hematopoietic and stromal MyD88-deficient, chimeric mice (Figures 2D and 2E). However, unlike in the aortic root lesions, there was significantly more reduction in the lesion coverage in the en face aorta preparations from mice deficient for MyD88 in both the stromal and hematopoietic cells than either deficiency alone, indicating a greater sensitivity to MyD88 signaling in the aorta during HFD-induced atherogenesis in Apoe−/− mice.

TLR4 AND MYD88 ARE REQUIRED IN BOTH HEMATOPOIETIC AND STROMAL CELLS FOR CIRCULATING PROINFLAMMATORY CYTOKINE LEVELS DURING ATHEROREGENESIS. Our data indicate that TLR4 and MyD88 play a role in both hematopoietic cells and stromal cells in HFD-induced atherogenesis. However, we do not know whether their effects are in the arterial wall, a general proinflammatory response, or both. To understand their systemic impact, we measured key cytokines, IL-6 and IL-12p40, in the serum of our chimeric animals. Concordant to the reduction that we observed in the aortic lesion size, we also found that serum IL-6, IL-12p40, and the chemokine MCP-1, were also significantly reduced in animals lacking Myd88 in either hematopoietic or stromal cell chimeric mice (Figures 3A to 3C). Similar to our Myd88−/− chimeras, TLR4 deficiency in either stromal or hematopoietic cells also led to a significant reduction in circulating serum concentrations of IL-6, IL-12p40, and MCP-1 (Figures 3D to 3F). These results suggest that TLR4 and MyD88 in both hematopoietic and stromal cells can act as proinflammatory inducers during dyslipidemia in HFD-mediated atherogenesis.

MYD88 SIGNALING IN CD11C+ MYELOID CELLS, INCLUDING DC EXACERBATES DIET-INDUCED ATHEROSCLEROSIS. DC are thought to play a critical role during the development of atherosclerotic lesions, and like macrophages, DC also migrate into atherosclerotic plaques (47-49) and are able to present antigens by clustering with T cells especially in rupture-prone regions of the plaques (49). We have previously reported that acceleration of atherosclerosis induced by CP infection is accompanied by increased numbers of activated DC in aortic sinus plaques but, as was the case with lesion size and circulating cytokine levels, this was significantly blocked in Apoe−/− mice that lacked TLR2, or TLR4, or MyD88 (33). To further dissect the role of MyD88 signaling in CD11c+ myeloid cells, including DC in atherosclerotic plaque development in Apoe−/− mice, we generated the Cd11c-Myd88-Tg (44) mice and placed them on HFD for 16 weeks. These mice are fully deficient for MyD88 except in CD11c+ myeloid cells (all on Apoe−/− background). CD11c is a common marker for DC as well as some types of peripheral macrophages. Whereas Myd88−/−/Apoe−/− mice had very little lesion development as reported before, the Cd11c-Myd88-Tg/Apoe−/− mice had a moderately significant increase in aortic root lesion size as well as in the aortic arch (Figures 4A and 4B). Interestingly, there were no significant changes in the lipid content at both the aortic root lesions as well as the aortic arch, but there was a trend for greater lipid in the aortic arch (Figure 4B). Finally, the presence of Myd88 gene in only CD11c+ myeloid cells led to a significant increase in the lesion area of the whole aorta en face (Figure 4C). Chemokines are critical in the formation and progression of atherosclerosis plaques (50), thus a mouse chemokine protein array was used to screen for alterations in the levels of chemokines in mouse serum. Under these conditions, C10, MCP-2, and macrophage inflammatory protein 1-a/b trended toward an increase in C10/MCP-2 mice compared with Myd88−/− mice, but less than WT mice after 16 weeks of HFD (Online Figure 2). These data suggest that Myd88 signaling in CD11c+ myeloid cells, including DC, plays an important role in atherosclerotic lesion development, but perhaps not in lipid accumulation.
CD11c-MYD88-Tg MICE HAVE INCREASED MACROPHAGE AND T-CELL INFILTRATION IN AORTIC SINUS PLAQUES. Because MyD88 signaling in CD11c+ myeloid cells, including DC alone, was enough to increase the lesion size in Apoe−/− mice, we next examined the effect of MyD88 only in DC on the cellular composition of atherosclerotic plaques and the extent of monocyte/macrophage and T-cell infiltration into the aortic root lesions. Aortic sinus plaques of Cd11c-Mydd88-Tg/Apoe−/− mice on HFD exhibited increased numbers of monocyte/macrophages (F4/80+) (Figures 5A and 5B) as well as T cells (CD3+) compared with Myd88−/−/Apoe−/−.
**FIGURE 3** TLR4 and MyD88 Are Required in Both Hematopoietic and Stromal Cells for Normal Circulating Cytokine Levels During Atherogenesis

**A** Interleukin (IL)-6 in Myd88–/– chimeras. **B** IL-12p40 in Myd88–/– chimeras. **C** Monocyte chemotactic protein (MCP)-1 in Myd88–/– chimeras. **D** IL-6 in Tlr4–/– chimeras. **E** IL-12p40 in Tlr4–/– chimeras. **F** MCP-1 in Tlr4–/– chimeras. n = 8 to 9. Data are presented as mean ± SEM. Significance was determined using 2-way analysis of variance with Bonferroni post hoc test. **p < 0.01; ***p < 0.001. Abbreviations as in Figures 1 and 2.

TLR4–/– and MyD88–/– bone marrow chimeras (all on Apoe–/– background) were assessed for serum cytokine levels after 16 weeks on high fat diet. Cytokine levels were measured by enzyme-linked immunosorbent assay. **(A)** Interleukin (IL)-6 in Myd88–/– chimeras. **(B)** IL-12p40 in Myd88–/– chimeras. **(C)** Monocyte chemotactic protein (MCP)-1 in Myd88–/– chimeras. n = 8 to 9. **(D)** IL-6 in Tlr4–/– chimeras. **(E)** IL-12p40 in Tlr4–/– chimeras. n = 8 to 9. Data are presented as mean ± SEM. Significance was determined using 2-way analysis of variance with Bonferroni post hoc test. **p < 0.01; ***p < 0.001. Abbreviations as in Figures 1 and 2.
mice (Figures 5A and 5C), but we did not observe a significant difference in the numbers of Foxp3+ Treg (Figure 5D). In agreement with these data, circulating serum levels of IL-6, IL-12p40, and interferon γ were also increased in Cd11c-Myd88− mice compared with Myd88−/− mice (Figures 5E to 5G). These results suggest that MyD88 in Cd11c+ myeloid cells (including DC) plays an important role in chronic inflammation, driving myeloid cellular recruitment and cytokine production during development of atherosclerosis.

TLR4 AND MyD88 ARE REQUIRED IN BOTH HEMATOPOIETIC AND STROMAL CELLS FOR CP INFECTION-INDUCED ACCELERATION OF ATHEROSCLEROSIS. We previously reported that TLR2, TLR4, and MyD88 signaling were required for CP infection-induced acceleration of atherosclerosis.
FIGURE 5  Cd11c-MyD88-Tg Mice Have Increased Macrophage and T-Cell Infiltration in Aortic Sinus Plaques

All mice are on Apoe\(^{-/-}\) background. MyD88\(^{-/-}\) and Cd11c-MyD88-Tg MyD88\(^{-/-}\) mice were fed for 16 weeks with high fat diet. (A) F4/80 and CD3 staining in the aortic sinus of MyD88\(^{-/-}\) and Cd11c-MyD88-Tg mice. Quantification F4/80 per plaque area, \(n = 7\) to 8 (B); CD3 T cells, \(n = 7\) to 8 (C); and Foxp3 regulatory T cells (Treg), \(n = 7\) to 8 (D). Serum levels of IL-6, \(n = 9\) to 10 (E); IL-12p40, \(n = 9\) to 10 (F); interferon (IFN)-\(\gamma\), \(n = 9\) to 10 (G). Data represent mean ± SEM. Statistical significance was determined using 2-tailed Student’s t-test. *\(p < 0.05\); **\(p \leq 0.01\). Abbreviations as in Figures 1 and 3.
Atherosclerosis in Apoe−/− mice (33). However, the relative contribution of TLR/MyD88 signaling specifically in BM-derived hematopoietic cells versus non-hematopoietic stromal cells in CP infection-induced acceleration of lesion formation was unknown. To address this specific question, we used the generated Tlr4−/− BM chimeras (again all on Apoe−/− background) and infected them with CP and put them on HFD for 16 weeks (Online Figure 1). Infection with CP significantly increased the lesion size in both the aortic sinus (Figure 6A) and the total lesion area in the en face aorta (Figure 6B) in mice that received WT Tlr4+/+/Apoe−/− BM, but not with Tlr4−/−/Apoe−/− BM. Similarly, the lesion area of the aortic sinus and aorta en face did not increase after CP infection in Tlr4−/−/Apoe−/− mice that received WT Tlr4+/+/Apoe−/− BM (Figures 6A and 6B). We repeated this experiment using Myd88−/−/Apoe−/− chimeras and found that loss of MyD88 in either stromal or hematopoietic cells also resulted in a failure of CP infection-induced acceleration of atherogenesis (Figures 6C and 6D). Additionally, lack of MyD88 in either compartment led to a reduction in circulating IL-6 and IL-12p40 (Online Figure 3). Thus, CP infection-induced acceleration of atherosclerosis also requires intact TLR4/MyD88 in both hematopoietic and stromal cell components and a lack in either cellular compartment is enough to diminish its effect on acceleration of lesion formation.
MyD88 GENE TRANSGENICALLY EXPRESSED ONLY IN CD11c+ MYELOID CELLS, INCLUDING DC, IS SUFFICIENT FOR CP-INFECTION-INDUCED ACCELERATION OF ATHEROSCLEROSIS. Myd88-cd11c-Tg/Apoe−/− mice and the full Myd88−/−/Apoe−/− control mice were infected with 5 × 10⁴ CP inclusion-forming units intranasally 3 × 1 week apart (or mock infection with buffer) and fed HFD for 16 weeks. As expected, WT Myd88−/−/Apoe−/− mice infected with CP had significantly larger lesions in the aortic sinus and aorta en face as well as increased lipid accumulation at the aortic root lesions compared with uninfected control mice (Figures 7A to 7C). CP infection of Myd88−/−/Apoe−/− mice did not develop accelerated atherosclerosis, as we previously published (33). However, CP infection of Cd11c-Myd88-Tg/Apoe−/− mice, compared with Myd88−/−/Apoe−/− mice, did result in larger aortic sinus lesions and aorta en face plaques (Figures 7A and 7C), but intriguingly did not result in increased lipid composition of the aortic root lesions (Figure 7B). These data suggest that MyD88 signaling in DC can partially facilitate CP infection-induced acceleration of atherosclerotic lesion size, but it may not affect the lipid composition of the lesion.

DISCUSSION

Several studies now suggest that inflammatory and innate immune mechanisms that are activated by dyslipidemia and infectious agents play a key role in the development and progression of the atherosclerotic plaque (1-3). Results in experimental animal models of atherosclerosis have produced clear evidence that TLR signaling and therefore innate immunity plays a significant role in the development of atherosclerosis (51). MyD88 is utilized by most TLR, but TLR-induced signaling pathways are subdivided into MyD88-dependent and MyD88-independent TIR-domain-containing adapter-inducing interferon-β signaling pathways, which can lead to very distinct responses (5,6,52,53). We reported the role of TLR in atherosclerosis (7) and that TLR4/MyD88 signaling plays a critical proatherogenic role in HFD-induced as well as in CP infection-induced acceleration of atherosclerosis (33). Whereas TLR4/MyD88 signaling clearly plays a role in dyslipidemia-induced atherogenesis, these pathways are also essential for innate immune responses to infections (Central Illustration).

The lifetime pathogen burden is generally considered to play an important role in various chronic inflammatory diseases, including atherosclerosis, but the mechanisms remain unresolved (54,55). Indeed, for some infections such as influenza, vaccination is considered prophylactic for an adverse
Atherosclerotic event (56,57). The innate immune system plays a key role in the inflammatory processes implicated in atherosclerotic progression through TLR and the NLRP3 inflammasome (58). Abnormal inflammasome activation and the consequent increase in the circulating IL-1β and IL-18 levels correlate with increased macrophage recruitment to lesions, accelerated foam cell formation, and plaque progression (59). These studies all support the concept that the NLRP3-generated inflammatory cytokines, IL-1β and IL-18, are central to lesion progression.

In the current study, we observed that TLR4 and MyD88 signaling in both stromal and hematopoietic cells contribute to atherogenesis, and we found a key role of MyD88-dependent signaling in CD11c+ myeloid cells, in a both HFD-induced and CP infection-mediated atherosclerosis in mice. TLR4 and MyD88 signaling are key in the production of IL-1β and IL-18, and MyD88 signaling is also required for...
cellular responses to IL-1β and IL-18. The recently published CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial reported that neutralizing IL-1β led to a modest but significantly lower rate of recurrent cardiovascular events in high-risk patients with high inflammatory burden with previous myocardial infarction (60) and has helped strengthen the case for the inflammatory basis of human atherosclerosis. However, the exact mechanisms by which circulating IL-1β ablation benefited these patients are not completely understood.

Infections and innate immune inflammatory pathways are intimately linked to cholesterol metabolism (61-63). Liver X receptors orchestrate body cholesterol homeostasis, especially macrophage cholesterol metabolism (61,64). Studies have shown that innate immune activation by microbial components and the acute phase response can suppress liver X receptors and its target genes, including ABCA1 (63), and may explain, in part, infection-induced acceleration of atherosclerosis. Interestingly, CP infection of macrophages has also been associated with diminished adenosine triphosphate-binding cassette transporter 1 expression, diminished cholesterol efflux, and promotion of foam cell formation (65–67). However, there are likely several other mechanisms that are currently unknown that may link infection-mediated NLRP3 activation and IL-1β production/release with cholesterol efflux in macrophages.

In the current study, we further define the role of TLR4 and MyD88 signaling (1 of the key pathways to induce IL-1β) in CP infection-induced acceleration of atherosclerosis to better understand the pathogenesis. We investigated the specific contribution of these innate immune signaling molecules in BM-derived versus stromal cells. Based on the ability of CP to infect monocytes/macrophages, T cells, and ECs, we also wished to investigate whether HFD induced dyslipidemia and CP infection during hyperlipidemia would engage the innate immune system in different or similar way. We found that, similar HFD-induced atherosclerosis, TLR4 and MyD88 were required in both the BM-derived hematopoietic component as well as stromal cell component for either HFD- or CP infection-induced cytokine up-regulation and acceleration of atherosclerosis in the apolipoprotein E (ApoE)-deficient mice. Our studies suggest that both CP infection and metabolic stress associated with dyslipidemia use the same innate immune response pathway with similar relative contributions in BM-derived hematopoietic cells and in stromal cells.

Prior studies have addressed the relative contribution of hematopoietic versus stromal cell contribution of TLR/MyD88 pathway in diet-induced atherosclerosis. The adhesion of monocytes to EC that are stimulated with minimally modified low-density lipoprotein is enhanced, but we reported that this does not occur if the EC are MyD88-deficient (9). Thus, if EC do not express MyD88, then monocyte adhesion and subsequent transendothelial migration may be reduced, which would be expected to reduce focal subendothelial inflammation in the developing plaque. Mullick et al. (10) showed that Tlr2+/Ldlr− mice had reduction in diet-induced atherosclerosis and BM chimera experiments in atherosclerotic mouse models revealed that for endogenous TLR2 ligands, stromal cells were important, whereas for exogenous recurrent microbial TLR2 ligand induced acceleration of atherosclerosis BM-derived cells were important (10). By using cell type-specific MyD88-deficient mice, Yu et al. (68) further demonstrated that MyD88 deficiency in EC or myeloid cells inhibits vascular lesion formation and atherosclerotic area in chow-fed ApoE-deficient mice. Our findings using BM chimeras now show TLR4 and MyD88 signaling were required in both the BM cells and stromal cells in both dyslipidemia and CP infection-mediated atherosclerosis. In addition to most TLR, MyD88 is also required for IL-1β signaling, which is a master regulator of inflammation, plays multiple roles in the development of atherothrombotic plaque such as promoting monocyte and leukocyte adhesion to vascular endothelial cells, growth of vascular smooth-muscle cells, and procoagulant activity (69-72). Considering this, one would suspect that loss of MyD88 would lead to a greater reduction in atherogenesis than loss of TLR4 would. Whereas we did not notice a significant difference at the level of the aortic root, the reduction in lesion coverage in the aorta en face was ~50% for total loss of TLR4 (Figure 1E) but was almost 80% with total loss of MyD88 (Figure 2E). These data suggest that MyD88 plays a larger role in atherosclerosis than just TLR4 signaling.

In this study, we focused on the distal signal from TLR in CP infection-mediated acceleration, and we did not focus on the upstream mechanisms of TLR4. Our studies show a key role of MyD88, in both stromal and BM-derived cells, particularly in CD11c+ DC (myeloid cells) in CP infection-mediated acceleration of atherosclerosis. We have shown that Myd88−/−/Apoe−/− as well as Tlr4−/−/Apoe−/− mice develop significantly less atherosclerosis than littermate WT control mice do for uninfected mice on HFD (9), and another group also reported that Cd14−/−/Apoe−/− mice were not protected (12), suggesting that the contribution of TLR4 does not require CD14, at least in the uninfected hypercholesterolemic mice. However,
these earlier studies did not test the impact of infection on atherosclerosis, thus it remains possible that CD14 could influence atherosclerosis in response to infectious agents. Nevertheless, we have also reported that CP infection of macrophages promotes foam cell formation, in the presence of oxidized low-density lipoprotein, via TLR4 through both a MyD88- and TIR-domain-containing adapter-inducing interferon-β-dependent manner (via interferon regulatory factor 3 by down-regulating the nuclear receptor family liver X receptors), thus shifting cholesterol transport toward profoam cell production, supporting the intimate connection between cholesterol metabolism and innate immunity (63).

Several DC subsets play a major role bridging innate and adaptive immunity. DC are identified in atherosclerotic arteries (47) and are particularly abundant in rupture-prone regions of the plaques (48,49). DC are present in both the normal and atherosclerotic intima (17,47) and the adventitia (18,19), and they have been implicated in all stages of atherosclerosis, including lipid uptake, antigen presentation, efferocytosis, and inflammation resolution (41,73–75). Besides inducing adaptive immune responses, maturing DC also secrete cytokines that recruit immune cells to the site of infection. Because DC maturation is necessary for the initiation of an adaptive immune response, TLR signaling thus provides the essential bridge between innate and adaptive immunity. DC provide instruction to naive T cells during an inflammatory response; however, they are also involved in the induction of Treg (76). Subramanian et al. (25) showed that MyD88 deficiency in CD11c+ DC in low-density lipoprotein–R−/− mice fed Western diet resulted in decreased T-effector cell activation in the periphery as well as decreased T-effector cells and Treg infiltration to the atherosclerotic lesions, with a net effect of enlarging the lesions. It was interpreted that this effect on the plaque was due to a dominant loss of atheroprotective Treg, which led to more MCP-1-induced inflammatory myeloid cell infiltration into the lesions (25). We now provide an additional aspect of the key role of CD11c+ DC (myeloid cells). Our data show that the MyD88 expression only in CD11c+ DC in cells (in otherwise full MyD88-deficient mice) is sufficient to promote larger plaques in the aortic sinus as well as the aorta, when compared with total MyD88−/− mice during CP infection. However, we did not observe significant changes in Treg numbers. The complexity of dissecting the cross talk between innate and adaptive immunity during atherosclerosis is related to the unaccounted variances in inflammatory signaling between 2 different mouse models where the loss of MyD88 only in CD11c+ cells (but not in any other immune cell types) and the presence of MyD88 only in CD11c+ cells is being addressed. Feedback loops, IL-1 and IL-18 signaling, as well as other TLR such as TLR2 and TLR9 could all be activated differently between the 2 transgenic models. The delicate balance between all these complex cross talks among various cell types is likely altered in different ways in these 2 models and underlies the discrepancy. In our current study, infection and HFD provided an infectious and metabolic trigger and were used in combination to accelerate the lesion formation, another important difference from the work previously completed by Subramanian et al. (25). Conventional DC and plasmacytoid DC are present in the aortic sinus plaques of Apoe−/− mice developing atherosclerosis and their numbers increase with CP infection to parallel the infection-mediated acceleration of atherosclerosis (41). Whereas CP infection could not accelerate atherosclerosis in Tlr4−/−/Apoe−/− and Myd88−/−/Apoe−/− chimeras, CP infection was able to induce larger plaques in mice that expressed MyD88 transgenically only in CD11c+ cells.

Finally, infectious agents such as CP infection may contribute to acceleration of atherosclerosis by direct or indirect mechanisms (30). Whereas a direct effect would be the ability of the organisms to infect vascular cells and/or lesion macrophages and indirect effect of infections could be the increase of inflammatory cytokines, including IL-1β (77). Activation of the NLRP3 inflammasome by microorganisms such as CP results in caspase 1-dependent processing and strongly induces the secretion of IL-1β (39,40,78). IL-1β is an important alarmin to infection that stimulates intrinsic, innate, and adaptive immune pathways. Excessive and chronic IL-1β activation is implicated in the pathogenesis of several inflammatory diseases, including rheumatoid arthritis, type 1 diabetes, and atherosclerosis (79). Accordingly, IL-1β inhibitor therapy has therapeutic benefits in these chronic inflammatory diseases including atherosclerosis as recently reported in the CANTOS trial, where 10,061 patients with previous myocardial infarction were enrolled in a randomized double-blind trial to receive a monoclonal antibody targeting IL-1β (60). In contrast to its pathological consequences, IL-1β is also protective of the host during most infections as it coordinates an immune response that antagonizes colonization, replication, invasion, and dissemination of the infecting microbial agents (80). A review of post-marketing surveillance and the U.S. Food and Drug Administration Adverse Event Reporting System data reveals that humans receiving IL-1β inhibitors have
disproportionally high reporting of infections, particularly invasive group A Streptococcus infections (81). Whereas CP infection leads to IL-1β release that induces the chronic inflammatory effects accelerating atherosclerosis, IL-1β secretion in return is also critical for immune host defenses to successfully clear the CP infection. It remains to be investigated whether the anti-IL-1β therapy in the CANTOS trial may have provided any benefit by blocking intercurrent or chronic infection-induced IL-1β and the related residual inflammation in high-risk patients. It is also unknown whether anti-IL-1β therapy had any detrimental effects by interfering with host immune clearance of infections and thus leading to the increased rate of fatal infections that was observed during the trial (60).

**STUDY LIMITATIONS.** In this study, we showed that lipids and microbial antigens trigger the same TLR/MyD88 signaling pathway that activates IL-1β and accelerate atherosclerosis in mice. Further studies are required to address the role of IL-1β in infection-mediated acceleration of atherosclerosis in cardiovascular patients.

**CONCLUSIONS**

Taken together, our data suggest a complex interplay between innate and adaptive immune signaling involving both hematopoietic cells and stromal cells during atherogenesis induced by CP infection during hypercholesterolemia. We show that both CP infection and lipids use the same innate immune response pathway, TLR4/MyD88 signaling, with similar relative contributions in BM-derived hematopoietic cells and in stromal cells. MyD88 expression only in CD11c+ DC (myeloid cells) in cells was sufficient in otherwise MyD88-deficient mice to induce CP infection-mediated acceleration of atherosclerosis, underlining the key role of MyD88 in CD11c+ DC (myeloid cells). Activation of TLR/MyD88 signaling that leads to NLRP3 inflammasome activation by microorganisms such as CP results in the secretion of IL-1β providing a possible mechanism for infection-mediated progression of atherosclerosis.

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**COMPETENCY IN MEDICAL KNOWLEDGE:**
CP infection and hyperlipidemia both engage a common TLR4/MyD88 immune signaling pathway that activates the NLRP3 inflammasome for IL-1β production, promoting inflammation and accelerating atherogenesis.

**TRANSLATIONAL OUTLOOK:** Strategies that target these common immunologic signaling pathways are emerging as novel approaches to prevention of atherosclerosis progression, but further investigation is required to clarify the mechanisms by which neutralizing IL-1β reduces the risk of recurrent cardiovascular events in patients with myocardial infarction.

**PERSPECTIVES**

**REFERENCES**


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APPENDIX For supplemental figures, please see the online version of this paper.