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Chlamydia and Lipids Engage a Common Signaling Pathway that Promotes Atherogenesis

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Abstract

Background—Recent studies indicate that TLR4 and MyD88 signaling promote the development of high fat diet-induced atherosclerosis in hypercholesterolemic mice.

Objective—Here, we investigated the role of TLR4/MyD88 signaling in hematopoietic and stromal cells in the development and infection mediated acceleration of atherosclerosis.

Methods—We generated bone marrow chimeras between WT and *Tlr4*$^{-/-}$ mice, as well as WT and *Myd88*$^{-/-}$ mice. All mice were on the *Apoe*$^{-/-}$ background and fed high fat diet. We 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*$^{-/-}$ BM 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+ DCs (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+ DCs (myeloid cells). While 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).

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Conclusions—We 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 system in various conditions so that more effective design DC-mediated atheroprotective vaccines and other therapeutic strategies.

Keywords

Atherosclerosis; MyD88; dendritic cells; TLR4; *Chlamydia pneumoniae*

Introduction

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). While 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 (TLRs) is 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 TLRs 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 to control $Apoe^{-/-}$ mice (9–12).

Dendritic cells (DCs) are a professional antigen-presenting cells that centrally direct innate immune responses and initiation of acquired immunity (13–16). Only mature DCs have high levels of surface expression of MHC and co-stimulatory molecules that are essential for efficient T cell stimulation. DCs 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 DCs become trapped in the adventitia, local tolerance is broken, and the mature DCs can then activate local CD4+ T cells and promote local inflammation (22). We have previously shown that DCs 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 DCs also secrete cytokines that recruit immune cells to the site of infection and this requires sufficient TLR stimulation. TLR signaling in DCs can also drive regulatory T cells (Treg) (24). Importantly, Subramanian et al. showed that MyD88 plays a key role in CD11c+ DCs (or myeloid cells) by promoting atheroprotective Treg generation

and the absence of MyD88 in CD11c+ DCs leads to loss of these atheroprotective Tregs. This results in a concomitant increase in pro-atherogenic effector T cells, and increase in MCP-1 mediated myeloid-derived inflammatory cells with a net effect of increase in lesion size (25). However, to fully understand the role of MyD88 signaling in CD11c+DCs in atherosclerosis, we have used mice that transgenically express MyD88 only in the CD11c+ DCs (or myeloid cells) in a MyD88 knock out (KO) background.

Inflammatory and immune mechanisms activated by dyslipidemia and metabolic stress and also by infectious agents can promote the development and/or destabilization of atherosclerotic plaque. For example, *Chlamydia pneumoniae* (CP) is a common respiratory pathogen that causes atypical pneumonia and has been suggested to trigger or promote several chronic inflammatory conditions including asthma and atherosclerosis (26). Indeed, a large body of evidence in mice and humans suggests that the intracellular organism, CP, has been identified in human atherosclerotic plaques (27–29), and shown to promote progression/exacerbation of atherosclerotic processes, but the molecular mechanisms engaged by CP remain unclear (26,30–37). Live CP (38–40) and excessive intracellular cholesterol (38–40) also activate the NLRP3 inflammasome, which leads to for the release of the central pro-inflammatory cytokine IL-1β.

In our previous studies, we showed that MyD88-dependent signaling from TLR2 and TLR4 pathways play a key role in high fat diet-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 DCs (mDCs) and by the presence of plasmacytoid DCs (pDCs) (41). Immunohistochemical staining revealed the presence of CP in DCs in the atherosclerotic plaques, thus supporting a likely role for DCs in bridging the pathogen-induced pro-inflammatory 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 derived cells (particularly macrophages or dendritic cells) versus the non-hematopoietic stromal cells and compare it to that seen in high-fat diet induced atherosclerosis.

In this study we show that TLR4 and MyD88 signaling in both stromal and hematopoietic cells contribute to atherogenesis, and found a key role of MyD88-dependent signaling in CD11c+ myeloid cells, in a both high fat diet-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 bone marrow derived or stromal compartments. However, to our surprise the transgenic expression of MyD88 only in CD11c+ DCs (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 (AAALAC 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 DHHS 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 a high fat diet containing 0.15% cholesterol (Harlan Teklad) starting at 8 weeks of age before infection and continuing until sacrifice unless otherwise noted. Atherosclerosis data was compared between males and females. As no significant differences were observed between the genders, male and female mice were pooled for these studies.

Bone-marrow chimeric mice

All mice (both donor and recipients) were on the Apoe$^{-/-}$ background. 8 weeks old recipient mice were irradiated 9.5 Gy (Gammacell 40 Cs γ-irradiation) to eliminate endogenous bone marrow (BM) stem cells and most of the BM-derived cells. A group of control mice received no transplantation. Bone marrow from donor mice was harvested by flushing the femurs and tibias with PBS (pH 7.4). Single-cell suspensions were prepared by passing the cells through a 70 μm cell strainer (BD, Breda, The Netherlands). Next, 2×10^6 donor BM cells were injected into the tail vein of the irradiated, recipient mice. The mice were kept in micro isolator cages for 6–8 weeks to allow full reconstitution of the BM. After recovery, chimeric mice were placed on high fat diet for additional 16 weeks. At the termination of each experiment, bone marrow was harvested, and mice were genotyped for the corresponding genes (Tlr4 or Myd88) by polymerase chain reaction (PCR) from the DNA obtained from BM derived macrophages, peritoneal macrophages, blood PBMCs (donor), and endothelial cells (ECs) (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) (44) were kindly provided by Ruslan Medzhitov (Yale University, New Haven, CT) and

were crossed with *Apoe*$^{-/-}$/*Myd88*$^{-/-}$ double KO mice that we have previously generated (9) to generate *Apoe*$^{-/-}$/*Cd11c–Myd88*-TG mice.

C. pneumoniae (CP) lung infection

We used the same protocol of CP infection that we previously described to investigate infection-mediated acceleration of atherosclerosis(33,45). Briefly, CP strain CM-1 (American Type Culture Collection) was propagated in Hep-2 cells as (33). Mice were anesthetized with isoflurane before intranasal application of 5×10^4 inclusion-forming units (IFU) 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 three times 1 week apart, high fat diet 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 concentrations were determined in duplicate by using a colorimetric assay (infinity cholesterol reagent, Sigma Diagnostics, St. Louis) as described earlier (9).

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 (Amrecso, Solon, OH). Whole aortas were opened longitudinally and mounted *en face*, then stained for lipids with Oil Red O. Hearts were embedded in OCT compound (Tissue Tek, Sakura, Torrance, CA) 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, MD). Image analysis was performed by a trained observer, who was blinded to the genotypes of mice as previously described (9,45,46). The lesion size area and composition of the lesions by lipid-stained areas in the aortic sinus were measured as described earlier (47). Lipid content in localization was measured by florescence microscopy with BZ-II analyzer (KEYENCE).

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, CA, US) or rat anti-CD3 (1:50, eBioscience, CA, US) for 1 hour at room temperature. After washing, the sections were incubated with Alexa Fluor 594-conjugated anti-Rat mAb (1:500, Life Technologies) for 2 hours. Serum free protein block reagent (Dako co. CA, US) was used for blocking and ProLong Gold Antifade Reagent with DAPI (Invitrogen, NY, US) 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 (eBiosciences) were detected by ELISA according to the manufacturer’s instructions. The chemokine expression profiles of

the serum were assessed using a mouse Chemokine array kit (cat. no. ARY020; R&D Systems, MN), 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-horseradish peroxidase and then exposed to the Chemi Reagent Mix. The reaction intensity was analyzed using BioSpectrum UVP Imaging system (Bio-Rad, CA).

Statistical Analysis

Results are reported as mean ± SE. All data were analyzed with the Prism 4.03 statistical program. Statistical differences were assessed by Student's t test between two groups and oneway ANOVA for three or more groups with Tukey's post-hoc test, and values of $p < 0.05$ were considered significant. For data with two independent variables two-way ANOVA was used with Bonferroni's post-hoc test.

Results

TLR4 and MyD88 expression in both hematopoietic and stromal cells play a role in high fat diet-induced atherogenesis in Apoe$^{-/-}$ mice

We have previously reported that both MYD88 and TLR4 deficiency lead to significantly reduced atherogenesis in *Apoe*$^{-/-}$ mice fed high fat diet, despite similar levels of hypercholesterolemia (9). Additionally, we showed that these animals have diminished numbers of macrophages and reduced COX-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 atherogenesis. We first investigated the relative contribution of TLR4 in hematopoietic cells versus non-hematopoietic or stromal cells during the development of atherosclerosis by generating bone marrow chimeras between *Tlr4*$^{-/-}$ mice and WT mice (*Tlr4*$^{+/+}$), all on the *Apoe*$^{-/-}$ background. The experimental schematic is shown in Online Figure 1.

After 16 weeks of high fat diet (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$^{-/-}$/Apoe$^{-/-}$ bone marrow compared to control BM chimeric mice (Tlr4$^{+/+}$/Apoe$^{-/-}$ BM → Tlr4$^{+/+}$/Apoe$^{-/-}$) (Figure 1A–C), despite similar blood cholesterol levels (Table 1). Tlr4$^{-/-}$/Apoe$^{-/-}$ mice that received WT (Tlr4$^{+/+}$/Apoe$^{-/-}$) bone marrow 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$^{-/-}$) (Figure 1A–C). Tlr4$^{-/-}$/Apoe$^{-/-}$ bone marrow 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 (Figure 1D, E).

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 high fat-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%) (Figure 2A–C), despite similar blood cholesterol levels (Table 1). *Myd88*$^{-/-}$/*Apoe*$^{-/-}$ mice that received WT *Myd88*$^{+/+}$/*Apoe*$^{-/-}$ bone marrow also demonstrated a reduction in the lesions size of the cross-sectional aortic root area (31%) as well as significantly less lipid content (36%) in aortic sinus plaques, when compared to controls (Figure 2A–C). As before, we found a reduction in the lesion coverage in *en face* aorta preparations from both hematopoietic and stromal MyD88-deficient, chimeric mice (Figure 2D, E). 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 high fat-induced atherogenesis in *Apoe*$^{-/-}$ mice.

TLR4 and MYD88 are required in both hematopoietic and stromal cells for circulating proinflammatory cytokine levels during atherogenesis

Our data indicate that TLR4 and MyD88 play a role in both hematopoietic cells and stromal cells in high fat diet-induced atherogenesis. However, we do not know if their effects are in the arterial wall, a general pro-inflammatory 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 (Figure 3A, B, C). 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 (Figure 3D–F). These results suggest that TLR4 and MyD88 in both hematopoietic and stromal cells can act as pro-inflammatory inducers during dyslipidemia in high fat diet-mediated atherogenesis.

MyD88 signaling in CD11c+ myeloid cells, including DCs exacerbates diet-induced atherosclerosis

DCs are thought to play a critical role during the development of atherosclerotic lesions, and like macrophages, DCs also migrate into atherosclerotic plaques (48–50) and are able to present antigens by clustering with T cells especially in rupture-prone regions of the plaques (50). We have previously reported that acceleration of atherosclerosis induced by CP infection is accompanied by increased numbers of activated DCs 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 DCs in atherosclerotic plaque development in *Apoe*$^{-/-}$ mice, we generated the *Cd11c-Myd88*-Tg (44) mice and placed then 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 DCs as well as some types of peripheral macrophages. While *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 (Figure 4A, B). Interestingly, there was 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 (51), thus a mouse chemokine protein array was used to screen for alterations in the levels of chemokines in mouse serum. Under these conditions C10, MCP2 and MIP1-a/b trended towards an increase in CD11c-Tg 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 DCs, 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

Since MyD88 signaling in CD11c+ myeloid cells, including DCs 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-Myd88*-Tg/*Apoe*$^{-/-}$ mice on high fat diet exhibited increased numbers of monocyte/macrophages (F4/80+) (Figure 5A, B) as well as T-cells (CD3+) compared with *Myd88*$^{-/-}$/*Apoe*$^{-/-}$ mice (Figure 5A, C), but we did not observe a significant difference in the numbers of Foxp3+ Tregs (Figure 5D). In agreement with these data, circulating serum levels of both IL-6, IL-12p40, and IFN-γ were also increased in *Cd11c-Myd88*-Tg: *Apoe*$^{-/-}$ mice compared with *Myd88*$^{-/-}$: *Apoe*$^{-/-}$ mice (Figure 5E–G). These results suggest that MyD88 in CD11c+ myeloid cells (including DCs) 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 Chlamydia pneumoniae-infection induced acceleration of atherosclerosis

We previously reported that TLR2, TLR4, and MyD88 signaling were required for *Chlamydia pneumonia* (CP) infection-induced acceleration of atherosclerosis in *Apoe*$^{-/-}$ mice (33). However, the relative contribution of TLRs/MyD88 signaling specifically in bone marrow derived hematopoietic cells versus non-hematopoietic stromal cells in CP infection-induced acceleration of lesion formation was unknown. The address this specific question, we used the above generated *Tlr4*$^{-/-}$ bone marrow chimeras (again all on *Apoe*$^{-/-}$ background), and infected them with CP and put them on high fat diet 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 (Figure 6 A, B). 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 to acceleration of

atherogenesis (Figure 6 C, D). Additionally, lack of MyD88 in either compartment let 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 DCs, is sufficient for CP-induced acceleration of atherosclerosis

Myd88-cd11c-Tg/*Apoe*$^{-/-}$ mice and the full *Myd88*$^{-/-}$/*Apoe*$^{-/-}$ control mice were infected with 5×10^4 CP IFU intra nasal three times one week apart (or mock infection with buffer) and fed a high fat diet for 16 weeks as above. 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 controls (Figure 7A–C). 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 did result in larger aortic sinus lesions and aorta *en face* plaques compared to *Myd88*$^{-/-}$/*Apoe*$^{-/-}$ mice (Figures 7A, C), but intriguingly not to increased lipid composition of the aortic root lesions (Figure 7B). These data suggest that MyD88 signaling in DCs can partially facilitate CP infection-induced acceleration of atherosclerotic lesion size, but 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. 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 (52). MyD88 is utilized by most TLRs, but TLR-induced signaling pathways are subdivided into MyD88-dependent and MyD88-independent (TRIF) signaling pathways, which can lead to very distinct responses (5,6,53,54). We reported the role of TLRs in atherosclerosis (7) and that TLR4/MyD88 signaling plays a critical pro-atherogenic role in high fat diet-induced as well as in CP infection-induced acceleration of atherosclerosis (33). While 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 (55,56). Indeed, for some infections such as Influenza, vaccination is considered prophylactic for an adverse atherosclerotic event (57,58). The innate immune system plays a key role in the inflammatory processes implicated in atherosclerotic progression through TLRs and the nucleotide binding domain and leucine-rich repeat (NLR) pyrin domain containing 3 (NLRP3) inflammasome (59). 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

(60). 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 observe that TLR4 and MyD88 signaling in both stromal and hematopoietic cells contribute to atherogenesis, and found a key role of MyD88-dependent signaling in CD11c+ myeloid cells, in a both high fat diet-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 (61), 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 (62–64). Liver X receptors (LXRs) orchestrate body cholesterol homeostasis, especially macrophage cholesterol metabolism (62,65). Studies have shown that innate immune activation by microbial components and the acute phase response can suppress LXR and its target genes, including ABCA1 (64), and may explain, in part, infection-induced acceleration of atherosclerosis. Interestingly, CP infection of macrophages has also been associated with diminished ABCA1 expression, diminished cholesterol efflux and promotion of foam cell formation (66–68). 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 (one 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 bone marrow derived versus stromal cells. Based on the ability of CP infecting monocytes/macrophages, T cells and endothelial cells, we also wished to investigate whether high fat diet induced dyslipidemia and CP infection during hyperlipidemia would engaged the innate immune system in different or similar way. We found that, similar for the high fat diet-induced atherosclerosis, TLR4 and MyD88 were required in both the bone marrow derived hematopoietic component as well as stromal cell component for either high-fat diet- or CP infection induced cytokine upregulation and acceleration of atherosclerosis in the 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 bone marrow derived hematopoietic cells and in stromal cells.

Prior studies have address the relative contribution of hematopoietic versus stromal cell contribution of TLR/MyD88 pathway in diet induced atherosclerosis. The adhesion of monocytes to ECs that are stimulated with MM-LDL (minimally modified LDL) is enhanced, but we reported that this does not occur if the ECs are MyD88-deficient (9). Thus, if ECs 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 showed that *Tlr2*$^{-/-}$/*Ldlr*$^{-/-}$ mice had reduction in diet-induced atherosclerosis and bone marrow 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 further demonstrated that MyD88-deficiency in endothelial or myeloid cells inhibits vascular lesion formation and atherosclerotic area in chow-fed ApoE-deficient mice (69). Our findings using bone marrow 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 TLRs, 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 pro-coagulant activity (70–73). Considering this, one would suspect that loss of MyD88 would lead to a greater reduction in atherogenesis that loss of TLR4. While 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 than in atherosclerosis than just TLR4 signaling.

In this study, we focused on the distal signal form TLRs in CP infection mediated acceleration, and did not focus on the upstream mechanisms of TLR4. Our studies show a key role of MyD88, in both stromal and BM derived cell, particularly in CD11c+ DCs (myeloid cells) in CP infection mediated acceleration of atherosclerosis. While we have shown that *Myd88*$^{-/-}$/*Apoe*$^{-/-}$ as well as *Tlr4*$^{-/-}$/*Apoe*$^{-/-}$ mice develop significantly less atherosclerosis than littermate WT controls for uninfected mice on high fat diet (9), 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 *C. pneumoniae* infection of macrophages promote foam cell formation, in the presence of ox-LDL, via TLR4 through both a MyD88- and TRIF-dependent manner (via IRF3 by down regulating the nuclear receptor family LXRs), thus shifting cholesterol transport toward pro-foam cell production, supporting the intimate connection between cholesterol metabolism and innate immunity (74).

Several Dendritic cells (DC) subsets play a major role bridging innate and adaptive immunity. DCs are identified in atherosclerotic arteries (48) and are particularly abundant in rupture-prone regions of the plaques (49,50). DCs are present in both the normal and atherosclerotic intima (17,48) and the adventitia (18,19), and have been implicated in all stages of atherosclerosis including, lipid uptake, antigen presentation, efferocytosis, and inflammation resolution (41,75,76,77). Besides inducing adaptive immune responses, maturing DCs also secrete cytokines that recruit immune cells to the site of infection. Since DC maturation is necessary for the initiation of an adaptive immune response, TLR signaling thus provides the essential bridge between innate and adaptive immunity. DCs

provide instruction to naive T-cells during an inflammatory response, however, they are also involved the induction of regulatory T cells (Tregs) (78). Subramanian et al, showed that MyD88 deficiency in CD11c+ DCs in LDL-R$^{-/-}$ mice fed Western diet resulted in decreased T effector cell activation in the periphery as well as decreased T effector cells and Tregs infiltration to the atherosclerotic lesions, with a net effect of enlargening the lesions. It was interpreted that this effect on the plaque was due to a dominant loss of atheroprotective Tregs, which lead 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+ DCs (myeloid cells). Our data show that the MyD88 expression only in CD11c+ DCs 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 crosstalk between innate and adaptive immunity during atherosclerosis is related to the unaccounted variances in inflammatory signaling between two 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 TLRs such as TLR2 and TLR9, could all be activated differently between the two transgenic models. The delicate balance between all these complex cross-talks amongst various cell types is likely altered in different ways in these two models and underlies the discrepancy. In our current study, infection and high fat diet 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. Conventional DCs and plasmacytoid DCs 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). While 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). While 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 (79). Activation of the NLRP3 inflammasome by microorganisms like CP results in caspase-1 dependent processing and strongly induces the secretion of IL-1β (39,80,81). 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 I diabetes and atherosclerosis (82). 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 (61). 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 (83). A review of post-

marketing surveillance and the FDA Adverse Event Reporting System (FAERS) data reveals that humans receiving IL-1β inhibitors have disproportionally high reporting of infections, particularly invasive group A Streptococcus infections (84). While 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 (61).

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 bone marrow derived hematopoietic cells and in stromal cells. MyD88 expression only in CD11c+ DCs (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+ DCs (myeloid cells). Activation of TLR/MyD88 signaling that leads to NLRP3 inflammasome activation by microorganisms like CP results in the secretion of IL-1β providing a possible mechanism for infection-mediated progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TLRs	Toll-like receptors
MyD88	Myeloid Differentiation factor 88
DCs	Dendritic cells
CP	*Chlamydia pneumoniae*
BM	Bone marrow

HFD	high fat diet
WT	wild type

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CLINICAL PERSPECTIVES

Competency in Medical Knowledge

Chlamydia pneumoniae infection and hyperlipidemia both engage a common TLR4/MyD88 immune signaling pathway that activates the NLRP3 inflammasome for IL-β production, promoting inflammation and accelerating atherogenesis.

Translational Outlook

Strategies that target these common immunological signalling 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.

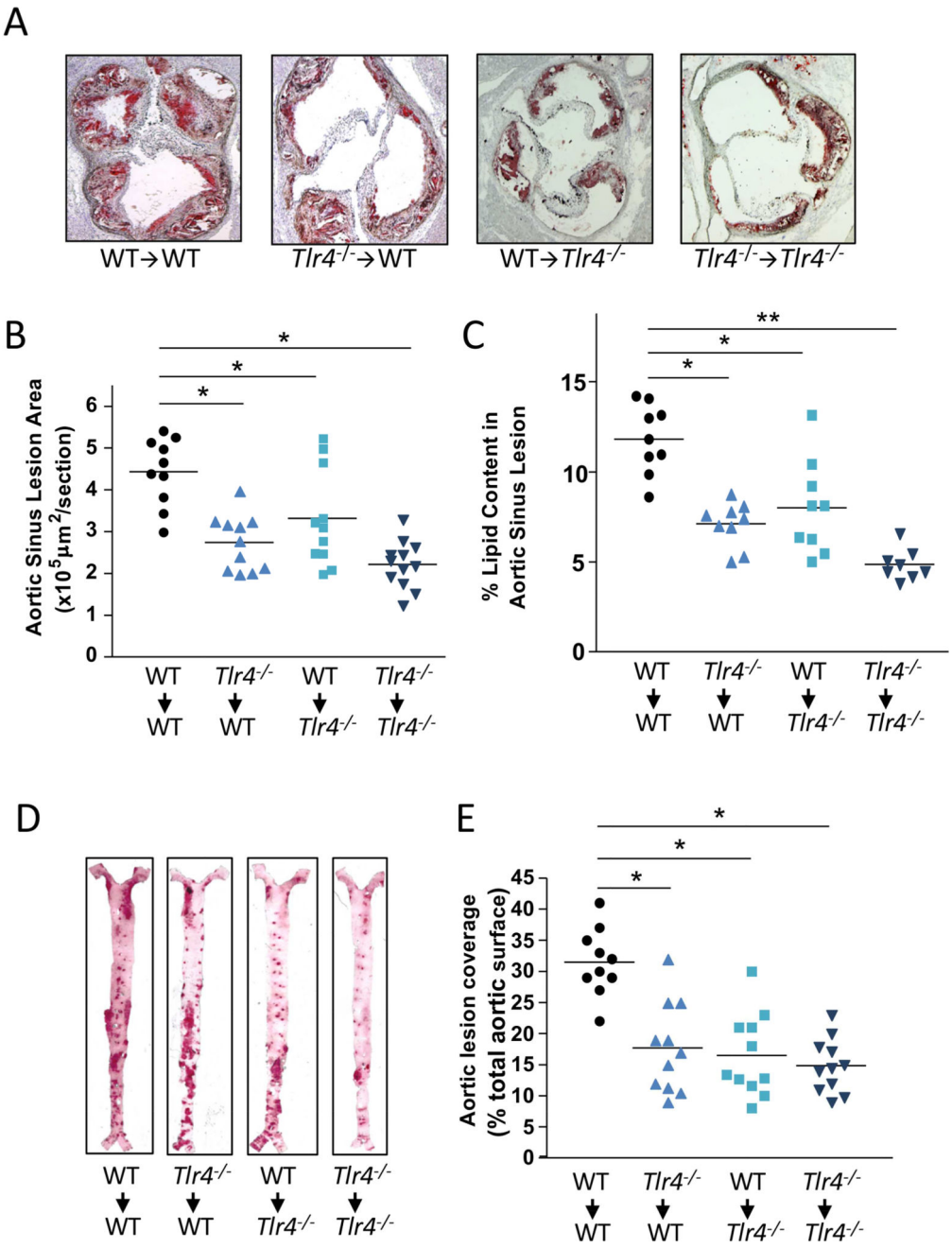


Figure 1. TLR4 deficiency in hematopoietic and stromal cells protects against atherosclerosis in *Apoe*$^{-/-}$ mice

All mice are on *Apoe*$^{-/-}$ background. Irradiated WT and *Tlr4*$^{-/-}$ mice received WT or *Tlr4*$^{-/-}$ bone marrow cells. After 8 weeks reconstitution, the mice were fed HFD for 16 weeks. A) Representative Oil Red O staining of aortic sinus. B) Quantification of area of aortic sinus plaques. C) lipid content of aortic sinus plaques. D) Representative Oil Red O staining of aortic *en face*. E) Aortic lesion coverage. n=10–12. Data are presented as mean values ± SEM. Significance was determined using two-way ANOVA with Bonneferroni's posthoc test (B, C and E). *p<0.05, **p<0.01.

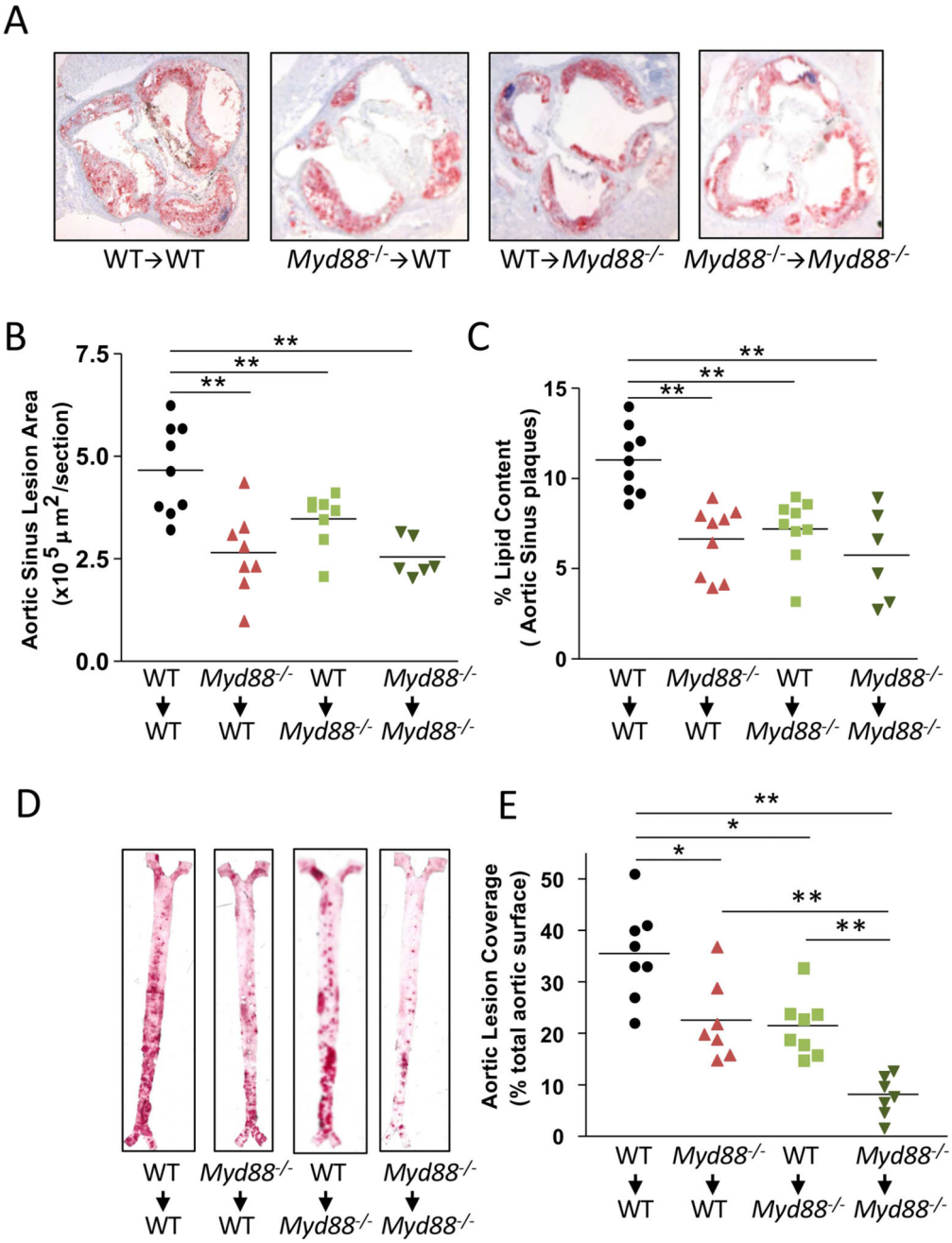


Figure 2. MYD88 deficiency in hematopoietic and stromal cells protects against atherosclerosis in *Apoe*$^{-/-}$ mice

All mice are on *Apoe*$^{-/-}$ background. Irradiated WT and *Myd88*$^{-/-}$ mice received WT or *Myd88*$^{-/-}$ bone marrow cells. After 8 weeks reconstitution, the mice were fed HFD for 16 weeks. A) Representative Oil Red O staining of aortic sinus. B) Quantification of area of aortic sinus plaques. C) lipid content of aortic sinus plaques. D) Representative Oil Red O staining of aortic *en face*. E) Aortic lesion coverage. n = 7–9. Data are presented as mean values ± SEM. Significance was determined using two-way ANOVA with Bonneferroni's posthoc test (B, C and E). *p<0.05, **p<0.01.

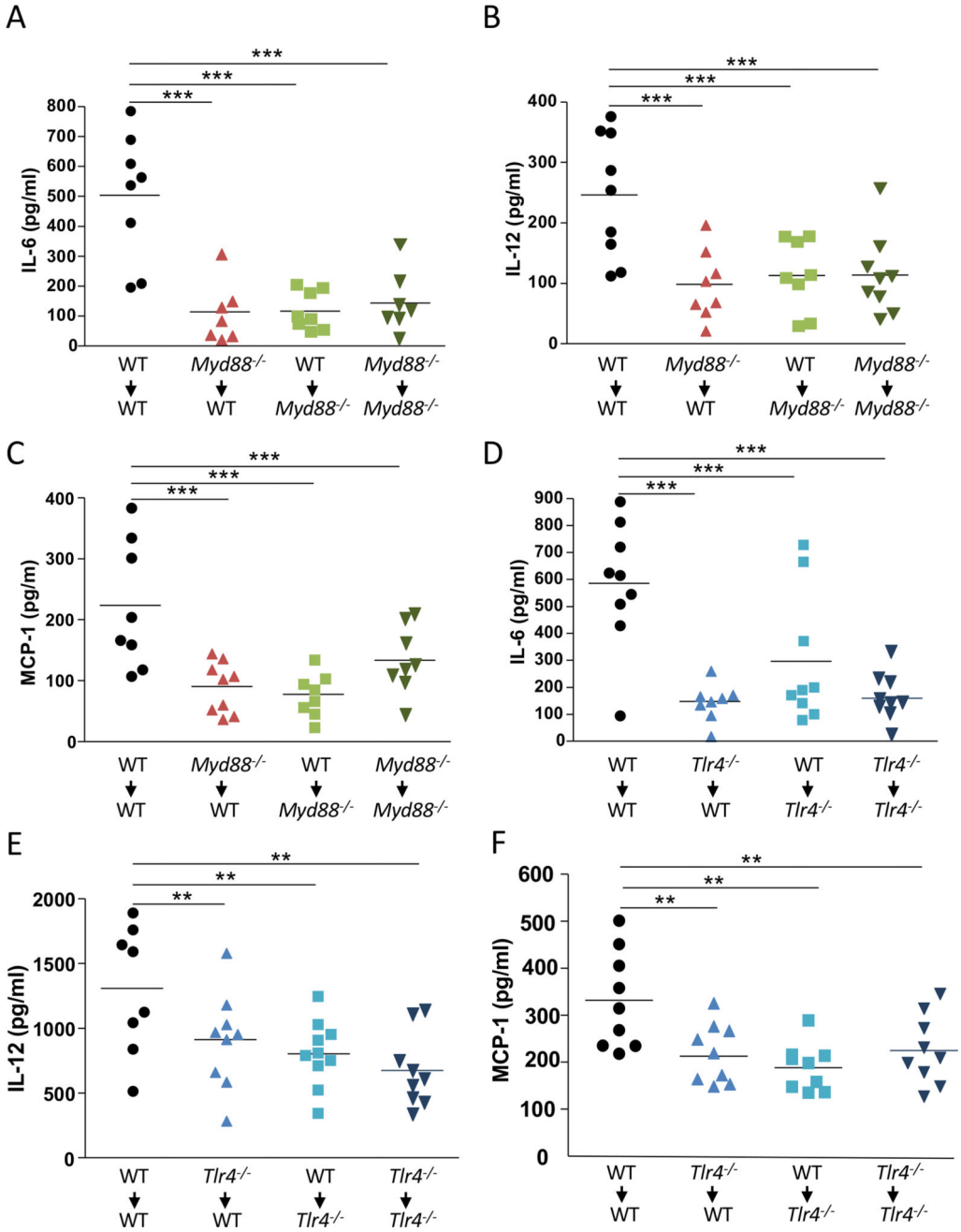


Figure 3. TLR4 and MYD88 are required in both hematopoietic and stromal cells for normal circulating cytokine levels during atherogenesis

Tlr4$^{-/-}$ and *Myd88*$^{-/-}$ bone marrow chimeras (all on *Apoe*$^{-/-}$ background) were assessed for serum cytokine levels after 16 weeks HFD. Cytokine levels were measured by ELISA. A) IL-6 in *Myd88*$^{-/-}$ chimeras. B) IL-12p40 in *Myd88*$^{-/-}$ chimeras. C) MCP-1 in *Myd88*$^{-/-}$ chimeras. n=8–9. D) IL-6 in *Tlr4*$^{-/-}$ *chimeras*. E) IL- 12p40 in *Tlr4*$^{-/-}$ *chimeras*. n=8–9. F) MCP-1 in *Tlr4*$^{-/-}$ chimeras. n=8–9. Data are presented as mean values ± SEM, Significance was determined using two-way ANOVA with Bonneferroni's post-hoc test. **, $p < 0.01$ ***, $p < 0.001$.

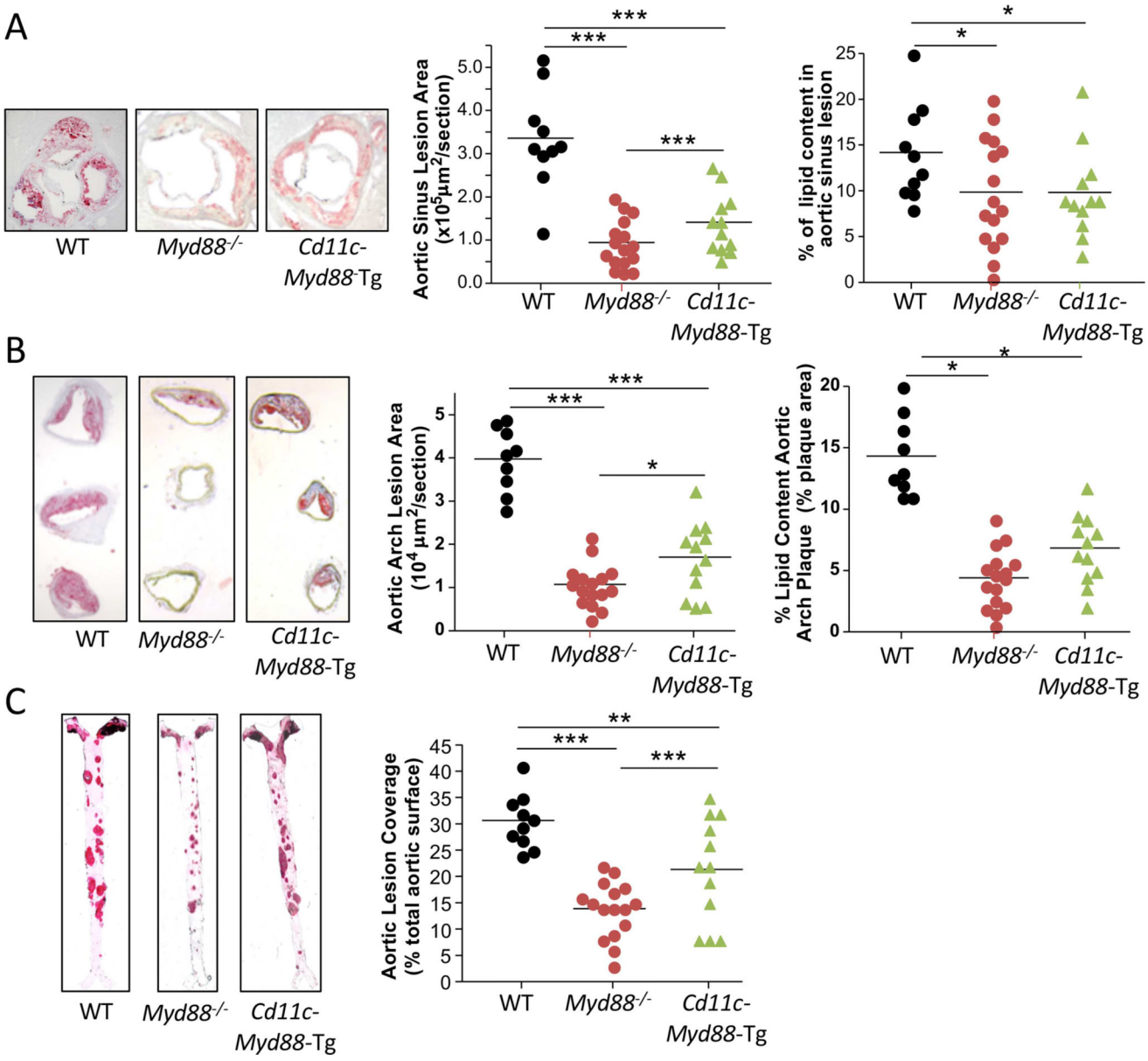


Figure. 4. MYD88 signaling in *CD11c*+ DCs exacerbates diet-induced atherosclerosis

All mice are on *Apoe*$^{-/-}$ background. *Myd88*$^{-/-}$ and *CD11c-Myd88*-Tg *Myd88*$^{-/-}$ mice were fed for 16 weeks with HFD. A) Representative Oil Red O staining of aortic sinus. Quantification of area of aortic sinus plaques. Lipid content of aortic sinus plaques. B) Representative Oil Red O staining of aortic arch. Quantification of area of aortic arch plaques. Lipid content of aortic arch plaques. C) Representative Oil Red O staining of aortic *en face*. Aortic lesion coverage. n= 10–16. Data are presented as mean values ± SEM. One-Way ANOVA with Tukey's post-hoc test, denoted as *p<0.05, **p<0.01, ***p<0.001.

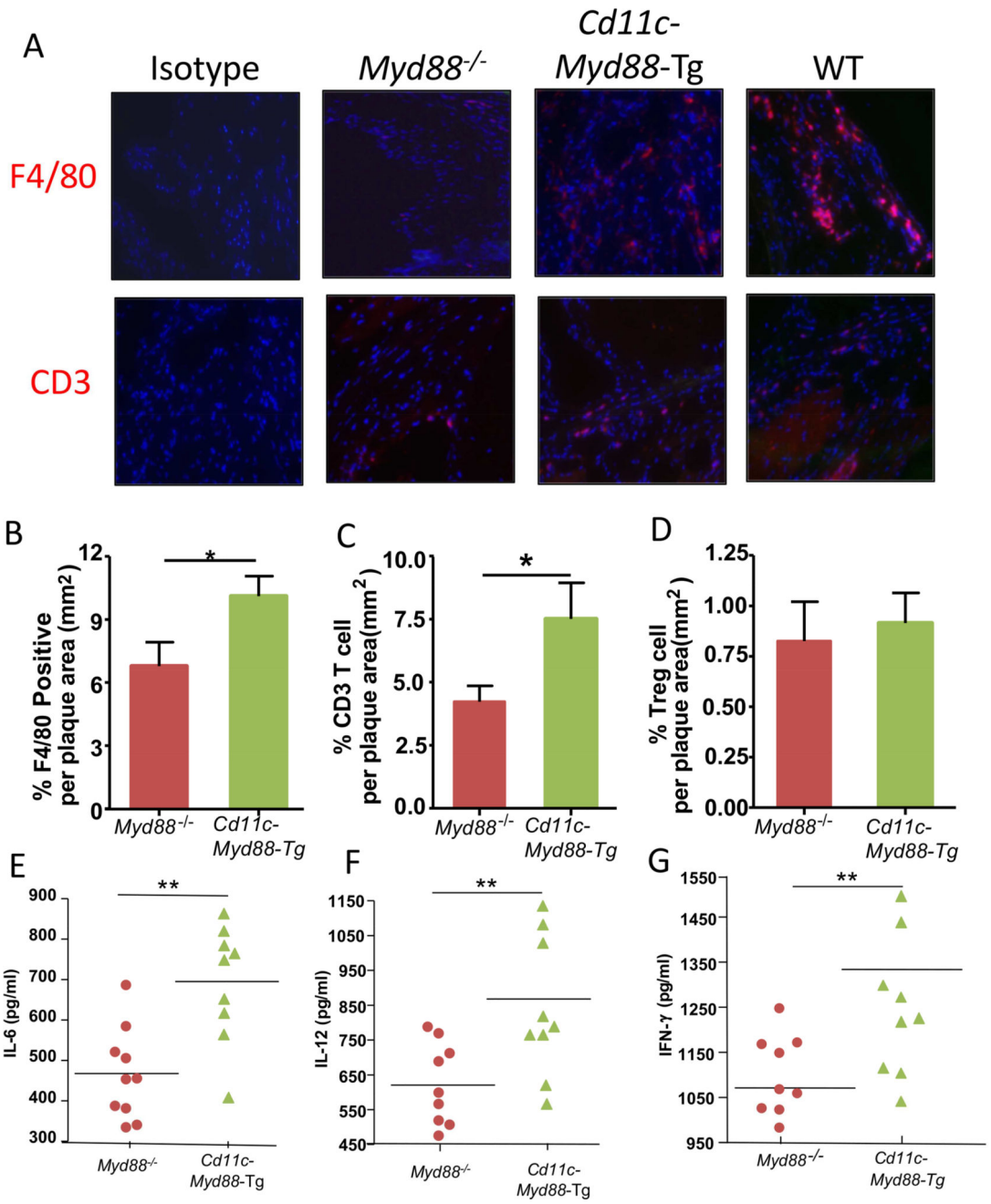


Figure 5. *Cd11c-Myd88*-Tg mice have increased macrophage and T-cell infiltration in aortic sinus plaques

All mice are on *Apoe*$^{-/-}$ background. *Myd88*$^{-/-}$ and *CD11c-Myd88*-Tg *Myd88*$^{-/-}$ mice were fed for 16 weeks with HFD. (A) F4/80 and CD3 staining in the aortic sinus of Myd88$^{-/-}$ and Cd11c-Myd88-Tg mice. Quantification F4/80 per plaque area (B) n=7–8, CD3 T cells (C) n=7–8. Foxp3 Treg (D) n=7–8. (E) Serum IL-6, n=9–10. (F) IL-12p40, n=9–10 (G) IFN-γ levels, n=9–10. Data represent means + SEM. Statistical significance was determined using 2 tails Student's t-test, *, $p < 0.05$; **, $p \leq 0.01$.

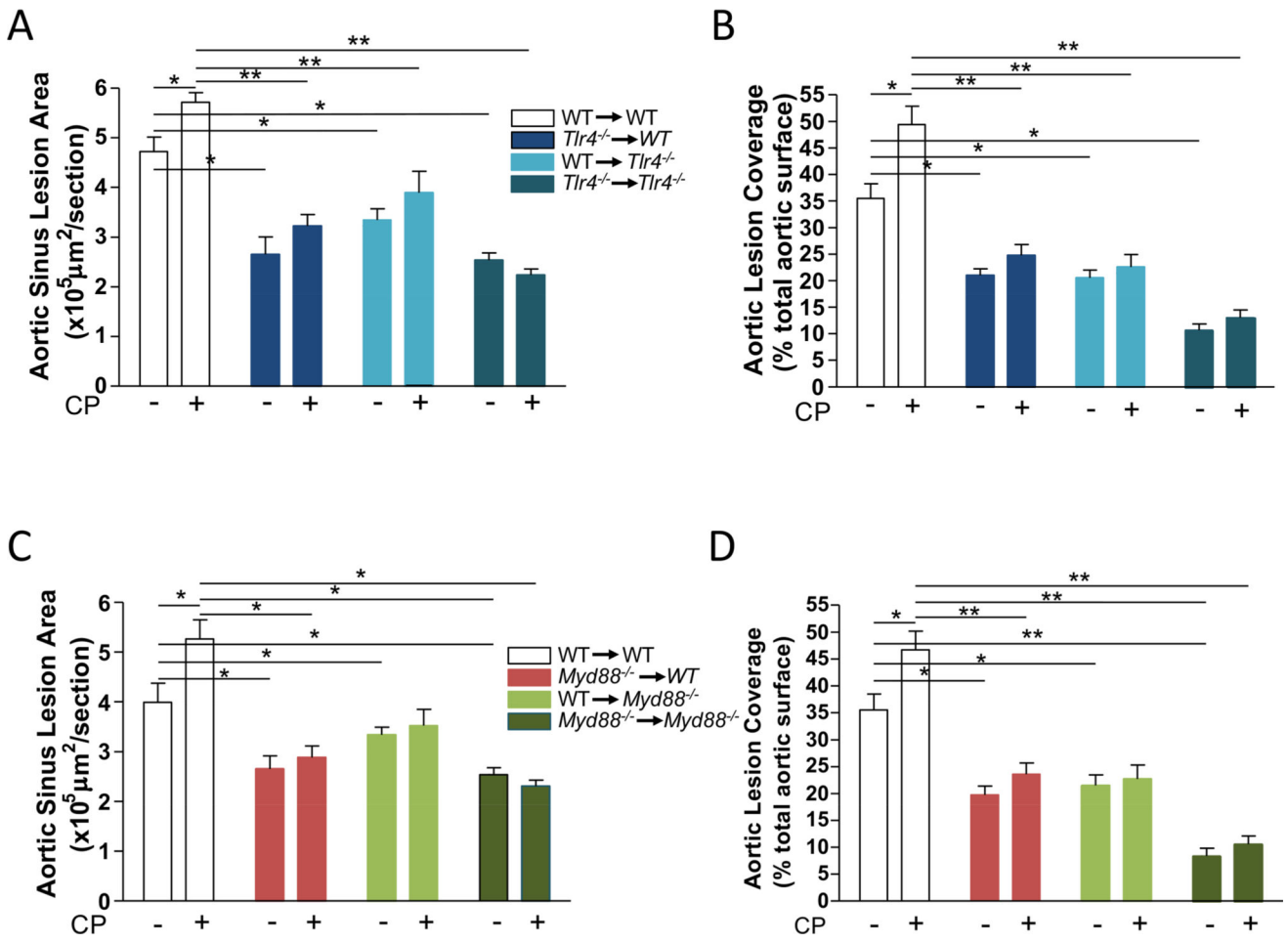


Figure 6. TLR4 and MyD88 are required in both hematopoietic and stromal cells for *C. pneumoniae*-induced acceleration of atherosclerosis

All mice are on *Apoe*$^{-/-}$ background. Irradiated WT, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice received WT, *Tlr4*$^{-/-}$, *or Myd88*$^{-/-}$ bone marrow cells. After 8 weeks reconstitution, the mice were infected with 5×10^4 IFU CP intranasal (once a week for three weeks) and fed HFD for 16 weeks. A) Quantification of area of aortic sinus plaques in *Tlr4*$^{-/-}$ chimeras. B) Aortic lesion coverage in *Tlr4*$^{-/-}$ chimeras. C) Quantification of area of aortic sinus plaques in *Myd88*$^{-/-}$ chimeras. D) Aortic lesion coverage in *Myd88*$^{-/-}$ chimeras. n=10–11. Data are presented as mean values ± SEM. Significance was determined using two-way ANOVA with Bonneferroni's post-test. *p<0.05, **p<0.01.

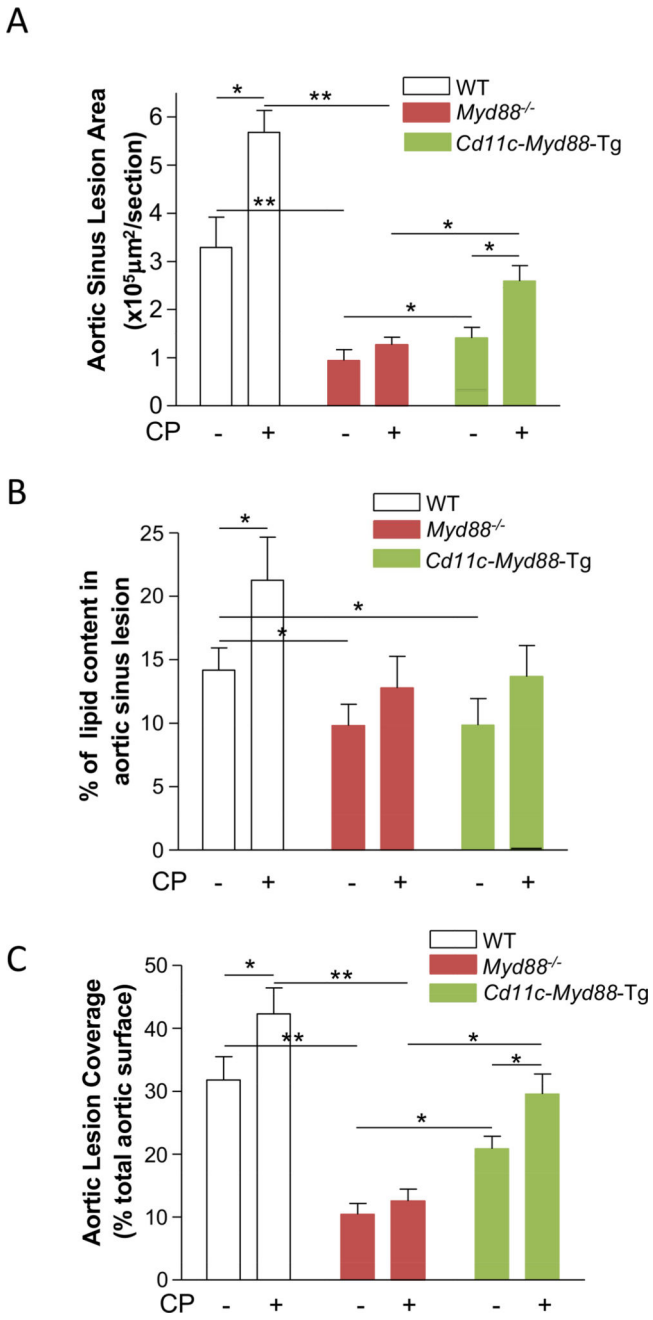
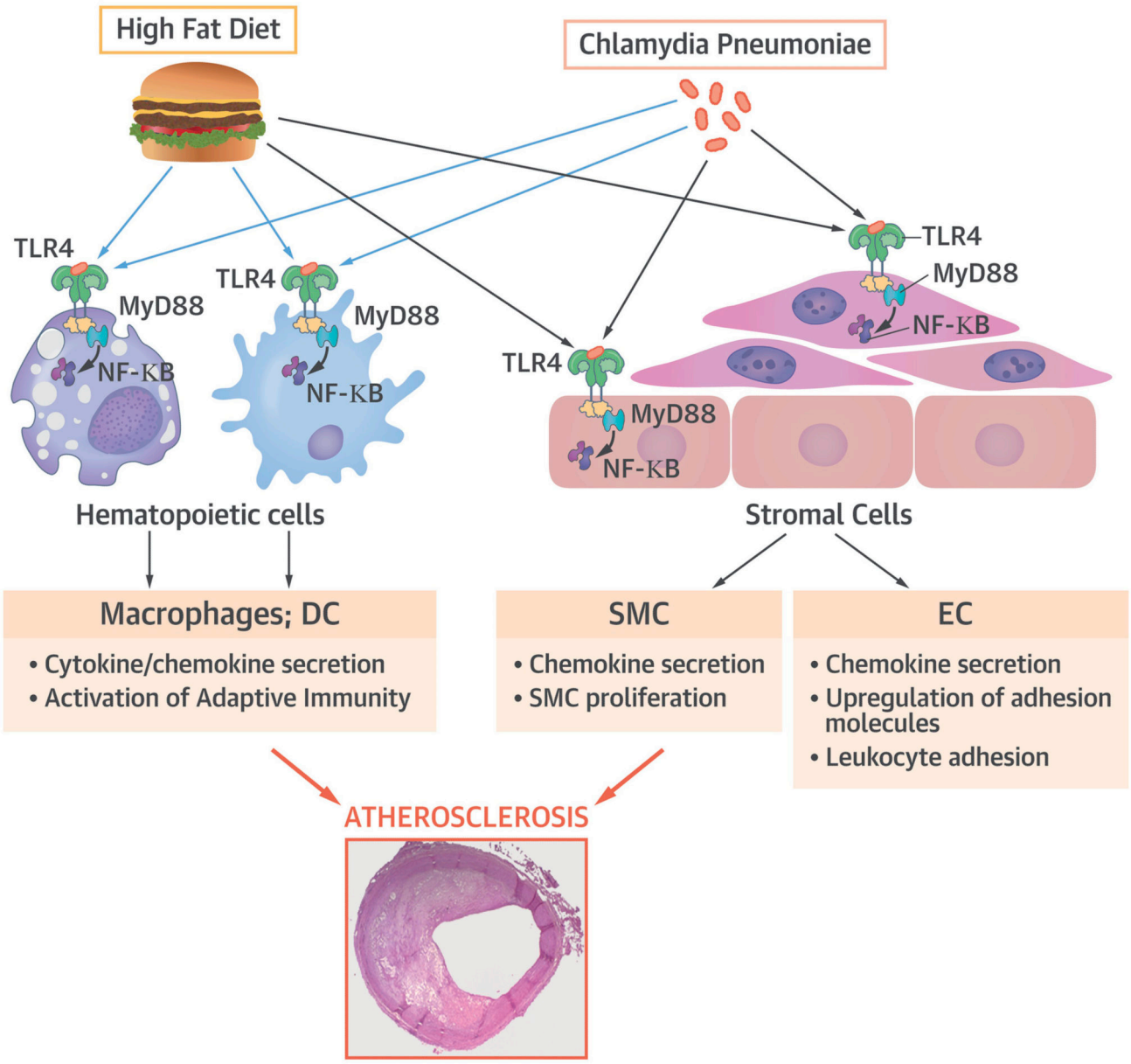


Figure 7. MyD88 in CD11c+ myeloid cells, including DCs is sufficient for *C. pneumoniae*-induced acceleration of atherosclerosis in otherwise MyD88-deficient mice

All mice are on *Apoe*$^{-/-}$ background. *Myd88*$^{-/-}$ and *CD11c-Myd88*-Tg/*Myd88*$^{-/-}$ mice were infected with 5×10^4 IFU CP intranasal (once a week for three weeks) and fed HFD for 16 weeks. A) Quantification of area of aortic sinus plaques. B) Lipid content of aortic sinus plaques. C) Aorta *en face* lesion coverage. n=10–11. Data are presented as mean values ± SEM. Significance was determined using two-way ANOVA with Bonneferroni's post-test. *p<0.05, **p<0.01.



Central Illustration. TLR4 and MYD88 in both bone marrow and stromal cells are required for atherosclerosis

TLR4/MyD88 expression by both stromal and hematopoietic cells plays an important role in diet-induced as well as CP infection-mediated acceleration of atherosclerosis in *Apoe*$^{-/-}$ mice.

Table 1

Total Cholesterol Level, lipoprotein profile and Triglyceride concentrations in Serum (mg/dl) chimeric mice

	Donor (BM) to Recipient		**Donor (BM) to Recipient**	
TC	WT to *Apoe*$^{-/-}$	913±67	WT to Apoe$^{-/-}$	956±82
	Tlr4$^{-/-}$ to Apoe$^{-/-}$	892±53	*MyD88*$^{-/-}$ to Apoe$^{-/-}$	937±46
	WT to Apoe$^{-/-}$; *Tlr4*$^{-/-}$	947±61	WT to Apoe$^{-/-}$; *MyD88*$^{-/-}$	908±76
	Tlr4$^{-/-}$ to Apoe−/−; *Tlr4*$^{-/-}$	879±84	*MyD88*$^{-/-}$ to Apoe$^{-/-}$; *MyD88*$^{-/-}$	941±58
HDL	WT to Apoe$^{-/-}$	61±15.6	WT to Apoe$^{-/-}$	48±15.4
	Tlr4$^{-/-}$ to Apoe$^{-/-}$	57±13.7	*MyD88*$^{-/-}$ to Apoe$^{-/-}$	52±13.1
	WT to Apoe$^{-/-}$; *Tlr4*$^{-/-}$	51±17.2	WT to Apoe$^{-/-}$; *MyD88*$^{-/-}$	57±16.5
	Tlr4$^{-/-}$ to Apoe−/−; *Tlr4*$^{-/-}$	64±18.6	*MyD88*$^{-/-}$ to Apoe$^{-/-}$; *MyD88*$^{-/-}$	67±17.9
LDL	WT to Apoe$^{-/-}$	98±18.1	WT to Apoe$^{-/-}$	112±15.4
	Tlr4$^{-/-}$ to Apoe$^{-/-}$	105±20	*MyD88*$^{-/-}$ to Apoe$^{-/-}$	107±16.7
	WT to Apoe$^{-/-}$; *Tlr4*$^{-/-}$	116±17.4	WT to Apoe$^{-/-}$; *MyD88*$^{-/-}$	97±19.8
	Tlr4$^{-/-}$ to Apoe−/−; *Tlr4*$^{-/-}$	109±16.7	*MyD88*$^{-/-}$ to Apoe$^{-/-}$; *MyD88*$^{-/-}$	118±17.9
TG	WT to Apoe$^{-/-}$	128±26	WT to Apoe$^{-/-}$	138±17.6
	Tlr4$^{-/-}$ to Apoe$^{-/-}$	132±19.1	*MyD88*$^{-/-}$ to Apoe$^{-/-}$	125±15.1
	WT to Apoe$^{-/-}$; *Tlr4*$^{-/-}$	141±16.9	WT to Apoe$^{-/-}$; *MyD88*$^{-/-}$	129±18.5
	Tlr4$^{-/-}$ to Apoe−/−; *Tlr4*$^{-/-}$	123±15.6	*MyD88*$^{-/-}$ to Apoe$^{-/-}$; *MyD88*$^{-/-}$	135±20.5

TC: Total Cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: Triglyceride; BM: bone marrow