Genetic inhibition of CARD9 accelerates the development of experimental atherosclerosis through CD36 dependent-defective autophagy

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Abstract

Macrophage-mediated innate immune responses contribute to the initiation, progression and complications of atherosclerosis. However, the underlying pathways linking activation of macrophages to atherosclerotic plaque development are still poorly understood. We hypothesized that activation of caspase recruitment-domain containing protein 9 (CARD9) plays a determinant role in pro-atherogenic responses in macrophages. We showed that global deletion of Card9 in male Apoe−/− mice as well as hematopoietic deletion of Card9 in female Ldlr−/− mice increased atherosclerosis. Card9−/− chimeric animals displayed more inflammatory atherosclerotic plaques and decreased systemic Th17 responses when compared to Card9+/+ chimeric mice. The acceleration of atherosclerosis was also observed in Apoe−/−Rag2−/−Card9−/− mice lacking T, B, and NKT cells, ruling out a role for the adaptive immune system in the pro-atherogenic effect of Card9 deficiency. Card9 deficiency altered macrophage phenotype with increased production of pro-inflammatory cytokines, improved lipid uptake, higher cell death susceptibility and defective autophagy. Rapamycin or metformin, two autophagy inducers, abolished intracellular lipid overload, restored macrophage survival and autophagy flux in vitro and finally abolished the pro-atherogenic effects of Card9 deficiency in vivo. Card9 deficiency up-regulated Cd36 expression in macrophages, which blocked AMPK phosphorylation, a key inducer of autophagy. In the absence of Cd36, the pro-atherogenic effects of Card9 deficiency were blunted both in vitro and in vivo. Transcriptomic analysis of human monocytes isolated from CARD9-deficient patients confirmed the pathogenic signature identified in murine models. In summary, we identified CARD9 signaling as a key protective pathway in atherosclerosis, modulating macrophage CD36-dependent inflammatory responses, lipid uptake and autophagy.

Introduction

Atherosclerosis is a chronic inflammatory disease of large and medium sized arteries that develops in response to subendothelial retention and modification of ApoB containing low-density lipoproteins (LDL) 1. Inflammatory activation of endothelial cells orchestrates the recruitment of different subsets of circulating leukocytes, notably monocytes, into the vascular wall. Recruited monocytes significantly contribute to the pool of intimal macrophages 2, which promote the growth of atherosclerotic plaque after differentiation, activation and proliferation. Macrophage activation, an instrumental step in the development of atherosclerosis, is associated with the upregulation of pattern recognition receptors for innate immunity, including scavenger receptors (SR-A, CD36) and pathogen recognition receptors such as Toll-like receptors (TLRs) and Dectin receptors 3–5. A broad range of molecules and particles bearing danger-associated molecular patterns, including oxidized LDL, can be taken up by macrophages, leading ultimately to the formation of pro-inflammatory foam cells 6. A large body of evidence suggest that, cumulative metabolic/inflammatory signals and impaired efferocytosis foster foam cell apoptosis and next apoptotic foam cells undergo secondary necrosis contributing to the growth of the necrotic core and progression of atherosclerosis 7. However, the critical downstream pathways that drive both macrophage activation and conversion into foam cells are still poorly understood.
Here, we investigated the role of Caspase recruitment-domain containing protein 9 (Card9) in atherosclerosis. Card9 is an adaptor protein that integrates pattern recognition receptor downstream signals in macrophages and dendritic cells. Card9 is particularly involved in response to fungi via C-type lectin sensing, but also in response to bacteria by mediating nucleotide-binding oligomerization domain 2 (NOD2)-dependent p38/JNK signaling and TLR signaling. Card9 is required to mount appropriate immune responses, through the production of interleukin (IL)-6, IL-17A, IFN-γ, and IL-22. The role of Card9 in inflammatory diseases is ambiguous, being pathogenic in post-ischemic cardiac remodeling but protective in experimental colitis.

Here, we show that Card9 signaling pathway in macrophages regulates cytokine production, lipid upload and cell survival. Global, as well as hematopoietic deletion of Card9, markedly accelerates the development of atherosclerosis, independently of the adaptive immune system. Mechanisms of the pro-atherogenic effects of Card9 deficiency mainly involve CD36-dependent defective autophagy.

**Methods**

**Human carotid plaques.** Formalin-fixed and paraffin-embedded arterial tissue sections were used after antigen retrieval by heating in microwave oven in buffer pH9. For single labeling, CARD9 rabbit polyclonal antibody (Abcam, Cambridge, UK) was used at 1:500 dilution, incubated for 1 hour and revealed using ABC-peroxidase technique (Vector Laboratories, Burlingame, CA, USA). For double labeling, the sections were first incubated with mouse monoclonal anti-CD68 antibodies diluted at 1:50 (to detect macrophages) or anti-a smooth muscle actin antibodies diluted at 1:100 (to detect smooth muscle cells), both from Dako-Agilent (Trappes, France). Sections were then incubated with species-specific secondary antibodies (1:500 dilution, 45 min at room temperature) conjugated to AlexaFluor 488 or 594 (Fischer Scientific) and mounted on microscope slides using the Prolong Antifade Diamond kit (ThermoFisher). Image acquisition was performed on a laser scanning confocal microscope (Leica TCS SP8, Leica Microsystems). Immunostaining studies were performed on normal aorta, atherosclerotic plaques after endarterectomy or on carotid artery obtained post-mortem at autopsy (Ethical Committee CPP Ile de France 2013-13-19).

**Animals.** Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and were approved by the Ethical Committee of INSERM and the French Ministry of Agriculture (agreement A75-15-32). Card9−/− and Rag2−/−Card9−/− mice (null for the Card9 gene), obtained from Dr. Harry Sokol (Centre de Recherche Saint-Antoine UMR_S 938, Paris, France) have been previously described. They were backcrossed for over 10 generations into a C57BL/6J background. Cd36−/− mice (null for the Cd36 gene) C57bl/6 Cd36−/− mice were generated in Dr. Roy Silverstein's laboratory (Febbraio et al JBC 1999) and were crossed with Card9−/− mice to generate Card9−/−Card36−/− mice. Ten-week old male C57BL/6J Ldlr−/− mice were subjected to medullar...
aplasia by lethal whole-body irradiation (9.5 grays). The mice were repopulated with an intravenous injection of bone marrow (BM) cells isolated from femurs and tibias of sex-matched C57BL/6J Card9−/− mice or Card9+/+ littermates. After 4 weeks of recovery, mice were fed a pro-atherogenic high-fat diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 8 weeks. During the first 14 days following BM cell transfer, animals were administered an antibiotic in their drinking water (Baytrilâ, Enrofloxacin).

**Pharmacological in vivo treatment**

Eight-week old male Apoe−/− Card9+/+ and Apoe−/− Card9−/− mice were treated with daily intraperitoneal injections of rapamycin (4 mg/kg body weight) for 6 weeks and were put on a high fat diet. Eight-week old male Apoe−/− Card9+/+ and Apoe−/− Card9−/− mice were treated with metformin (300 mg/kg body weight, drinking water) for 6 weeks and were put on a high fat diet.

**Extent and composition of atherosclerotic lesions.** Plasma cholesterol was measured using a commercial cholesterol kit (DiaSys® Cholestérol FS*). Quantification of lesion size was performed as described previously. Briefly, the basal half of the ventricles and the ascending aorta were perfusion-fixed in situ with 4% paraformaldehyde, then transferred to a PBS-30% sucrose solution, embedded in frozen OCT and stored at −80°C. Serial 10-μm sections of the aortic sinus with valves (80 per mouse) were cut on a cryostat, as previously described. One section out of 5 was used for plaque size quantification after Oil red O staining. In total, 16 sections spanning over 800 mm of the aortic root were used to determine the mean lesion area for each mouse. After PBS flushing, the aorta from the root to the iliac bifurcation was removed and fixed with 10% neutral-buffered formalin. After thorough PBS washing, the adventitial tissue was removed and the aorta was longitudinally opened to expose the luminal surface for en-face visualization of atherosclerotic lesions after Oil Red O staining. Quantification of Oil Red O positive surface area was performed by a blinded operator. Aortic collagen content was detected using Sirius red staining. Necrotic core surface was quantified after Masson’s Trichrome staining. At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Morphometric studies were performed using Histolab software (Microvisions). For immunostaining on mouse atherosclerotic plaques, we used antibodies raised against Card9 (AA 274-530), MOMA-2 (MAB1852, Merck Milliporeâ) and CD3 (A0452, Dakoâ). The presence of macrophages was determined using specific antibodies, as previously described. TUNEL (Terminal dUTP nick end-labeling) staining was performed using In Situ Cell Death Detection Kit (histochemistry staining) and TMR Red kit (Fluorescent staining) (Roche).

**Splenocyte culture.** Splenocytes were cultured in RPMI 1640 supplemented with Glutamax, 10% fetal calf serum (FCS), 0.02 mM b-mercaptoethanol and antibiotics. For cytokine measurements, splenocytes were
stimulated with LPS (1 mg/ml) and IFN-γ (100 UI/ml) for 24 hours. IL-1β, IL-10 and TNF-α production in the supernatants were measured using specific ELISA immunoassay kits (BD Biosciences).

**Spleen cell recovery and purification.** Spleen cells were purified according to standard protocols as follows. CD4+ T cells were negatively selected using a cocktail of anti-CD8a, anti-CD11b, anti-CD45R, anti-DX5, anti-ter 119 antibody-coated magnetic beads, yielding CD4+ cells with >95% purity (Miltenyi Biotech). CD11c+ cells were positively selected with biotin-conjugated anti-CD11c mAb (7D4, PharMingen) and captured with streptavidin microbeads (Miltenyi Biotech) followed by 2 consecutive magnetic cell separations using LS columns (Miltenyi Biotech), yielding CD11c+ cells with >80% purity.

**CD4+ T cell culture and cytokine assays.** Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM β-mercaptoethanol and antibiotics. For cytokine measurements, CD4+ T cells were cultured at 1x10^5 cells/well for 48 hours in anti-CD3-coated microplates (5 mg/ml). In some experiments, CD4+ T cells were stimulated with purified soluble CD3-specific antibody (1 mg/ml, Pharmingen) in the presence of antigen-presenting cells that were purified on CD11c-coated magnetic beads (Miltenyi Biotech). Secretion of IL-17A, IL-22, IL-10 and IFN-γ secretion in the supernatants was measured using analyte-specific ELISAs (BD Biosciences and R&D Systems). T cell proliferation was measured using the Quick Cell proliferation Assay Kit II (Abcam).

**Macrophage experiments**

Primary macrophages were derived from mouse BM cells (BMDMs). Tibias and femurs of C57Bl6/J male mice were dissected and their marrow flushed out. Cells were grown in RPMI 1640 medium, 10% FCS, and 15% Macrophage–Colony-Stimulating Factor (M-CSF)-rich L929-conditioned medium for 7 days at 37°C. To analyze oxidized LDL uptake, BMDMs were exposed to human oxidized LDL (25mg/ml) for 24 hours (see oxidation method below). Cells were then washed, fixed and stained using Bodipy (493/503, Thermofischer Scientific D3922). Foam cells were quantified blindly on 6-8 fields and the mean was recorded. To analyze apoptosis susceptibility, macrophages were incubated with OxLDL (200 mg/ml) for 24 h or in a FCS-poor medium (1% FCS, starvation) or with TNF-α (10 ng/ml) and cycloheximide (10 mmol/l) for 6 h or with etoposide (50 mmol/l). Apoptosis was determined by independent experiments using Annexin V- (FITC) apoptosis detection kit with 7-AAD (PerCP) (BD Biosciences) according to the manufacturer’s instructions. Intracellular cholesterol (total and ester) quantification was done using Amplex® Red Cholesterol Assay Kit (Invitrogen A12216).

**Cholesterol efflux assays**

BMDMs were obtained by differentiation of BM cells in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20% L929 cell-conditioned media (as a
source of M-CSF), and penicillin-streptomycin for 5 days. BMDMs were loaded with 50 µg/ml [3H]cholesterol-labeled acetylated LDL (acLDL, 1 µCi/mL) for 48 hours in serum-free DMEM supplemented with 50 mM glucose, 2 mM glutamine, 0.2% BSA (RGGB) and 100 µg/ml penicillin / streptomycin. The labeling medium was then removed and cells were washed twice in PBS and then equilibrated in RGGB for additional 16-24 hours. To measure cholesterol efflux, cells were incubated 4 hours at 37°C in the presence of 60µg/ml lipid-free apoAI (Sigma), or 30 µg/ml HDL-PL (density=1.063-1.21 g/ml), isolated from normolipidemic plasma by preparative ultracentrifugation, as cellular cholesterol acceptor. Finally, culture media was harvested and cleared of cellular debris by brief centrifugation. Fractional cholesterol efflux (expressed as a percentage) was calculated as the amount of radio-label detected in the supernatants divided by total radio-label in each well (radioactivity in the supernatant plus radioactivity in the cells) obtained after lipid extraction from cells in a mixture of 3:2 hexane:isopropanol (3:2 vol/vol). The background cholesterol efflux obtained in the absence of any acceptor was subtracted from the efflux obtained with samples.

**Flow cytometry**

Blood and spleen samples were collected at sacrifice for analysis of leukocyte subsets. Myeloid cells were identified as CD45+CD11b+. Monocytes were identified as CD11b+CD115+. Among them, classical monocytes were Gr1<sup>high</sup> (or Ly6C<sup>high</sup>) and non-classical monocytes were Gr1<sup>low</sup> (or Ly6C<sup>low</sup>). Neutrophils were identified as CD11b+CD115-Gr1+ (or CD11b+CD115-Ly6G+). B220+IgM+ B lymphocytes, CD4+ and CD8+ T lymphocyte subsets were also analyzed. Antibodies raised against CD11b, CD115, Gr1 (Ly6C and G), B220, CD4, CD8a, NK1.1, CD45, F4/80, CD3<sup>ε</sup>, MHC II, IgM, CD11c and CD36 were used for immunostaining and are listed in supplementary table 1.

Forward scatter (FSC) and side scatter (SSC) were used to gate live cells excluding red blood cells, debris, and cell aggregates in total blood cells and splenocytes preparations. Cells were acquired using a BD LSRII Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo™ (TreeStar, Inc.).

**Quantitative real-time PCR.**

Quantitative real-time PCR was performed, after Trizol RNA extraction and RT-PCR, on an ABI Prism 7700 (Applied Biosystems™) in triplicate for each sample. Expression of all genes has been normalised to the expression level of Gapdh. Relative expression was calculated using the 2-delta-delta CT method followed by geometric average, as recommended<sup>15,16</sup>. The following primer sequences were used: Card9 (F: 5’- GAC CCT CTT AGT CCC AAT CTG -3’; R: 5’- CTC GTC GTC ATT CTC ATA GTC TG -3’), Mrs1 (F: 5’- CCG TGA ATC TAC AGC AAA GCA -3’; R: 5’- CCC AGT CCT TCA GTC TGA GG -3’), Scarb1 (F: 5’- CCT CTC
Transcriptomic analysis on human monocytes

The study was approved by the Committee for the Protection of Human Subjects in Biomedical Research (C10-14 Prédisposition génétique aux infections fongiques sévères). RNA sequencing libraries were prepared from 100 to 200 ng of total RNA using the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero Plus library preparation kit, which allows performing a strand specific sequencing. This protocol includes a first step of enzymatic depletion of abundant transcripts from multiple species (including human cytoplasmic & mitochondria rRNA, mouse rRNA, rat rRNA, bacteria Gram +/- rRNA, human beta globin transcripts) using specific probes. cDNA synthesis was then performed and resulting fragments were used for dA-tailing followed by ligation of RNA Index Anchors. PCR amplification with indexed primers (IDT for Illumina RNA UD Indexes) was finally achieved, with 13 cycles, to generate the final cDNA libraries. Individual library quantification and quality assessment were performed using Qubit fluorometric assay (Invitrogen) with dsDNA HS (High Sensitivity) Assay Kit and LabChip GX Touch using a High Sensitivity DNA chip (Perkin Elmer). Libraries were then equimolarly pooled and quantified by qPCR using the KAPA library quantification kit (Roche). Sequencing was carried out on the NovaSeq 6000 instrument from Illumina using paired-end 2 x 100 bp, to obtain around 100 million clusters (200 million raw paired-end reads) per sample. Raw and normalized counts are provided in Supplementary Table 2.

We performed the gene set enrichment analysis using clusterProfiler v4.0.5 with selected pathways from Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and Benjamini-Hochberg correction was applied. Used keywords were: “apopto*”, “atheroscleros*”, “NF-kappa B”, “TNF”. All pathways with adjusted p-value below 0.05 were considered as significantly enriched. For each category, median expression of all gene included in core enrichment were calculated for each patient before plotting the heatmap.

Single-cell analysis of Card9/CARD9 expression patterns

The murine scRNA-seq dataset was generated by integrating single-cell gene expression data of aortic cells from steady-state or atherosclerotic vessels from several published studies. Data were integrated using canonical correlation analysis in Seurat v3. Human scRNA-seq data were mined from reference and correspond to single-cell analysis of total cells from atherosclerotic coronary arteries dissected from explanted hearts of transplant recipients (n=4 patients) and analyzed in Seurat v3. Expression of Card9/CARD9 was mapped using the FeaturePlot function in Seurat. For clarity, minimum
and maximum gene expression cutoffs were applied, and cells with detectable transcripts were brought to the front of plots using the “order=TRUE” argument within the Seurat v3 FeaturePlot function.

**Western-Blot**

Proteins from BMDMs were extracted by pipetting in ice-cold lysis buffer (NaCl 150 mM, HEPES 20 mM, EDTA 1 mM, EGTA 1 mM, NP-40 0.25% (Vol/Vol)) supplemented with protease (1 tab/10ml, Roche) and phosphatase (Na$_4$VO$_3$ 2 mM) inhibitors). Protein extracts were briefly sonicated (5 pulses, amplitude 20, Vibracell 75021) and quantified by BCA (Thermofisher Scientific). Equal protein amounts were separated by SDS-PAGE (10% acrylamide) and transferred onto nitrocellulose membrane (0.2 μm, Biorad). For LC3b detection, SDS-PAGE separated proteins were transferred onto polyvinylidene difluoride membranes.

Membranes were then blocked with Tris Buffered Saline supplemented with 0.1% Tween-20 (TBST) and 2% BSA (2h at room temperature), then incubated with primary antibodies (overnight, 4ºC) diluted following the manufacturer's recommendations. After three washes in TBST, membranes were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies (1:8,000 dilution, 45 min at room temperature). After three washes in TBST, the peroxidase activity was detected using Clarity Western ECL Substrate (Biorad) using Cytiva's ImageQuant Fluor 800. The migration position of transferred proteins was compared to the PageRuler Prestain Protein Ladder (10 to 170 kDa, Thermo Fisher Scientific). Densitometric analysis was performed using ImageJ software (NIH). Phosphorylated protein signals were normalized on total protein levels, whereas non-phosphorylated proteins were normalized on β-actin protein levels. Primary and HRP-coupled secondary antibodies used for immunoblotting experiments are listed in supplementary table 1.

**Microbiota analysis**

**Stool collection and DNA extraction**

Fecal samples were homogenized and 0.2g aliquots were stored at −80°C for further analysis. DNA was extracted from fecal samples as previously described. Briefly, following microbial lysis by mechanical and chemical methods, nucleic acids were precipitated in isopropanol for 10 min at room temperature, incubated for 15 min on ice and centrifuged at 20,000g for 30 min at 4°C. Pellets were resuspended in 450 μl of PBS and 50 μl of potassium acetate. After RNase treatment and DNA precipitation, nucleic acids were recovered via centrifugation at 20,000g for 30 min at 4°C. The pelleted DNA was resuspended in 80 ml of trypsin-EDTA buffer.

**Sequencing**
Microbiota analysis was performed by amplicon sequencing of the V3-V4 region of the 16S ribosomal RNA gene. This region was amplified using the following primers – 16S sense 5′-TACGGRAGGCAGCAG-3′ and anti-sense 5′-CTACCNGGGTATCTAAT-3′ – according to an optimized and standardized 16S amplicon library preparation protocol (Metabiote, GenoScreen, Lille, France). Briefly, PCR of the 16S DNA was performed with 5ng of genomic DNA according to the manufacturer’s protocol (Metabiote), with bar-coded primers (Metabiote MiSeq Primers) to a final concentration of 0.2 µmol/l, with an annealing temperature of 50°C for 30 cycles. Purification of the PCR products was performed with Agencourt AMPure XP-PCR purification system (Beckman Coulter, Brea, CA, USA), and quantified following the manufacturer’s instructions. The samples were multiplexed at equal concentrations. Sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a 250 bp paired-end sequencing protocol at GenoScreen. Raw paired-end reads were subjected to the following processes: (1) quality filtering using the PRINSEQ-lite PERL script, by truncating the bases from the 3′ end, that did not exhibit a quality <30, based on the Phred algorithm and (2) searching for and removing both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in the primer sequences. Only sequences where perfect matching forward and reverse primers were detected were included.

16S sequence analysis

Sequences were quality filtered using the dada2 software package (version 1.12.1) in the R programming language (R version 3.6.1) to produce amplicon sequence variants (ASVs). Taxonomic classification was performed using the Silva reference database (version 132). Bacterial ASVs that could not be assigned to Phylum-level taxonomy were excluded. Alpha diversity was estimated using the number of observed species and the Shannon diversity index. Raw sequence data are accessible in the Sequence Read Archive (accession number pending). Beta diversity analysis was performed on proportion-normalized data using the Bray-Curtis index. Assessment for significant differences between clusters was performed using PERMANOVA with the adonis function in the vegan package (version 2.5-6) in R with 99999 permutations.

Differential abundance was tested using linear discriminant analysis with effect size (Lefse) using default settings.

LDL isolation and oxidation

LDL from normal human pooled sera was prepared by ultracentrifugation and dialyzed against PBS containing 100 µM EDTA. The LDL pool was then diluted to 2 g/l with PBS into a final volume of 3 ml. LDLs were mildly oxidized by UV-C for 2 h in the presence of 5 µM CuSO4 as previously reported. Oxidized LDL contained 4.2 to 7.4 nmoles of TBARS (thiobarbituric acid-reactive substances) /µg apoB.
Relative electrophoretic mobility (REM) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) reactive amino groups were 1.2-1.3 times and 85-92% of native LDL, respectively.

**Statistical analysis.** Graphs and statistical analyses were performed using Prism software (Graphpad). Values are expressed as mean ± s.e.m. Differences between values were examined using the non-parametric Mann-Whitney test. When 3 or more experimental groups were compared, Kruskal-Wallis test was used. Statistical significance was reached when P<0.05.

**Results**

**Genetic invalidation of Card9 accelerates atherosclerosis in Apoe-/- mice**

To gain insight into the immune cells expressing Card9, single-cell analysis of total cells from mouse atherosclerotic plaques was performed. Card9 transcript was detected in myeloid cells, and macrophages in particular (Figure 1a-b). Immunofluorescence staining confirmed that Card9 was expressed in atherosclerotic lesions of Apoe-/- mice at both early (Supplementary fig.1) and advanced stages of atherosclerosis (Figure 1c) and mainly co-localized with MOMA+ macrophages (Figure 1c).

To investigate the role of Card9 in this experimental setting, we generated Apoe-/-Card9-/- mice. Card9 deficiency was confirmed by qPCR in peritoneal macrophages (Figure 1d) and by immunostaining of atherosclerotic plaques (Supplementary fig.2). Body weight was slightly increased in Card9 deficient mice, but no significant differences in plasma cholesterol levels were observed between Apoe-/-Card9+/+ and Apoe-/-Card9-/- mice (Supplementary fig.3a-b). At 8 weeks of age, animals were put on a high-fat diet for 6 weeks to accelerate plaque formation. Apoe-/-Card9-/- mice showed a significant increase in atherosclerotic lesion size in the aortic sinus (368 ± 64 vs 278 ± 87. 10³ mm², P<0.05) (Figure 1e).

Card9 deletion in Apoe-/- mice induced a switch toward a more inflammatory plaque phenotype with a significant increase in macrophage accumulation (Figure 1f) and necrotic core size (Figure 1g). Collagen content was increased in plaques of Apoe-/-Card9-/- mice (Supplementary fig.3c), but T cell accumulation was similar (Supplementary fig.3d).

**Dampened systemic pro-inflammatory cytokine signature in Apoe-/-Card9-/- mice**

Because Card9 is known to modulate cytokine production and T cell polarization ¹¹, we next investigated the immuno-inflammatory response in Apoe-/-Card9+/+ and Apoe-/-Card9-/- mice. Leukocyte populations were analyzed by flow cytometry in both blood and spleen at sacrifice. We did not observe any significant difference in leukocyte percentages in blood between groups. We only found a slight increase in
neutrophil and classical monocyte counts in the blood of Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice, compared to control Apoe<sup>−/−</sup>Card9<sup>+/+</sup> mice (Supplementary fig.4-5). Splenocyte number was significantly higher in Card9 deficient mice but the proportion of myeloid and lymphoid populations was not different between groups (Supplementary fig.6-8). Splenocytes from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice stimulated with IFN-g and LPS produced less TNF-a (Figure 1h) than those from control mice, but the production of IL-10 and IL-1b was not different. We then purified splenic CD4<sup>+</sup> T cells from control Apoe<sup>−/−</sup>Card9<sup>+/+</sup> and Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice and performed functional tests. In vitro, the proliferation of CD4<sup>+</sup> T cells from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice was significantly increased compared with control cells (Supplementary fig.9), and their production of IFN-g and IL-17A was increased. There were no differences in IL-10 and IL-22 production (Figure 1i).

**Hematopoietic Card9 deficiency increases atherosclerosis in Ldlr<sup>−/−</sup> mice**

To confirm our result in another mouse model of atherosclerosis, we performed bone marrow (BM) transplantation experiments using either Card9<sup>+/+</sup> or Card9<sup>−/−</sup> BM cells to repopulate lethally irradiated Ldlr<sup>−/−</sup> mice (Supplementary fig.10). We confirmed that Card9 gene expression was almost abolished in peritoneal macrophages of chimeric Ldlr<sup>−/−</sup>Card9<sup>−/−</sup> (Figure 2a). Chimeric Ldlr<sup>−/−</sup> mice were then fed a high-fat diet for 8 weeks. We observed no difference in body weights (Figure 2b) or serum cholesterol levels (Figure 2c) between the 2 groups of chimeric mice. In blood, myeloid populations were not different between groups, but we observed a significant increase in circulating CD4<sup>+</sup> T and B cell counts in chimeric Ldlr<sup>−/−</sup>Card9<sup>−/−</sup> mice (Supplementary fig.11-12). As shown in Figure 2, hematopoietic Card9 deficiency was associated with a significant increase in lesion development compared with controls, in the thoracoabdominal aorta (Figure 2d), and in the aortic sinus (Figure 2e). In addition, Card9 deletion induced a more inflammatory plaque phenotype with a significant increase in both macrophage accumulation (Figure 2f) and necrotic core size (Figure 2g). We also observed an increase in collagen content in plaques of chimeric Ldlr<sup>−/−</sup>Card9<sup>−/−</sup> mice (Supplementary fig.13). T cell accumulation in plaques was similar in the 2 groups (Supplementary fig.13). Stimulated splenocytes from Ldlr<sup>−/−</sup>Card9<sup>−/−</sup> mice produced less TNF-a, IL-1b, and IL-10 than splenocytes from Ldlr<sup>−/−</sup>Card9<sup>+/+</sup> mice (Figure 2h). Splenic CD4<sup>+</sup> T cells isolated from Ldlr<sup>−/−</sup>Card9<sup>−/−</sup> mice produced less IL-17A than CD4<sup>+</sup> T cells from control Ldlr<sup>−/−</sup>Card9<sup>+/+</sup>mice. IL-10, IFN-g and IL-22 production was not affected in our experimental conditions (Figure 2i).

**Gut microbiota does not participate to atherosclerosis development in Card9 deficient animals**

Previous studies have shown that Card9 plays a critical role in gut microbiota homeostasis. Card9<sup>−/−</sup> mice have been shown to display dysbiosis<sup>33</sup>, which has been implicated in atherosclerosis development<sup>34</sup>. To evaluate a potential impact of Card9-induced dysbiosis in our experimental conditions, we analyzed
the bacterial microbiota was performed using 16S rRNA based sequencing. While there were no significant differences in alpha diversity between Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice (Supplementary fig.14a-b), beta diversity analysis showed a significant difference between the 2 groups, as demonstrated by the PCoA plot of Bray-Curtis distance (Supplementary fig.14c). To determine which taxonomic groups accounted for these differences, we performed linear discriminant analysis with effect size (Lefse) 31. Compared to Apoe<sup>-/-</sup>Card9<sup>+/+</sup> mice, Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice displayed an increase in the pathobiont Helicobacter with a concomitant decrease in beneficial members of the Firmicutes phylum, including the order Clostridiales, as well as in Candidatus arthromitus, segmented filamentous bacteria. These bacteria are essential for Th17 maturation in the murine gut 35, and in the genus Akkermansia, a genus associated with a lean body type and favorable metabolic outcomes 36 (Supplementary fig. 14d).

Given the marked dysbiosis in Card9 deficient Apoe<sup>-/-</sup> mice, we aimed to determine whether this dysbiosis was also observed in Ldlr<sup>-/-</sup> mice transplanted with Card9<sup>-/-</sup> bone marrow cells. As found in Apoe<sup>-/-</sup> mice, 16S rRNA based sequencing showed no significant difference in alpha diversity among the 2 groups, but the beta diversity analysis showed significant differences in mice transplanted with Card9<sup>-/-</sup> BM cells (Supplementary fig. 15a-b). Of note, because of transient aplasia and increased risk of sepsis chimeric Ldlr<sup>-/-</sup> mice received antibiotics during 14 days following lethal irradiation and BM cell transplantation. The administration of antibiotics caused marked changes in the microbiota in the 2 groups. In particular, at genus level, Parasutterella was increased in Ldlr<sup>-/-</sup>Card9<sup>-/-</sup> mice and the sulfate-reducing bacteria Desulfovibrio was enhanced in Ldlr<sup>-/-</sup>Card9<sup>+/+</sup> mice. Only one bacterial family, Clostridiaceae_1 that was increased in Ldlr<sup>-/-</sup>Card9<sup>+/+</sup> mice, was concordantly altered in the two sets of experiments (Supplementary fig.15c-d). Taken together, these data confirm an effect of the Card9 on the gut microbiota composition. However, the highly divergent microbiota composition between Apoe<sup>-/-</sup> and Ldlr<sup>-/-</sup> genetic backgrounds despite similar Card9 effects on atherosclerosis strongly suggest that the gut microbiota did not mediated the Card9-related impacts on atherosclerosis.

**Pro-atherogenic effect of Card9 deficiency is not dependent on adaptive immunity.**

As described above, Card9 deficiency had major effects on CD4<sup>+</sup> T cell proliferation and polarization. In order to evaluate the role of adaptive immunity in the acceleration of atherosclerosis observed in Card9 deficient mice, we backcrossed Apoe<sup>-/-</sup>Rag2<sup>-/-</sup> mice with Rag2<sup>-/-</sup>Card9<sup>-/-</sup> to generate athero-prone lymphocyte (T, B, NKT)-deficient Apoe<sup>-/-</sup>Rag2<sup>-/-</sup>Card9<sup>-/-</sup> mice. Eight-week old control Apoe<sup>-/-</sup>Rag2<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Rag2<sup>-/-</sup>Card9<sup>-/-</sup> mice were fed a high-fat diet for 6 weeks. Card9 deficiency was confirmed by qPCR in peritoneal macrophages (Supplementary fig.16a). There was no significant difference in body weight between groups (Supplementary fig.16b) but plasma cholesterol levels were slightly increased in Card9 deficient mice (Supplementary fig.16c). Apoe<sup>-/-</sup>Rag2<sup>-/-</sup>Card9<sup>-/-</sup> mice exhibited a significant increase in atherosclerotic lesion size in the thoracoabdominal aorta (Supplementary fig.16d) and in the aortic sinus (Supplementary fig.16e). In addition, Card9 deletion in Apoe<sup>-/-</sup>Rag2<sup>-/-</sup> mice induced
a switch toward a more inflammatory plaque phenotype with increased macrophage accumulation (Supplementary fig.16f) and necrotic core size (Supplementary fig.16g). Stimulated splenocytes from Apoe<sup>−/−</sup>Rag2<sup>−/−</sup> Card9<sup>−/−</sup> mice produced less TNF-a, IL-1b, and IL-10 than those from Apoe<sup>−/−</sup>Rag2<sup>−/−</sup> Card9<sup>+/+</sup> mice (Supplementary fig.16h). Altogether, these results suggest that the adaptive immune system was not involved in the pro-atherogenic effects of Card9 deficiency.

*Card9* deficiency upregulates CD36 expression and increases foam cell formation

Next, we speculated that the marked increase of acellular area in atherosclerotic plaques of Card9 deficient mice might be related to increased foam cell formation. To explore this hypothesis, we performed in vitro experiments investigating the uptake of oxidized LDL (ox-LDL) by BM-derived macrophages and their ability to accumulate intracellular lipids. Interestingly, foam cell formation was significantly increased in Card9 deficient macrophages, compared with control macrophages, after 6 and 24 hours of incubation with ox-LDL (Figure 3a-b). Total cholesterol and cholesterol ester content, after ox-LDL exposure, were significantly increased in macrophages from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice, confirming the intracellular cholesterol overload (Figure 3c-d). Next, we investigated the mechanisms that could drive lipid overload in the absence of Card9. We measured a significant increase in Abca1, Abcg1 and Scarb1 mRNA levels in macrophages from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice exposed to ox-LDL, compared to those from Apoe<sup>−/−</sup>Card9<sup>+/+</sup> mice (Figure 3e). Cholesterol transfer to HDL and to ApoA1 was also enhanced in Apoe<sup>−/−</sup>Card9<sup>−/−</sup> macrophages (Figure 3f). This finding highly suggests that increased foam cell formation in Card9 deficient macrophages was not due to impaired cholesterol efflux. Next, we investigated the expression of scavenger receptors involved in lipid uptake. We found no difference in Msr1 mRNA content between groups (Figure 3g) but Cd36 mRNA levels were markedly increased in macrophages from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice exposed to ox-LDL (Figure 3g), which was confirmed at the protein level by immunofluorescent staining (Figure 3h). In agreement with our in vitro experiments, Cd36 mRNA levels were higher in aortas from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice, compared with control mice (Supplementary fig.18). We also found increased nuclear translocation of RelB in Card9<sup>−/−</sup> macrophages (Supplementary Fig.17), which suggests that the mechanism responsible for CD36 up-regulation observed in the absence of Card9 could be related to ox-LDL signaling deviation towards the non-canonical NF-κB pathway. 37,38

As intracellular lipid overload modulates cell phenotype, we investigated cytokine production by macrophages and apoptosis susceptibility. Interestingly, we observed that Card9 deficiency led to a phenotypic switch towards a more pro-inflammatory macrophage phenotype characterized by higher secretion of IL-1b and lower secretion of IL-10 following in vitro stimulation (Figure 3i). In addition, apoptosis was increased in macrophages from Apoe<sup>−/−</sup>Card9<sup>−/−</sup> mice exposed to ox-LDL (Figure 3j & Supplementary fig.19). On the same note we found that the number of TUNEL+ cells was increased in atherosclerotic plaques of Card9 deficient mice (Figure 3K & Supplementary fig.20), which might also
account for the presence of large necrotic core in plaques of Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice (Figure 1g & Figure 2g & supplementary fig.16).

**Acceleration of atherosclerosis in Card9 deficiency is due to impaired autophagy**

CD36 is a multifunctional membrane protein that facilitates lipid uptake, but also participates in the regulation of AMPK-mediated autophagy. Based on our previous observations showing an upregulation of CD36 on Card9 deficient macrophages, we next focused on autophagy, a compensatory survival mechanism involved in atherosclerosis<sup>39</sup>. Macrophages isolated from Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice were cultured and stressed to activate autophagy. Interestingly, we found a significant decrease in AMPK phosphorylation in Card9 deficient macrophages (Figure 4a) but no difference in CHOP levels as well as Beclin-1 and LKB-1 phosphorylation (Supplementary Fig. 21). In addition, we found higher P62 protein content in macrophages from Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice compared to Apoe<sup>-/-</sup>Card9<sup>+/+</sup> mice, suggesting that autophagy was impaired in the absence of Card9 (Figure 4a). The accumulation of P62 in Card9 deficient macrophages was confirmed in vitro using immunostaining (Figure 4b), as well as in vivo, as revealed by the higher number of P62+ MOMA+ macrophages in atherosclerotic plaques of Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice in reference to plaques of Apoe<sup>-/-</sup>Card9<sup>+/+</sup> mice (Figure 4c & Supplementary fig.22). To evaluate the causative link between impaired autophagy and acceleration of atherosclerosis in Card9 deficient mice, we used rapamycin, a pharmacological activator of autophagy. In vitro, rapamycin treatment restored autophagy flux (Figure 4b), abolished intracellular lipid overload (Figure 4d), and attenuated cell death susceptibility observed in Card9 deficient macrophages (Figure 4e).

To further validate the physiopathological relevance of our findings, Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice were fed a high-fat diet for 6 weeks and received daily rapamycin treatment. There was no difference in body weight and in plasma cholesterol levels between Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> groups treated with rapamycin (Supplementary fig. 23a-b). Interestingly, rapamycin treatment abolished the pro-atherogenic effect of Card9 deficiency. Histological analysis revealed a significant decrease in atherosclerosis in the aortic sinus of rapamycin-treated Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice, compared to rapamycin-treated Apoe<sup>-/-</sup>Card9<sup>+/+</sup> mice (Figure 4f). In addition, macrophage content and necrotic core size and P62 accumulation were also significantly reduced in atherosclerotic plaques of rapamycin-treated Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice (Figure 4g-h), as well as the accumulation of P62 (Figure 4i). To further confirm that impaired autophagy was involved in the pro-atherogenic effect of Card9 defect, we used an alternative pharmacological approach to restore autophagy. Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice under a high-fat diet for 6 weeks, were treated with metformin, a well-known activator of autophagy through AMPK stimulation<sup>40</sup>. There was no significant difference in body weight and in plasma cholesterol levels between Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> groups treated with metformin (Supplementary Fig.23c-d). Metformin treatment abolished the acceleration of atherosclerosis observed in Card9 deficiency, with no difference in plaque size (Figure 4j), plaque composition (Figure 4k-l) and P62 accumulation (Figure 4m) between the 2 treated groups.
Card9 deficiency-mediated acceleration of atherosclerosis is dependent on CD36

To address the direct role of CD36, we generated Cd36-deficient Card9+/+ and Card9−/− mice. Following in vitro challenge, AMPK phosphorylation (Figure 5a), P62 accumulation (Figure 5b), lipid uptake (Figure 5c & supplemental figure 24) and cell death susceptibility (Figure 5d) were not different between macrophages from Cd36−/−Card9+/+ and Cd36−/−Card9−/− mice. Furthermore, increased production of IL-1b observed in Card9−/− macrophages was also abolished in the absence of Cd36 (Figure 5e).

To investigate the effect of Card9 deficiency on atherosclerosis in the absence of CD36, we performed BM transplantation experiments using either Cd36−/−Card9+/+ or Cd36−/−Card9−/− littermate BM to repopulate lethally irradiated Ldlr−/− mice. After 4 weeks of recovery and additional 8 weeks of high-fat diet, animals were sacrificed. We confirmed that Cd36 and Card9 gene expression was almost abolished in the spleen (Figure 5f-g) and in peritoneal macrophages of chimeric Ldlr−/− (Supplemental fig.25). Cholesterol levels were not different between both groups (Figure 5h). Atherosclerosis plaque size and composition were no longer different between Ldlr−/−Cd36−/−Card9+/+ and Ldlr−/−Cd36−/−Card9−/− chimeric groups (Figure 5i-k), indicating that increased expression of CD36 was directly responsible for increased atherosclerosis in Card9 deficient animals.

CARD9-related pathways in human

In order to evaluate the clinical relevance of our experimental results, we compared the transcriptomic profile of blood monocytes from CARD9 deficient patients (n=3) with those of controls (n=4) (figure 6a & Supplementary table 2). Genetic investigations in CARD9 deficient patients identified a homozygous c.865C>T in exon 6 of CARD9 leading to a premature stop codon at position 289 (p.Q289*) (N=2) and a homozygous c.52C>T missense mutation in exon 2 of CARD9, resulting in the replacement of the arginine in position 18 with a tryptophan residue (p.R18W) (N=1) 41-43. Differential analysis of these RNA-Seq revealed 256 differentially expressed genes (Figure 6b): 211 were up-regulated and 45 were down-regulated in CARD9 deficient patients. Up-regulated genes included inflammatory cytokines, such as IL-1b [log2(FC) = 2.2, adjusted p-value = 0.035] and IL-6 [log2(FC) = 4.3, adjusted p-value = 1.52e-05] (Figure 6c). In gene set enrichment analysis (GSEA), several pathways previously identified in murine models had significant enriched score: apoptosis (34 pathways, 330 genes), autophagy (1 pathway, 1 gene), atherosclerosis (2 pathways, 69 genes), NF-kB signaling (1 pathway, 25 genes) and TNFα signaling pathway (1 pathway, 20 genes) (Figure 6d & detailed selected pathways and core enrichment genes in Supplementary table 3).

Finally, we examined CARD9 expression in atherosclerotic plaques from human carotid arteries. CARD9 was not detected in normal aorta (Supplementary fig.26). However, CARD9 was detected in fatty streak lesions (Figure 6d) and in lipid-rich areas surrounding the necrotic core of advanced atherosclerotic
plaque (Figure 6e). Fluorescent staining confirmed that CARD9 expression was mostly confined to CD68+ intimal macrophages (Figure 6d-e), and analysis of previously published single cell RNA-seq revealed CARD9 mRNA expression specifically in macrophages from atherosclerotic human coronary arteries (Figure 6f).

Discussion

Using several in vitro and in vivo complementary approaches, we demonstrated that Card9 deficiency significantly accelerated atherosclerosis in mice throughout the aorta (aortic sinus, ascending and descending aorta) and induced a more inflammatory plaque phenotype, characterized by increased macrophage infiltration and necrotic core size. Card9 deficient macrophages are characterized by increased pro-inflammatory cytokine release, enhanced lipid uptake and elevated cell death susceptibility. Such pro-atherogenic phenotype resulted from CD36-mediated impaired autophagy, since this was reversed by Cd36 gene deletion and by autophagy-inducing treatments with rapamycin and metformin. Importantly, the human relevance of our findings in mice was confirmed using transcriptomic analysis of monocytes isolated from extremely rare CARD9 deficient patients and ScRNA seq analysis in human atherosclerotic plaques.

The role of CARD9 in atherosclerosis still remains debated. In two recent studies, one reported increased lesion size in chimeric Ldlr−/− Card9−/− mice, whereas the other found that deletion of haematopoietic Card9 did not affect the atherosclerosis in chimeric Ldlr−/− mice under hyperglycaemic conditions. In our study, we provided strong evidence that Card9 deficiency promoted atherosclerosis. We confirmed this finding in two different atheroprone models, Apoe−/− mice and chimeric Ldlr−/− mice under high-fat diet. The pro-atherogenic role of Card9 deficiency was not dependent on gender as we observed similar accelerated vascular disease in both male Apoe−/− and female chimeric Ldlr−/− mice with Card9 deficiency. The acceleration of atherosclerosis, which is known to be an inflammatory disease, in the absence of Card9 might be counterintuitive since Card9 is a downstream adaptor of fungal- and bacteria-induced activation of TLRs, as well as activation of ITAM-containing non-TLRs and Dectin-1. Card9 engagement and Card9-Bcl-10-MALT1 complex formation lead to NF-κB transcription and subsequent secretion of pro-inflammatory cytokines. Decreased spleen production of TNF-α is consistent in all murine models and in line with previous studies. Decreased IL-10 levels is also consistent in all the models and explained by the fact that Card9 regulates spleen tyrosine kinase (SYK), a key driver for IL-10 production. The acceleration of atherosclerosis in Card9 deficient animals was unlikely due to decreased production of IL-10, since macrophages from Cd36−/− Card9−/− animals, produced less IL-10 than those from Cd36−/− Card9+/+ mice, but plaque size was similar in both groups. The effect of Card9 deficiency on IL-1β production is more complex. We found decreased IL-1β production by stimulated mixed immune cells in the spleen, but higher production by murine Card9−/− macrophages as well as higher IL-1β transcripts in monocytes from CARD9 deficient patients. In the context of Salmonella infection, it has also been reported that CARD9 negatively regulates IL-1β by fine-tuning pro-IL-1β expression, SYK-mediated NLRP3 activation and repressing inflammasome-associated caspase-8 activity.
Higher production of IL-1β might be involved, at least in part, in the acceleration of atherosclerosis in Apoe−/−Card9−/− mice and might be due to increased CD36 expression. Liu et al have shown that CD36 promoted the expression of NLRP3 and consecutive IL-1β production through ROS generation in ox-LDL-stimulated macrophages. In our study, we found that Card9 deficiency had significant effects on the adaptive immune system and particularly on T cell polarization. We observed discrepancies in cytokine production by CD4 + T cells between male Apoe−/− and female chimeric Ldlr−/−, which could be due to gender or background difference, Apoe having by itself immune-modulatory functions. In chimeric Ldlr−/− mice, Card9 deficient CD4+ T cells produced less IL-17A than control CD4+ T cells, which is consistent with previous studies in normocholesterolemic mice. However, our findings of increased atherosclerosis in immune-deficient Apoe−/−Rag2−/−Card9−/− mice rules out the possibility that the acceleration of atherosclerosis in the absence of Card9 was mediated by a modulation of the adaptive immune system.

Given the marked increase in aortic atherosclerosis in the absence of hematopoietic Card9 and the colocalization between Card9 + macrophages and lipid-rich areas in both mouse and human plaques, we then focused on the role of Card9 in macrophage foam cell formation. We found a marked increase in ox-LDL uptake and lipid accumulation in Card9−/− macrophages. Among the receptors that govern foam cell formation in macrophages, Card9 deletion selectively increased both Cd36 gene expression and cell surface protein levels, in vitro, as well as in vivo in plaque macrophages. The effect of Card9 deficiency on the up-regulation of CD36 expression and lipid uptake might account for the accelerated atherosclerosis in Card9 deficient mice. Several studies have previously reported a pro-atherogenic role of CD36 in Apoe−/− and Ldlr−/− mice. We found higher RelB nuclear translocation in Card9 deficient macrophages, which might account for increased Cd36 gene transcription.

We reported increased apoptosis susceptibility of Card9−/− macrophages exposed to ox-LDL, which is consistent with the increase number of TUNEL + cells found in plaques of Apoe−/−Card9−/− mice. Apoptosis susceptibility of Card9 deficient macrophages might be due to intracellular lipid overload. In the context of atherosclerosis, other mechanisms, including autophagy, could account for enhanced apoptosis susceptibility of macrophages in the absence of Card9. Notably, we found that autophagy was impaired in Card9 deficient macrophages in vitro, as well as in atherosclerotic plaques of Apoe−/−Card9−/− mice, as shown by p62 accumulation. Defective autophagy has been shown to promote atherosclerosis. Autophagy blockade in LysMCre+Atg5lox/loxLdlr−/− mice led to increased lesion size and larger necrotic core, which phenocopies the genetic loss of Card9 in Apoe−/− mice. Ohman et al. previously suggested a link between the Card9 signaling pathway and autophagy, and found that Curdlan, an activator of the Dectin-1 receptor, induced LC3I conversion into LC3II in cultured macrophage. More recently, Rubicon, a Beclin-1-binding partner, was identified as a physiological feedback inhibitor of Card9-BCL10-MALT1-mediated PRR signaling. Our study showed that impaired autophagy in Card9 deficient macrophages was related to AMPK blockade. We focused on AMPK because CD36 is known to inhibit AMPK phosphorylation and also because AMPK is an upstream regulator of autophagy through several
mechanisms, including Ulk1 activation \(^{61}\) and mTORC1 inhibition \(^{62}\). In Card9-deficient macrophages, oxLDL-induced AMPK phosphorylation was abolished and P62 accumulated in cell cytoplasm. Interestingly, AMPK phosphorylation and autophagy were restored in Cd36\(^{-/-}\)Card9\(^{-/-}\) cells, supporting a critical role of CD36 in the regulation of autophagy and subsequently atherosclerotic plaque development. Rapamycin and metformin, two pharmacological drugs that activate autophagy, respectively through mTOR blockade and AMPK activation, also abolished the pro-atherogenic effect of Card9 deficiency. The beneficial effects of rapamycin on atherosclerosis development and on autophagy in the context of Card9 deficiency were stronger than those of metformin, which could be due, at least in part, by differences in pharmacological activities of the two drugs. Rapamycin inhibits both mTORC1 and mTORC2, whereas metformin only blocks mTORC1 \(^{63}\). Further investigations are required to study the interactions between Card9 and mTORC1/2 pathways. Experiments performed on Cd36\(^{-/-}\) animals confirmed that CD36 upregulation was involved in the acceleration of atherosclerosis in Card9\(^{-/-}\) animals. However, how the two functions of CD36 (lipid uptake versus autophagy) contribute to the pro-atherogenic impact of Card9 deficiency remains unknown. Addressing this issue is very challenging because autophagy, by itself, can regulate Cd36 levels \(^{64}\). Finally, in our study, cholesterol efflux was not impaired in Card9-deficient macrophages suggesting that autophagy-independent mechanisms such as neutral lipolysis \(^{65,66}\) were activated in Card9\(^{-/-}\) macrophages to limit lipid overload.

**Conclusion**

Altogether, our studies identify CARD9 as a major protective pathway in the development and complications of atherosclerosis. Pro-apoptotic and pro-atherogenic effects of Card9 deficiency are mediated by CD36-dependent defective autophagy that can be reversed by rapamycin and metformin.

**Declarations**

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Authors Contributions

Study concept and design, all authors. Acquisitions of data YZ, MV, ISZ, JJ, AL, MG, BE, CH, OL, JRL, LL, CV, PB and HAO. Drafting of the manuscript YZ, OL, AT, ZM, ST, HS and HAO. Critical revision of manuscript, all the authors. Statistical analysis, YZ, MD and HAO.

Competing Financial interests

none

Disclosures

None

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**Figures**
Figure 1

Global Card9 deficiency accelerates atherosclerosis in Apoe<sup>-/-</sup> mice. scRNA-seq analysis of Card9 transcript expression in murine arteries with 26,910 immune and non immune cells from the aorta of atherosclerotic mice (integrated analysis of 13 datasets). A, UMAP representation of single-cell RNA-seq gene expression data and cellular lineage identification. B, Card9 expression in single cells projected onto the UMAP plot. C, Card9 (Red) and MOMA-2 (Green) immunofluorescent staining in plaques of 20-week old Apoe<sup>-/-</sup> mice. Scale bar 50 mm. D, Card9 mRNA expression by peritoneal macrophages isolated from Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice (n=5/group). E, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic sinus of Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice after 6 weeks of fat diet (2 experiments pooled, n=10/group); Scale bar 200 mm. F, representative photomicrographs and quantitative analysis of macrophage accumulation (MOMA staining, red) in atherosclerotic lesions of Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice after 6 weeks of fat diet (2 experiments pooled, n=10/group); Scale bar 100 mm. G, Representative photomicrographs and quantitative analysis of acellular area (Masson's Trichrome) of Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice after 6 weeks of fat diet (2 experiments pooled, n=10/group); Scale bar 100 mm. H, Cytokine production (ELISA in the supernatant) by Lps/Ifng-stimulated splenocytes from Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> mice (n=10/group); I, cytokine production (ELISA in the supernatant) of purified splenic CD4<sup>+</sup> T cells from Apoe<sup>-/-</sup>Card9<sup>+/+</sup> and Apoe<sup>-/-</sup>Card9<sup>-/-</sup> animals after 48 hours of coated anti-CD3 stimulation (n=6/group). *, P<0.05, **, P<0.01, ***, P<0.001.
Figure 2

Hematopoietic Card9 deficiency accelerates atherosclerosis in Ldlr−/− mice. A, Card9 mRNA expression by peritoneal macrophages isolated from chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice (n=5/group). B, body weight at sacrifice after 8 weeks of fat diet (n=14/group). C, plasma cholesterol levels at sacrifice after 8 weeks of fat diet (n=14/group). D, representative photomicrographs and quantitative analysis of atherosclerotic lesions on the thoraco-abdominal aortas from chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice after 8 weeks of fat diet (n=6-7/group). E, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic sinus of chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice after 8 weeks of fat diet (2 experiments pooled, n=14/group); Scale bar 200 mm. F, representative photomicrographs and quantitative analysis of macrophage accumulation (MOMA staining, red) in atherosclerotic lesions of chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice after 8 weeks of fat diet (2 experiments pooled, n=14/group); Scale bar 100 mm. G, Representative photomicrographs and quantitative analysis of acellular area (Masson's Trichrome) of chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice after 8 weeks of fat diet (2 experiments pooled, n=14/group); Scale bar 100 mm. H, Cytokine production (ELISA in the supernatant) by Lps/Ifng-stimulated splenocytes isolated from chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− mice (n=5-6/group). I, cytokine production (ELISA) of purified splenic CD4+ T cells from chimeric Ldlr+/−Card9+/+ and Ldlr−/−Card9−/− animals after 48 hours of coated anti-CD3 stimulation (n=6/group). *, P<0.05, **, P<0.01, ***, P<0.001.
Card9 deficiency increased CD36 expression in macrophages and promotes lipid uptake and foam cell formation. A, Representative photomicrographs and quantitative analysis (B) of Bodipy+ foam cells after incubation of BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice with oxLDL during 6 and 24 hours (2 pooled experiments, n=8/group/timepoints). C, quantification of intracellular cholesterol content of BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after exposure to ox-LDL (n=6/group/timepoint). D, quantification of intracellular cholesterol ester of BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after exposure to ox-LDL at 3, 6, 12 and 24 hours (n=6/group/timepoint). E, quantification of Abca1 and Abcg1 and Scarb-1 mRNA expression in BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after stimulation with oxLDL (n=6/group). F, quantification of cholesterol efflux in presence of ApoAI or HDL (n=4/group). G, quantification of Mrs-1 and Cd36 mRNAs in BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after stimulation with oxLDL (n=6/group). H, representative immunostaining and quantification of Cd36 expression by BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after 24-hour stimulation with ox-LDL (n=12-22/group). I, cytokine production by BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after stimulation (ELISA in the supernatant, n=5/group). J, Flow cytometry quantification of AnnexinV+ BMDMs from Apoe\(^{-/-}\) Card9\(^{+/+}\) and Apoe\(^{-/-}\) Card9\(^{-/-}\) mice after 24-hour stimulation with oxLDL (100 mmol/l) (n=5/group). K, Representative photomicrographs and
quantiﬁcation of TUNEL+ cells in plaques from Apoe−/−Card9+/+ and Apoe−/−Card9−/− mice (n=10/group). Scale bar 20 mm. *, P<0.05, **, P<0.01, ***, P<0.001.

Figure 4

Pro-atherogenic effects of Card9 deﬁciency are mediated by impaired autophagy. A, Quantitation of AMPK phosphorylation and P62 content in macrophages from from Apoe−/−Card9+/+ and Apoe−/−Card9−/− mice after exposure to oxLDL (n=5-8/group). B, quantitative analysis and representative photomicrographs of P62 accumulation (Green) in macrophages from Apoe−/−Card9+/+ and Apoe−/−Card9−/− mice after 8-hour exposure to oxLDL with or without Rapamycin (200 mMol) (n=6/group). C, Quantitative analysis and representative photomicrographs of p62 accumulation (Red) in MOMA-2+ macrophages (Green) in atherosclerotic lesions of Apoe−/−Card9+/+ and Apoe−/−Card9−/− mice after 6 weeks of fat diet (2 experiments pooled, n=10-12/group); Scale bar 200 mm. D, quantiﬁcation of intracellular cholesterol (total and ester) of BMDMs from Apoe−/−Card9+/+ and Apoe−/−Card9−/− mice after exposure to ox-LDL with...
or without Rapamycin (200 mMol) (n=5/group). E, Flow cytometry quantification of necrosis (7AAD+ Annexin V+) susceptibility of macrophages from Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 24-hour stimulation with high concentration of oxLDL (100 mmol/l) without and with rapamycin (200 mMol) (n=5/group). F, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic sinus of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by rapamycin (n=7/group); Scale bar 200 mm. G, representative photomicrographs and quantitative analysis of macrophage accumulation (MOMA staining, red) in atherosclerotic lesions of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by rapamycin (n=7/group; Scale bar 100 mm). H, representative photomicrographs and quantitative analysis of acellular area (Masson's Trichrome) of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by rapamycin (n=7/group; Scale bar 100 mm.). I, representative photomicrographs and quantitative analysis of P62 content in plaques of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by rapamycin (n=7/group; Scale bar 100 mm.). J, representative photomicrographs and quantitative analysis of atherosclerotic lesions in the aortic sinus of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by metformin (n=7-8/group); Scale bar 200 mm. K, representative photomicrographs and quantitative analysis of macrophage accumulation (MOMA staining, red) in atherosclerotic lesions of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by metformin (n=7-8/group; Scale bar 100 mm). L, representative photomicrographs and quantitative analysis of acellular area (Masson's Trichrome) of Apoe\(^{-/-}\)/Card9\(^{+/+}\) and Apoe\(^{-/-}\)/Card9\(^{-/-}\) mice after 6 weeks of fat diet and treated by metformin (n=7-8/group; Scale bar 100 mm.). *, P<0.05, **, P<0.01, ***, P<0.001.
Figure 5

chimeric Ldlr⁻/⁻Cd36⁻/⁻Card9⁺/+ and Ldlr⁻/⁻Cd36⁻/⁻Card9⁻/⁻ mice after 8 weeks of fat diet (n=10/group); Scale bar 100 mm. *, P<0.05, **, P<0.01, ***, P<0.001.

Figure 6

**CARD9 related pathways in human.** A, protocol to obtain transcripts after isolation of monocytes from controls and CARD9 deficient patients. B, Volcano-plot of the differentially expressed genes between monocytes from patients with CARD9 mutation and control patients. Red dots represent up-regulated genes and blue dots down-regulated genes (adjusted p-value < 0.05). C, Heatmap of mean expression in each patient of the leading edge genes contributing to the enrichment of indicated pathways in the GSEA. Immunofluorescent micrograph of human healthy (D) and atherosclerotic (E) carotid artery sections stained for CARD9 (green), a-actin+ smooth muscle (red) or CD68 (red) showing that CARD9 was strongly expressed by cells that engulf lipids and cholesterol crystals and of giant lipid-laden foam cells but not by smooth muscle cells. Magnitude X20 (D), X2.5 (E), X40 (E). F, CARD9 expression in 10,934 total human atherosclerotic coronary artery cells from 4 patients (data from Wirka et al. 26) with UMAP representation of single-cell RNA-seq gene expression data (left) and cellular lineage identification (right) where CARD9 expression in single cells projected onto the UMAP plot. (For clarity, expression cutoff have been applied and cells with detectable Card9/CARD9 transcripts were brought to the front of the plot).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- Supplementaryfigures.ZhangetalJune22.pptx