Macrophages define a key component of immune cells present in atherosclerotic lesions and are central regulators of the disease. Since epigenetic processes are important in controlling macrophage function, interfering with epigenetic pathways in macrophages might be a novel approach to combat atherosclerosis. Histone H3K27 trimethylation is a repressive histone mark catalyzed by polycomb repressive complex with EZH2 as the catalytic subunit. EZH2 is described to increase macrophage inflammatory responses by suppressing the suppressor of cytokine signaling, Socs3. We previously showed that myeloid deletion of Kdm6b, an enzyme that in contrast to EZH2 removes repressive histone H3K27me3 marks, results in advanced atherosclerosis. Because of its opposing function and importance of EZH2 in macrophage inflammatory responses, we here studied the role of myeloid EZH2 in atherosclerosis. A myeloid-specific Ezh2 deficient mouse strain (Ezh2<sup>del</sup>) was generated (LysM-cre+ x Ezh2<sup>fl/fl</sup>) and bone marrow from Ezh2<sup>del</sup> or Ezh2<sup>wt</sup> mice was transplanted to Ldlr<sup>-/-</sup> mice which were fed a high fat diet for 9 weeks to study atherosclerosis. Atherosclerotic lesion size was significantly decreased in Ezh2<sup>del</sup> transplanted mice compared to control. The percentage of macrophages in the atherosclerotic lesion was similar, however neutrophil numbers were lower in Ezh2<sup>del</sup> transplanted mice. Correspondingly, the migratory capacity of neutrophils was decreased in Ezh2<sup>del</sup> mice. Moreover, peritoneal Ezh2<sup>del</sup> foam cells showed a reduction in the inflammatory response with reduced production of nitric oxide, IL-6 and IL-12. In Conclusion, myeloid Ezh2 deficiency impairs neutrophil migration and reduces macrophage foam cell inflammatory responses, both contributing to reduced atherosclerosis.

Keywords: atherosclerosis, epigenetic, histone modification, H3K27, macrophage, polycomb, PRC2
INTRODUCTION

Atherosclerosis is a chronic lipid-driven inflammatory disorder of the arteries. Macrophages define a key component of immune cells present in atherosclerotic lesions and are important regulators of disease development and outcome (1). Unraveling pathways that are involved in the control of macrophage inflammatory responses in atherosclerosis will lead to a better understanding of disease. Since epigenetic processes are important modulators of macrophage function, we postulate that interference with epigenetic enzymes in macrophages might be a novel approach to modify macrophage function and influence atherosclerosis development (2, 3).

Histone H3K27 trimethylation (H3K27me3) is a repressive histone mark generated by the polycomb repressive complex 2 (PRC2), which core consists of Embryonic ectoderm development (EED), SUZ12, RpAp46/48 and Enhancer of the zeste homolog 1 (EZH1) or EZH2 (4). EZH1/2 contain a SET domain, which is necessary for the methyltransferase activity of the PRC2 complex (5, 6). Both EZH1 and EZH2 homologs can form similar PRC2 complexes but their differential function remains unclear. PRC2-EZH2 catalyzes mainly H3K27me2/3 methylation and Ezh2 knockdown affects global H3K27me3 levels, while EZH1 in the PRC2 complex performs this function weakly (7). EZH2 has been studied mainly in relation to developmental biology and cancer research. In addition, EZH2 controls stem cell proliferation and differentiation and it was demonstrated that EZH2 regulates T cell differentiation and plasticity (8).

EZH2 is a strong regulator macrophage inflammatory responses (9, 10). Macrophage Ezh2 deficiency was shown to inhibit pro-inflammatory gene expression via upregulation of suppressor of cytokine signaling 3 (SOCS3). Consequently, mice lacking Ezh2 in their macrophages showed improved outcome in models for autoimmune inflammation, experimental autoimmune encephalomyelitis (EAE) and colitis. The H3K27 demethylase KDM6b (also known as Jmjd3) removes repressive histone marks and is also an important regulator of macrophage activation (11–17). We previously showed that myeloid Kdm6b deficiency in mice results in advanced atherosclerosis (18). Since the H3K27 methyltransferases have opposing effects on this histone mark and the fact that EZH2 controls macrophage inflammatory responses, we hypothesized that inhibition of the H3K27 methyltransferase EZH2 in myeloid cells improves atherosclerosis outcome. To test this, we generated myeloid specific Ezh2 knockout mice and studied atherosclerosis progression. We found that atherosclerosis was significantly reduced in Ezh2-deficient mice, correlating with impaired neutrophil migration and reduced foam cell inflammatory responses.

MATERIALS AND METHODS

Atherosclerosis Experiment

Low-density lipoprotein receptor knock out mice (Ldlr−/−) (C57BL/6 background, Jackson laboratories) were used to study atherosclerosis, since these mice are prone to develop atherosclerosis in the presence of a high fat diet (HFD). A bone marrow transplantation (BMT) was performed with either LysM-creEzh2fl/fl mice (Ezh2fl/fl) or LysM-creEzh2−/− littermates (Ezh2−/−). Ezh2−/− mice were described before and crossbreeding with LysM-cre was performed in our mouse facility (19). Briefly, 40 (20 per group), 10-week-old female Ldlr−/− mice were randomly divided over filter-top cages and provided with antibiotics water [autoclaved tap water with neomycin (100 mg/l, Sigma) and polymyxin B sulfate (60,000 U/l, Invitrogen)] from 1 week pre-BMT till 5 weeks post-BMT. The animals received 2 × 6 Gy total body irradiation on two consecutive days. Bone marrow was isolated from 4 Ezh2Elof and 4 Ezh2+ mice (EZH2 is a strong regulator macrophage inflammatory responses in atherosclerosis will lead to a better outcome. To test this, we generated myeloid specific Ezh2 knockout mice and studied atherosclerosis progression. We found that atherosclerosis was significantly reduced in Ezh2-deficient mice, correlating with impaired neutrophil migration and reduced foam cell inflammatory responses.

MATERIALS AND METHODS

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Thermo Fisher Scientific) and incubated for 1 h at room temperature (RT). Nuclei were stained with DAPI (1:5,000, Thermo Fisher Scientific) and incubated for 15 min at RT. Slides were mounted and fluorescence was measured using a Leica DM300 microscope. Neutrophil numbers were counted manually and corrected for lesion size. Necrosis area was measured based on Toluidine Blue staining by our pathologist and corrected for total lesion area.

**Flow Cytometry**

150 µl of blood was withdrawn from mice via tail vein incision before the start of the diet and four days prior to sacrifice and added to 20 µl of 0.5 M EDTA (Sigma-Aldrich). Blood was withdrawn from mice which were fasted for 3 h. The blood was centrifuged (10 min, 4°C, 2,000 rpm) to separate the plasma from blood cells and plasma cholesterol and triglyceride levels were enzymatically measured according to the manufacturer’s protocol (Roche). Blood was further used for flow cytometry to assess relative leukocyte counts. Red blood cells were lysed by adding 5 ml of erythrocyte lysis buffer (8.4 g of NH₄Cl, 0.84 g of NaHCO₃, and 0.37 g of EDTA in 1 L MilliQ) for 15 min at RT. PBS was added to stop the reaction and cells were centrifuged. This was repeated until the pellet was not red anymore. White blood cells were used for flow cytometry. First Fc receptors were blocked with CD16/CD32 blocking antibody (1:100, eBioscience) in FACS buffer (0.5% BSA, 2mM EDTA in PBS). Hereafter cells were incubated with the appropriate antibodies for 30 min at RT (Supplemental Table 1). Cells were washed once and resuspended in FACS buffer. Fluorescence was measured with a BD Canto II and analyzed with FlowJo software. Immune cells were gated based on CD45+ expression and the following cell types were distinguished: Monocytes (CD11b+, Ly6G+), Neutrophils (CD11b+ and Ly6G+), B cells (CD19+), and T cells (CD3+).

**Bone Marrow-Derived Macrophage Culture**

Bone marrow was isolated from femurs and tibia of Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> mice by flushing. The cells were cultured in RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine (Life Technologies) which was supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) (all Gibco) and 15% L929-conditioned medium. Cells were cultured for 8 days to generate bone marrow-derived macrophages (BMDMs) on bacteriologic plastic plates. On day 8, macrophages were resuspended at a density of 10^6 cells/ml and plated in suspension culture plates allowing adherence for 6 h (Greiner). Next, cells were stimulated with LPS (10 ng/ml) for 6 h or left unstimulated where after the supernatant was collected for neutrophil migration experiments. Cultured BMDMs were also used for qPCR analysis, western blots and the H3K27 histone methyltransferase activity assay.

**RNA Isolation and Quantitive PCR Analysis**

RNA from BMDMs or PEMs was isolated with the High Pure RNA isolation kits (Roche) from 500,000 cells. 400 ng of RNA was used for cDNA synthesis with iScript (BioRad). qPCR was performed with 4 ng of cDNA using Sybr Green Fast on a ViiA7 PCR machine (Applied Biosystems). Ezh1, Ezh2, Cxcl1, and Cxcl2 gene expression was normalized to the mean of the two housekeeping genes Ppia and Rplp0. Primer sequences are available on request.

**Western Blot Analysis**

NP40 lysis buffer (Invitrogen) was used for whole cell lysates, supplemented with protease inhibitor cocktail (1:25; Roche). Cells were lysed for 30 min on ice. Hereafter, cells were spun down for 10 min, 4°C at maximum speed and the supernatant was collected and used for western blot analysis for EZH2 protein expression. Histone extractions were used for H3K27Me3 Western blotting. For the histone extractions, cells were resuspended in Triton Extraction Buffer [PBS containing 0.5% Triton X 100 (v/v) and 0.02% (w/v) NaNa₃ supplemented with protease inhibitor cocktail (1:25; Roche)] and lysed on ice for 10 min. Cells were spun down for 10 min at 4°C to 2000 rpm. The supernatant was discarded and the pellet was resuspended in 0.2N HCl and histones were extracted overnight at 4°C on a rotator. Samples were centrifuged for 10 min, 4°C at 2000 rpm and the supernatant was used. Whole cell lysates were diluted with 6x reducing sample buffer (374 mM Tris, 6% SDS, 0.05% Bromophenol Blue, 20% Glycerol, 10% β-mercaptoethanol) and histone extracts with 2x reducing sample buffer (4% SDS, 20% glycerol, 0.125 M Tris-HCl, 10% β-mercaptoethanol, and 0.004% Serva Blue, pH 6.8) and boiled for 10 min at 95°C. Hereafter samples were loaded on a NuPAGE® Novex 4-12% Bis-Tris protein gel and ran for 1.5 h in MOPS buffer for EZH2 western blotting or MES buffer for H3K27Me3 western blotting (NuPAGE® MOPS or MES SDS Running Buffer 20x (Invitrogen) in demi water) at 100 V. The gel was transferred to a nitrocellulose membrane (Bio-Rad) and blotted for 2 h at 30 V in transfer buffer (20x transferbuffer NuPAGE® (Invitrogen), 20% Methanol in demi water). The membrane was blocked in 5% milk (Milk powder (Elk) in TBS-T) for 1 h and hereafter the blot for EZH2 was cut and incubated with the primary antibodies overnight in 1% milk at 4°C. The primary antibodies we used were anti-EZH2 (1:1,000; Bioke) and anti-α-tubulin (1:5,000; Sigma) and for histone extracts anti-H3K27Me3 (1:1,000; bioconnnect) and anti-H3 (1:5,000; Cell Signaling Technology). The next day, blots were washed and incubated for 1 h at RT with the appropriate HRP-conjugated secondary antibody in 1% milk (1:5000 Dako). Blots were visualized using ECL substrate kit (Thermo Scientific).

**H3K27 Methyltransferase Activity Assay**

The H3K27 histone methyltransferase activity assay was performed with the EpiQuikTM assay kit on nuclear lysates following manufactures instructions (Epigentek). Absorbace was read on a microplate reader at 450 nm. HMT activity was calculated by the following formula: Activity (OD/h/mg)=(OD(sample-blank)/protein amount (µg) x incubation time substrate (h)) × 1,000.

**Neutrophil Migration**

Neutrophils were isolated from female mouse bone marrow of Ezh2<sup>wt</sup> and Ezh2<sup>del</sup> mice using a Ly6G-specific antibody (anti-mouse GR-1 APC, clone 1A8, 1:200, eBioscience). Cells were
incubated with anti-APC beads and separated by MACS cell separation columns following manufacturer’s instruction (Miltenyi Biotec). Neutrophils were labeled with calcein-AM (life technologies) and chemotaxis was measured in response to supernatants of unstimulated or 6 h LPS stimulated BMDMs (5× diluted) in a transwell chemotactic assay over 3-µm pore size fluoroblok filters, as described previously (20).

**Peritoneal Foam Cells**

Five mice per group from the atherosclerosis experiment were injected with thioglycollate medium (3%, Fischer). Four days after injection, mice were sacrificed and the peritoneum was flushed once with 10 ml ice-cold PBS to collect peritoneal macrophages (PEMs). Since these mice are on a HFD, these elicited macrophages are fully loaded with lipids and can thus be considered foam cells (21). Flushed thioglycollate-elicited foam cells were pooled per group and cultured at a density of 5 × 10⁵ cells/well in 24-well tissue culture plates in triplicate (Greiner Bio-One) and cultured in RPMI-1640 with 25 mM HEPES, 2 mM L-glutamine, 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml) (all Gibco) and allowed adherence for 3 h. Hereafter the cells were washed and stimulated for 24 h with LPS (10 ng/ml), LPS plus IFNγ (100 U/ml), IL-4 (20 ng/ml), or left unstimulated.

**ELISA and Nitric Oxide Assay**

Antibody pair kits were used to measure TNF, IL-6 and IL-12p40 (Life technologies) with ELISA following manufactures instructions. The following sample dilutions were used, TNF 1:10; IL-6 and IL-12p40 1:20. Streptavidin HRP was used to detect bound antibodies and TMB (Thermo scientific) was used as a substrate for the color reaction. This reaction was stopped with 1.8 M H₂SO₄. Absorbance was measured on a microplate reader (Victor) at a wavelength of 450 nm. For the nitric oxide (NO) assay, 50 µl of undiluted samples was used on a flat bottom 96-wells plate. A standard curve was made with NaNO₂ in culture medium. Next 50 µl of Griess reagent (2.5% H₃PO₄, 1% Sulfanylamide, 0.1% Naphthylene diamine in MilliQ) was added to the samples and absorbance was immediately measured on the microplate reader at a wavelength of 550 nm.

**Statistical Analysis**

Data is presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 5.0 software using an unpaired t-test or two-way ANOVA with bonferroni post-hoc analysis when comparing multiple groups. P-values < 0.05 were considered statistically significant.

**RESULTS**

**No Differences in the Lipid Levels and Immune Cell Populations After Bone Marrow Transplantation to Ldr⁻/- Mice**

A myeloid-specific Ezh2 deficient mouse strain (Ezh2del) was generated and cultured bone marrow-derived macrophages (BMDMs) showed a reduction of Ezh2 at both the mRNA as well as protein level, without changes in Ezh1 mRNA expression (Figures 1A, B). EZH1 is most likely not compensating for the loss of EZH2 in macrophages as global H3K27Me3 levels and H3K27 methyl transferase activity was strongly reduced in Ezh2-deficient BMDMs (Figures 1B, C). Bone marrow of Ezh2wt or Ezh2del mice was transplanted to Ldr⁻/⁻ mice, which were subsequently fed a high fat diet (HFD) for 9 weeks to induce atherosclerosis. Bone marrow of both Ezh2wt and Ezh2del mice was effectively transplanted to Ldr⁻/⁻ mice as chimerism was around 95% and not different between the groups (Figure 1D). Weight, cholesterol and triglyceride levels were similar after 8 weeks of HFD between Ezh2wt and Ezh2del transplanted mice (Figures 1E–G). Additionally, leukocyte levels in both blood and spleen were unaltered in Ezh2del transplanted mice compared to wildtype (Figures 1H, I). Overall, no differences in the lipid levels or composition of immune cell populations were seen after transplantation of Ezh2del bone marrow compared to wildtype, giving similar baseline characteristics.

**Atherosclerotic Lesion Size Is Reduced in Ezh2del Transplanted Mice**

Interestingly, we observed that atherosclerotic lesion size was significantly reduced in Ezh2del transplanted mice compared to Ezh2wt mice after 9 weeks of HFD (Figures 2A, B). A small but non-significant decrease in collagen content was seen in Ezh2del transplanted mice (P = 0.0528) (Figures 2C, D) and the percentage of macrophage and necrosis area were not different between Ezh2wt and Ezh2del transplanted mice (Figures 2E–H). Interestingly, the number of neutrophils was significantly lower in lesions of Ezh2del transplanted mice, even when corrected for lesion area (Figures 2I, J). These data indicate that myeloid Ezh2 deficiency leads to atherosclerotic lesions that are not only smaller but also less inflammatory.

**Ezh2del Neutrophils Are Less Migratory**

Since blood neutrophil numbers were unaltered (Figure 1E) but were significantly lower in Ezh2del lesions, our data suggest that the migration or recruitment of neutrophils to atherosclerotic lesions is reduced. We next investigated if this was due to differences in the chemotactic factors secreted by Ezh2del macrophages or due to intrinsic migration defects of neutrophils. We isolated neutrophils from bone marrow of Ezh2wt and Ezh2del mice and performed chemotaxis assays. We also assessed wild type neutrophil migration toward supernatants of stimulated Ezh2wt and Ezh2del BMDMs (Figure 3A). We observed no differences in the migration of wild type neutrophils toward supernatants of unstimulated and LPS activated Ezh2wt and Ezh2del BMDMs (Figure 3B, left). Furthermore, the expression of Cxcl1 and Cxcl2 expression as the main chemoattractants for neutrophils, was not different in aortic arches of Ezh2wt and Ezh2del transplanted mice, suggesting that these are similar in the lesion environment and not causing the difference in neutrophil accumulation (Figure 3C). Analysis of Cxcl1 and Cxcl2 mRNA expression in peritoneal foam cells and BMDMs was inconclusive, as expression was partly increased, which does not support the reduced neutrophil numbers in vivo.
Conversely, we did observe that the migration of Ezh2<sup>del</sup> neutrophils itself was reduced compared to Ezh2<sup>wt</sup> neutrophils in vitro (Figure 3B, right). These data suggest that neutrophils of Ezh2<sup>del</sup> mice are less migratory and therefore accumulate less in the atherosclerotic lesions.

**DISCUSSION**

We here studied the role of the H3K27 methyltransferase EZH2 in atherosclerosis development by use of a myeloid-specific Ezh2 knockout mice. Ezh2-deficient mice showed reduced levels of H3K27me3 and lower H3K27 methyltransferase activity, indicating that EZH1 is not compensation for the loss of EZH2. Ezh2 mRNA levels were partly reduced (± 60%) as deletion is never complete in the used floxed LysM-cre system and recombination efficiency dependent on the genomic location and distance between the LoxP sites (22–24). As our LoxP sites...
flank the catalytic SET domain of Ezh2, it is sufficient to strongly reduce H3K27Me3 levels and H3K27 methyltransferase activity.

We show that myeloid Ezh2 deficiency improves atherosclerosis outcome as lesion size was significantly reduced in Ezh2\textsuperscript{del} transplanted mice compared to wildtype. Overexpression of Ezh2 in all cell types was previously reported to promote macrophage foam cells formation and worsen atherosclerosis outcome (25). In line with their findings, we show that silencing of Ezh2 specifically in myeloid cells reduces atherosclerosis. Moreover, we show that atherosclerotic lesions of myeloid Ezh2-deficient mice are not only smaller but also contain less neutrophils. Neutrophils play an important role in all stages of atherosclerosis (26) and neutrophil depletion studies in atherosclerotic mice (ApoE\textsuperscript{-/-}) showed a reduction in early atherosclerosis (27). Since neutrophil numbers were similar in blood, this implies that the recruitment of neutrophils toward the lesion is impaired.

We studied neutrophil migration in vitro and indeed found that Ezh2-deficient neutrophils are less migratory. This phenomenon is consistent with studies showing that neutrophils derived from Ezh2 knockout stem cells have impaired migration together with increased cell death, decreased phagocytosis and overproduction of reactive oxygen species (ROS) (28). Accordingly, Gunawan et al. observed reduced neutrophil migration and in addition found reduced dendritic cell migration in cells lacking Ezh2 (29). They propose a mechanism by which EZH2 regulates migration, independent of its H3K27 methyltransferase activity (29). They show that EZH2 regulates integrin mediated migration via methylation of Talin1. EZH2 interacts with VAV1, causing methylation of Talin1. In turn, binding to filamentous actin (F-actin) was altered affecting adhesion and migration.

Likewise, we show that the inflammatory response of peritoneal foam cells is reduced in activated Ezh2\textsuperscript{del} cells compared to Ezh2\textsuperscript{wt} cells. IL-6, IL-12 and NO production, which are key cytokines induced upon macrophage activation, were reduced in Ezh2\textsuperscript{del} foam cells. Interestingly, the inflammatory cytokine TNF was increased in cells lacking Ezh2, indicating that not the full activation spectrum is impaired. Additionally, IL-4 responsive genes were not affected. The idea that inhibition of EZH2, a mediator of repression, results in enhancement of the inflammatory gene program in macrophages appears to be too simplistic. Zhang and colleagues nicely demonstrated one of the mechanisms by which the repressive EZH2 causes activation of inflammation in macrophages (10). By chromatin immunoprecipitation (ChIP) and RNA sequencing analysis they identified the suppressor of cytokine signaling 3 (Socs3) as being strongly upregulated in the absence of EZH2 in combination with EZH2 binding sites and reduced H3K27me3 levels in macrophages. They show that Ezh2 deficiency lowers Socs3 levels thereby enhancing Traf6 ubiquitination and degradation. The Myd88-NF-\kappaB signaling pathway is thereby suppressed, resulting in the reduced inflammatory responses. They illustrate how a repressor like EZH2 can cause activation of inflammation by repression of another repressor, in this case SOCS3 (9, 10). Additionally, Ezh2 deficiency improved the outcome on EAE and colitis, two inflammatory mouse models. We here show that myeloid Ezh2 deficiency also improves the outcome of atherosclerosis, another chronic inflammatory disease.

In conclusion, we show that myeloid Ezh2 deficiency limits atherosclerosis due to impaired neutrophil migration and modulation of foam cell inflammatory responses. Our findings support the thought that EZH2 is not only a target for various types of cancer but may also serve as novel target for autoimmune diseases. Several EZH2 inhibitors are available and the use of these inhibitors in mouse models of atherosclerosis would
FIGURE 3 | EZH2 regulates neutrophil migration. (A) Neutrophils were isolated from bone marrow of Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> mice. Neutrophils were fluorescently labeled and chemotaxis was measured in response to supernatants of unstimulated or LPS activated Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> BMDMs. (B) Neutrophil migration as the increase of RFU in time in relative fluorescence units (delta RFU). Presented is one out of three experiment of 2 pooled Ezh2<sup>wt</sup> and 2 pooled Ezh2<sup>del</sup> mice plated in duplo. Left is Ezh2<sup>wt</sup> neutrophil migration toward supernatant of Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> BMDMs and right is Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> neutrophil migration toward wildtype supernatants. (C) Cxcl1 and Cxcl2 mRNA expression in the aortic arch of Ezh2<sup>wt</sup> or Ezh2<sup>del</sup> transplanted mice. Statistics are performed on duplicates with a two-way anova with bonferroni correction. Data represent mean ± SEM. *P < 0.05. Panel (A) was made with use of smart servier medical art, licenced under a Creative Common Attribution 3.0 Unported License. https://smart.servier.com/.
be the next step to test the therapeutic potential of EZH2 inhibition to control atherosclerosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee for Animal Welfare of the Amsterdam UMC, location Academic Medical Center, University of Amsterdam (permit: DBC10AH).

AUTHOR CONTRIBUTIONS

AN and MW conceived and designed the experiments. AN, H-JC, MG, SV, MH, MB, JB, AT, and HM performed the experiments. AN, MG, and SV analyzed the data. AN, MG, EL, and MW were involved in the interpretation of the data. AN and MW wrote the manuscript. MG, JB, TB, and EL critically read and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by The Netherlands Heart Foundation [CVON 2011/B019 and CVON 2017-20: Generating the best evidence-based pharmaceutical targets for atherosclerosis (GENIUS I&II)]. AN received a Junior Postdoc grant from the Netherlands Heart Foundation (2020T029) and a postdoc grant from Amsterdam Cardiovascular Sciences. JB received a senior postdoctoral fellowship (2017T048) from the Netherlands Heart Foundation. MW is an established investigator of the Netherlands Heart Foundation (2007T067), is supported by a Netherlands Heart Foundation grant (2010B022), The Netherlands Heart Foundation and Spark-Holding BV (2015B002, 2019B016), the European Union (ITN-grant EPIMAC), Leducq Transatlantic Network Grant, ZonMW (Open competition 0912001190025).
ACKNOWLEDGMENTS

The Ezh2-floxed mice were a gift from Dr. van Loohuizen (Netherlands Cancer Institute) and originally generated by the laboratory of Dr. Orkin, S.H. (19).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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