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# Myeloid Kdm6b deficiency results in advanced atherosclerosis

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ABSTRACT

*Background and aims:* Atherosclerosis is a lipid-driven chronic inflammatory disorder of the arteries, and monocytes and macrophages play a central role in this process. Within the atherosclerotic lesion, macrophages can scavenge modified lipids and become the so-called foam cells. We previously reported that the epigenetic enzyme Kdm6b (also known as Jmjd3) controls the pro-fibrotic transcriptional profile of peritoneal foam cells. Given the importance of these cells in atherosclerosis, we now studied the effect of myeloid Kdm6b on disease progression.

*Methods:* Bone marrow of myeloid Kdm6b deficient (*Kdm6b*$^{del}$) mice or wild type littermates (*Kdm6b*$^{wt}$) was transplanted to lethally irradiated *Ldlr*$^{-/-}$ mice fed a high fat diet for 9 weeks to induce atherosclerosis.

*Results:* Lesion size was similar in *Kdm6b*$^{wt}$ and *Kdm6b*$^{del}$ transplanted mice. However, lesions of *Kdm6b*$^{del}$ mice contained more collagen and were more necrotic. Pathway analysis on peritoneal foam cells showed that the pathway involved in leukocyte chemotaxis was most significantly upregulated. Although macrophage and neutrophil content was similar after 9 weeks of high fat diet feeding, the relative increase in collagen content and necrosis revealed that atherosclerotic lesions in *Kdm6b*$^{del}$ mice progress faster.

*Conclusion:* Myeloid Kdm6b deficiency results in more advanced atherosclerosis.



## 1. Introduction

Atherosclerosis is a lipid-driven chronic inflammatory disorder of the arteries [1]. Monocytes and macrophages play a key role in the initiation and progression of atherosclerotic lesions [1–3]. Once monocytes enter the arterial wall and mature into macrophages, they can scavenge modified lipoproteins and thereby become foam cells [1]. Besides having foam cell characteristics, macrophages can adopt different activation states in response to their environmental triggers. Skewing macrophages to cells with anti-inflammatory features would be beneficial in chronic inflammatory diseases like atherosclerosis [4–6]. This can be accomplished by targeting epigenetic processes in macrophages since these are critical for monocyte and macrophage activation [7,8]. Histone modifications are one of the central epigenetic mechanisms of gene regulation referring to post-translational modifications at histone tails. Histone H3K27 trimethylation is a repressive histone mark catalyzed by the polycomb repressive complex 2 (PRC2) [9] and can be removed by the demethylases Jumonji-C domain 3 (Kdm6b;

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Jmjd3), Utx and Uty [10]. The role of these demethylases in macrophage polarization has been extensively studied and Kdm6b is regulated in response to numeral triggers regulating both inflammatory and anti-inflammatory responses [11–18]. We previously reported that peritoneal foam cells from Kdm6b deficient mice, have reduced expression of pro-fibrotic genes and pathways [19]. Fibrosis is also important in atherosclerosis. Advanced atherosclerotic lesions are characterized by a fibrous cap which is mainly built of smooth muscle cells and collagen [20]. Giving the fact that atherosclerosis is driven by immune cells like macrophages, and upon lesion progression a fibrous cap is built, we studied the involvement of myeloid Kdm6b in atherosclerosis. We found that atherosclerotic lesions of Kdm6b deficient mice were more advanced as they contained more collagen and exhibited more necrosis.

## 2. Materials and methods

### 2.1. Atherosclerosis experiment

For our experiments, we made use of C57BL/6 low density lipoprotein receptor knock out mice (*Ldlr*$^{-/-}$) since these mice are prone to develop atherosclerosis. *Ldlr*$^{-/-}$ mice were obtained from Jackson laboratories. A bone marrow transplantation (BMT) was performed with either LysM-cre$^{+}$ x *Kdm6b*$^{fl/fl}$ mice (*Kdm6b*$^{del}$) or LysM-cre$^{-}$ x *Kdm6b*$^{fl/fl}$ littermates (*Kdm6b*$^{wt}$). *Kdm6b*$^{fl/fl}$ mice were kindly provided by the laboratory of Stefanie Dimmeler [21]. Crossbreeding with LysM-cre was performed in our mice facility. Briefly, 40 (20 per group), 10-week old female *Ldlr*$^{-/-}$ mice were divided over filter-top cages and provided with water containing neomycin (100 mg/L, Sigma, Zwijndrecht, the Netherlands) and polymyxin B sulfate (60,000 U/L, Invitrogen, Bleiswijk, The Netherlands) from 1 week pre-BMT until 5 weeks post-BMT. The animals received 2 × 6 Gy total body irradiation on two consecutive days. Bone marrow was isolated from two *Kdm6b*$^{del}$ and two *Kdm6b*$^{wt}$ mice, resuspended in RPMI1640 (Gibco, Breda, The Netherlands) with 5 U/ml heparin and 2% iFCS (Gibco, Breda, the Netherlands), and $10^7$ cells were injected intravenously per irradiated mouse. Bone marrow transplantation efficiency was determined with qPCR for the *Ldlr* on DNA isolated from blood (GE Healthcare, Eindhoven, the Netherlands). Three mice were excluded from the analysis due to inefficient bone marrow transplantation (≤80%). Five weeks after the BMT, the mice were put on a high fat diet (0.15% cholesterol, 16% fat, Arie Blok Diets, The Netherlands) for 9 weeks. After sacrifice, hearts were taken out and frozen in Tissue-Tek (DAKO, Eindhoven, The Netherlands) for histology. Blood samples were taken before the start of the diet and before sacrifice for lipid profiling and immune cell flow cytometry. Two mice were sacrificed before the end of experiment as they reached the human endpoints. One additional mouse was excluded from the analysis due to insufficient tissue quality. A total of 16 *Kdm6b*$^{del}$ mice were compared to 18 *Kdm6b*$^{wt}$ mice for the statistical analysis. All animal experiments were conducted at the University of Amsterdam and approved (permit: DBC10AD) by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam.

### 2.2. Histochemistry

Atherosclerotic lesions from the heart were cut in sections of 7 μm on a Leica 3050 cryostat at −25 °C. Cross sections of every 42 μm were stained with Toluidin Blue (0.2% in PBS, Sigma-Aldrich, Gillingham, UK) to determine lesion size. Lesion size was measured by use of adobe photoshop CS4 and the sum of the three valves is presented. Lesion severity was scored by an experienced pathologist as early (intimal xanthoma), moderate (pathological intimal thickening) and advanced (fibrous cap atheroma) as described elsewhere [22]. Sirius red staining was performed for 30 min to measure collagen content (0.05% direct Red in saturated picric acid, Sigma, Zwijndrecht, the Nederlands). Images were obtained using a Leica DM3000 microscope and quantified with photoshop CS4 where collagen was quantified as the percentage of total lesion size. Cap thickness was defined as the thinnest part where cap is visible. For immunohistochemistry, slides were fixed in aceton and blocked with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, USA). Hereafter, cells were incubated with MOMA-2 (1:4000, AbD serotec, Uden, The Netherlands) to stain for macrophages, Ly6G (1:200, BD Pharmingen) for neutrophils and ER-MP58 (1:200, AbD serotec, Uden, The Netherlands) for incoming macrophages. T cells were stained with CD3 (1:250, AbD serotec, Uden, The Netherlands) and its subpopulations with FoxP3 (1:100, eBioscience), CD4 (1:100, biolegend) and CD8a (1:100, BD). Biotin-labeled rabbit anti–rat antibody (1:300, Dako, Eindhoven, the Netherlands) was used as a secondary antibody. Smooth muscle cells were stained with FITC-labeled alpha smooth muscle actin (Asma) (1:2000, Sigma, Zwijdrecht, The Netherlands). FITC-biotin-labeled Donkey-anti-mouse antibody (1:300, Brunschwig Chemie, Amsterdam, The Netherlands) was used as a secondary antibody. The signal was amplified using ABC kit (Vector Laboratories, Burlingame, USA) and visualized with the AEC kit (Vector Laboratories, Burlingame, USA). Necrosis area was measured based on Toluidin Blue staining by our pathologist and corrected for total plaque size. The EnzChek gelatinase assay kit was used to measure gelatinase activity (Molecular Probes, ThermoFisher scientific, Waltham, MA, USA). Briefly, the slides were fixed with 100% ethanol for 20 min. Next, the slides were incubated with the gelatin solution in a final concentration of 20 μg/ml covered with parafilm and incubated in a moister box for 2 h at 37 °C. To stop the reaction, samples were fixed in 3.7% formaldehyde for 10 min and stained with DAPI for 5 min. Fluorescence images were obtained using a Leica DM3000 microscope and quantified with photoshop CS4. Gelatinase activity (green fluorescence) was corrected for total plaque size.

### 2.3. Bone marrow-derived macrophage culture and collagen synthesis by VSMCs

Bone marrow was isolated from femurs and tibia of *Kdm6b*$^{wt}$ and *Kdm6b*$^{del}$ mice by flushing. The cells were cultured in RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine, which was supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 15% L929-conditioned medium as a source of M-CSF for 8 days. On day 8, cells were stimulated with 50 μg/ml oxLDL, 50 μg/ml acLDL (Alfa Aesar, Karlsruhe, Germany) or left unstimulated for 24 h. Supernatants were collected and used for collagen production by vascular smooth muscle cell (VSMCs) assays. Primary mouse VSMCs were isolated and cultured in DMEM/F12 with 20% FCS (Gibco) on 0.1% gelatin coated plates. $5^{*}10^{4}$ VSMCs were plated per well in a gelatin-coated 24-wells plate. After overnight adherence, cells were starved (0% FCS) for 48 h and next incubated with bone marrow-derived macrophage (BMDM) supernatants for 24 h. Hereafter the supernatant was removed and VSMCs were fixed in 3.7% formaldehyde and stained with 1% Sirius Red in 0.01 M HCl. Cells were lysed with 0.01 M NaOH and absorption was measured on a plate reader at 544 nm. Gelatin was used as a standard for quantification.

### 2.4. Chemotaxis assay

Chemotaxis of BMDMs was assessed using 24-well Transwell migration chambers (Costar; Corning) with a pore size of 5 μm in