Post-interventional vascular remodeling: novel insights and therapeutic strategies
Sluiter, T.J.

CHAPTER 5

Myeloid PHD2 conditional knock-out improves intraplaque angiogenesis and vascular remodeling in a murine model of venous bypass grafting

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ABSTRACT

Background: Intraplaque angiogenesis occurs in response to atherosclerotic plaque hypoxia which is mainly driven by highly metabolically active macrophages. Improving plaque oxygenation by increasing macrophage hypoxic signaling, thus stimulating intraplaque angiogenesis, could restore cellular function, neovessel maturation and decrease plaque formation. Prolyl hydroxylases (PHDs) regulate cellular responses to hypoxia. We therefore aimed to elucidate the role of myeloid PHD2, the dominant PHD isoform, on intraplaque angiogenesis in a murine model for venous bypass grafting.

Methods and Results: Myeloid PHD2 conditional knock-out (PHD2cko) and PHD2 WT mice on a Ldlr$^{-/-}$ background underwent vein graft surgery (n=11-15/group) by interpositioning donor caval veins into the carotid artery of genotype-matched mice. At Post-op day 28, vein grafts were harvested for morphometric and compositional analysis and blood was collected for flow cytometry. Myeloid PHD2cko induced and improved intraplaque angiogenesis by improving neovessel maturation, which reduced intraplaque hemorrhage. Intima/media ratio was decreased in myeloid PHD2cko vein grafts. In addition, PHD2 deficiency prevented dissection of vein grafts and resulted in an increase in vessel wall collagen content. Moreover, macrophage pro-inflammatory phenotype in the vein graft wall was attenuated in myeloid PHD2cko mice. *In vitro* cultured PHD2cko bone marrow-derived macrophages exhibited an increased pro-angiogenic phenotype compared to control.

Conclusion: Myeloid PHD2cko reduces vein graft disease and ameliorates vein graft lesion stability by improving intraplaque angiogenesis.

WHAT IS NEW?

- Intraplaque angiogenesis and intraplaque hemorrhage often coincide and have been previously thought to be intrinsically linked, but are now demonstrated to be two separate processes that can be disentangled;
- Evoking pseudo-hypoxic signaling by myeloid PHD2cko induces intraplaque angiogenesis via increased production of VEGF by macrophages, which improves vascular remodeling and ameliorates plaque stability by enhancing neovessel coverage and limiting intraplaque hemorrhage.

WHAT QUESTION SHOULD BE ADDRESSED NEXT?

- Whether targeted therapies aiming at restoring and promoting endothelial barrier function in atherosclerotic plaques increase neovessel maturation to inhibit intraplaque hemorrhage and if this favorable plaque phenotype could be further enhanced by induction of intraplaque angiogenesis whilst maintaining neovessel integrity.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease affecting the large to mid-sized arteries.[1] Leukocytes initially extravasate from the lumen into the vessel wall in response to inflamed or damaged endothelial cells (ECs) covering the luminal side of the vessel. In more advanced atherosclerotic lesions, neovessels invading the plaque from the adventitial layer become the major porte d'entrée.[2] This process of intraplaque angiogenesis is triggered by hypoxia.[3] These neovessels, however, are often immature and lack pericyte coverage, which leads to excessive extravasation of erythrocytes and leukocytes.[4] This in turn fuels inflammation, therefore creating a downward spiral of intraplaque angiogenesis and intraplaque hemorrhage (IPH) that renders an unstable plaque phenotype, which closely associates with plaque progression.[3,5]

Bypass surgery using venous grafts is a well-established treatment method to revascularize hypoxic tissue downstream occlusive atherosclerotic lesions. The patency rates of these vein grafts, however, are diminished by vein graft disease.[6] Similar to atherosclerosis, vein graft atherosclerotic lesions are hypoxic and exhibit intraplaque angiogenesis and IPH, which actively contribute to vein graft failure.[7]

Atherosclerotic plaque hypoxia directly affects macrophages, which comprise the major plaque leukocyte subset and aim to clear cholesterol and apoptotic cells in the vessel wall but are often unsuccessful. These macrophages ultimately transform into cholesterol laden "foam cells", exhibiting high metabolic activity and as a result, driving plaque hypoxia.[8] This compromises crucial (immune) functions and hampers macrophage function, which ultimately contributes to necrotic core formation.[9] Restoring their function by improving plaque oxygenation might therefore diminish atherogenesis. In a murine model for atherosclerosis, hyperoxia treatment alleviated plaque hypoxia and prevented necrotic core formation by enhancing efferocytosis.[10] In our model for vein graft atherosclerosis, it was shown that similar treatment improved vein graft patency and plaque stability as well as reducing macrophage accumulation. Increased exposure to oxygen did improve macrophage function, whereas no effect was found on lesion size, intraplaque angiogenesis and plaque hypoxia.[11] As these interventions did not impact quality or quantity of plaque neovessels, the question remains whether increased intraplaque angiogenesis can reduce lesion size.

Prolyl hydroxylases (PHD, official gene name Egl-9 Family Hypoxia Inducible Factor) 1,2 and 3 together with hypoxia inducible factor (HIF) 1α and 2α play a central role in hypoxic signaling. Under normoxic conditions, PHD enzymes use oxygen to hydroxylate HIF-1α and HIF-2α, which signals their ubiquitination and proteosomal degradation.[12] Upon hypoxia, PHD hydroxylation activity is reduced, leading to stabilization of HIF-α units, that translocate into the nucleus and ultimately transcriptionally activate downstream targets, including vascular endothelial growth factor (VEGF).[13,14] The expression of HIF-1α correlates with intraplaque angiogenesis and IPH in human lesions[3,15,16], but its causative effect on atherogenesis is controversial.[17,18] HIF-1α overexpression has been demonstrated to reduce atherosclerotic plaque size[17], whereas myeloid HIF-1α deletion reduced atherogenesis.[18] We recently reported that myeloid PHD deficiency exacerbated atherosclerosis and that expression of PHDs correlated with human plaque inflammation.[19] Interestingly, both HIF-1α and PHDs were predominantly expressed in macrophages.[15,19,20] The effects on intraplaque angiogenesis, however, remain unknown, since murine naïve atherosclerotic lesions do not present with leaky neovessels growing into the hypoxic plaque, despite previous work that has convincingly demonstrated hypoxia in these lesions.[10] This would suggest other factors affecting angiogenesis in naïve atherosclerosis in mice. In contrast, our murine atherosclerotic vein graft lesions exhibit substantial ingrowth of neovessels that are also immature, as we previously reported[21], thus yielding a suitable pre-clinical model to study hypoxic signaling, intraplaque angiogenesis and its effect on unstable atherosclerotic lesions. We therefore investigated the role of myeloid PHD2 on intraplaque angiogenesis in a murine model for venous bypass grafting.

MATERIALS AND METHODS

Mice

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Centre and Maastricht University Medical Centre. PHD2 conditional knock-out mice (PHD2cko)[22] and LysMCre transgenics,[23] were crossed to low-density lipoprotein receptor knock-out ($LDLr^{-/-}$) mice, obtained from an in-house breeding colony, originating from Charles River (Wilmington, MA, USA) and refreshed every 10 generations to avoid genetic drift. LysMCre $LDLr^{-/-}$ mice (hereafter referred to as PHD2 WT) served as control in all experiments with PHD2cko $LDLr^{-/-}$ mice (hereafter referred to as PHD2cko). Male mice were used as recipient while genotype-matched female mice were used as donor. All mice were crossed back on LDLrko C57Bl6/J background at least nine times. Animals were housed in the laboratory animal facility of Maastricht University under standard conditions. Food and water were provided *ad libitum* during the entire experiment. All animals were housed in individually ventilated cages (GM500, Techniplast) in groups of up to 5 animals per cage, with bedding (corncob, Technilab-BMI) and cage enrichment. Cages were changed weekly, reducing handling of the mice to one handling per week during non-intervention periods. Mice were fed a high-cholesterol diet *ad libitum* (0.25% cholesterol, SDS 824171) for 4 weeks following surgery until sacrifice.

Vein Graft Surgery

Vein graft surgery was performed by inter-positioning the caval vein of a donor mice into the arterial circulation of a recipient mice at the site of the right common carotid artery as previously described.[24] In brief, the thoracic caval vein was harvested from donor mice. Then, the right carotid artery of recipient mice was clamped and cut in the middle. The artery was thereafter everted around the cuffs which were placed on both ends of the artery and ligated with 8.0 sutures. The caval vein was then sleeved over both cuffs and ligated. Mice were put under anaesthesia with midazolam (5mg/kg, Roche Diagnostics, Basel, Switzerland), medetomidine (0.5 mg/kg, Orion, Espoo, Finland) and fentanyl (0.05 mg/kg, Janssen Pharmaceutical Beerse, Belgium). Response to toe pinching of the mice as well as monitoring breathing frequency was used to assess adequacy. After surgery, buprenorphine (0.1 mg/kg, MSD Animal Health, Keniworth, NJ, USA) was given for pain relief. At sacrifice, mice were anaesthetized and whole blood was drawn via the orbital sinus. Following exsanguination, the abdomen was opened and 5mL PBS was used to perfuse the mice from the left ventricle. Thereafter, 5mL formalin was perfused in a similar fashion to minimize morphological changes upon loss of hemodynamic forces after sacrifice.

Flow cytometry

Whole blood was added to BD Trucount Absolute Counting Tubes (340334) containing Fc receptor block solution (anti-CD16/32 antibody, eBioscience). Thereafter, blood was stained with an antibody mix to distinguish between leukocytes in general (CD45+, Biolegend), T cells (CD3ε+, NK1-1-; Miltenyi, eBioscience, resp.), B cells (B220+; BD), NK cells (NK1-1+), granulocytes (CD11bhigh Ly6Ghigh; BD, eBioscience, resp.) and monocytes (CD11bhigh Ly6Glow Ly6Chigh/intermediate/low; Miltenyi). Erythrocyte lysis buffer was added and samples were measured using a FACS Canto II (BD Biosciences) and analyzed with FACSDiva software (BD Biosciences). Gating strategy can be found in the **supplemental figure 1**.

Histological and Immunohistochemical assessment of Vein Grafts

Vein graft samples were embedded in paraffin, and cross sections (5µm thick) were made throughout the entire vein graft. The total vein graft was analyzed by minimum of 6 equally spaced sections. The plastic cuff functioned as starting point to mount sections onto glass slides. Slides stained according to MOVAT pentachrome staining were used for morphometric analysis. Lumen, intima (I) and media (M) area were measured in CaseViewer (3DHistech ltd. Version 2.4). From these parameters, I/M area ratios were calculated to quantify vein graft disease.

To quantify collagen content of the vein graft wall, Sirius Red staining (Klinipath 80,115) was used. Neovessels were stained with CD31 (1:4000, RM1006, Abcam Ab281583) and subsequent visualization with DAB substrate complex. Intraplaque angiogenesis was assessed by manually quantifying the amount of CD31+ neovessels in the vein graft wall (number / mm^2) as well as % area coverage.

Stained slides were scanned using Panoramic SCAN II (3D Histech). ImageJ was used for compositional analysis.

Macrophages were stained with either CCR2 (1:100, Alexa Fluor 647, clone SA203G11, Biolegend) or CD206 (1:100, Alexa Fluor 647 clone C068C2, Biolegend) in combination with MAC3 (1:100, CD107b, M3/84) to assess total macrophage presence and polarization. Mesenchymal cells were stained with alpha Smooth Muscle Actin (ACTA2) (1:100, AF647, SC32251, Santa Cruz) together with Ter119 (1:200, Alexa Fluor 488, 116202, Biolegend). VEGF visualized using a VEGF antibody directly conjugated to Alexa Fluor 647 (1:100, SC-7269, Santa Cruz). Images were obtained with laser scanning microscopy (LSM700, Zeiss). After deparaffinization, antigen retrieval, permeabilization and blocking of aspecific signal, the antibodies were diluted in 1% PBSA and 1% normal goat serum and incubated overnight at 4 °C. The next day, the slides were washed and incubated with secondary antibody (Streptavidin Cy3, 1:400) for 2 hours at 4 °C. Thereafter, slides were again washed and incubated for 5 min with Hoechst 34580 (1:800, Sigma) and mounted with Prolong gold. For analysis of macrophages, double-positive (CCR2&MAC3 or CD206&MAC3) determination was automatically done by a custom Python script run in Jupyter Notebook (Anaconda 3), after quantification of integrated density of MAC3, CCR2 and CD206 signal in ImageJ.

The first percentile of the integrated density distribution was set as a threshold to discard background.[25] Mesenchymal cells were quantified as ACTA2 positive area % in ImageJ. Intraplaque hemorrhage was scored by a blinded observer per quartile ranging from 0 (no hemorrhage) to, 1 (mild), 2 (moderate), 3 (severe hemorrhage) based on the number Ter119$^+$ spots: 0 = no Ter119$^+$ spots; 1 = <10 minor Ter119$^+$ spots; 2 = 10-20 minor and/or <3 major Ter119$^+$ spots; 3 = >20 minor and/or >3 major Ter119$^+$ spots.

Bone Marrow-Derived Cell Culture and ELISA

M-CSF differentiated bone marrow-derived macrophages were plated in 1500ul/well in 10% FCS, 1% PS in RPMI. Medium was either supplemented with 10 or 50 ng LPS (Sigma, K235) / ml. Cells were cultured for 24 hours and thereafter, medium was collected for ELISA as well as aortic ring assay, after which Trizol was added for RNA-isolation. Supernatant was stored at -80 °C. CCL2, IL-6, TNF-α and VEGF concentrations were determined by ELISA according to manufacturer's protocol (R&D systems, DY406, DY410, DY479 & DY493).

mRNA Expression analysis.

RNA was isolated from macrophages according to manufacturer's protocol and quantified using Nanodrop 1000 Spectrophotometer (Thermo Scientific). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. SybrGreen reagents (Qiagen Benelux, Venlo, the Netherlands, #204145) were used for the qPCR. qPCR was performed on ABI7500 Fast system. A list of all primers can be found in **supplementary table 1**, *Rplp0* was used as House Keeping Gene.

Analysis/Statistics

All variables were tested for normal distribution using Shapiro-Wilk normality test. Unpaired parametric t-test or Mann-Whitney test was used to assess statistical differences in continuous variables in Graph Pad Prism (Version 9.3.1). Fischer's exact test was used to assess statistical differences between two categorical variables. Data are represented as mean ± SEM unless indicated otherwise. $P<0.05$ were regarded as statistically significant. Significant differences are displayed as *$p<0.05$, **$p<0.01$, ***$p<0.005$ and ****$p<0.001$.

RESULTS

Increased VEGF production in PHD2cko macrophages

Having previously confirmed PHD2cko in macrophages and subsequent increased expression of HIF-1α[19], bone marrow-derived macrophages from myeloid PHD2 WT and cKO LDLr$^{-/-}$ mice were cultured *in vitro*, to assess the effect of PHD2 on production of pro-angiogenic factors by macrophages. After 24 hours, VEGF-production was increased by nearly 3-fold in the PHD2cko group upon stimulation with both 10 and 50 ng/ml LPS, mimicking the inflammatory environment in (vein graft) atherosclerotic lesions (**fig. 1A**, p<0.0001 and p=0.0004 respectively). In addition, expression of *Vegfa* mRNA was also increased upon PHD2 deficiency (**fig. 1B**, p=0.017 and p=0.03 respectively). Expression of other angiogenic genes (*Cxcl12 (C-x-c motif ligand 12)*, *Cxcr4 (C-x-c motif receptor 4)*, *Pdgf-a (platelet-derived growth factor-a)*, *Pdgf-b*, **supplemental fig. 2A-D**) was less prominently altered, identifying Vegf as the dominant angiogenic gene. Furthermore, no clear trend in excretion of inflammatory cytokines (C-C motif ligand 2 (CCL2), interleukin-6 (IL-6) and tissue necrosis factor-α (TNF-α)) could be observed between both groups (**supplemental fig. 2E-G**).

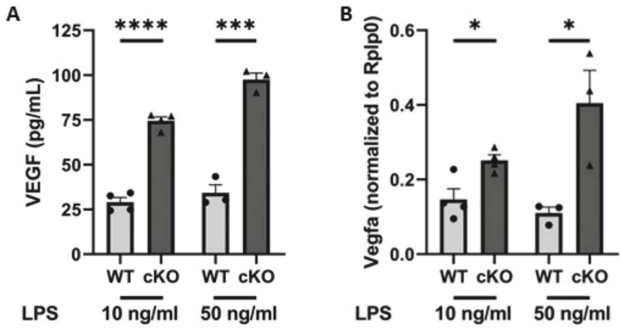

Figure 1. PHD2cko macrophages have a distinct angiogenic phenotype. Bone marrow-derived macrophages were cultured in vitro and stimulated with different concentrations of LPS for 24 hours.

(**A**) Concentration of VEGF in supernatant, (**B**) normalized expression of *Vegfa*. All data are presented as mean ± standard error of the mean (SEM). *p<0.05, ***P<0.001,****p<0.0001. LPS – lipopolysaccharide; PHD2cko – prolyl hydroxylase 2 conditional knock-out; VEGF – vascular endothelial growth factor.

Myeloid PHD2cko vein grafts display beneficial remodeling

Myeloid PHD2 WT and cKO LDLr$^{-/-}$ mice [19] underwent bypass surgery to assess the effect of myeloid PHD2 on vascular remodeling (n=11-15/group). (**fig. 2A**). A minor increase in body weight was observed in PHD2cko animals (**supplemental fig. 3**). After 28 days, the animals were sacrificed and tissue was collected for analysis.

Two vein grafts of PHD2 WT animals and one vein graft in PHD2cko group were excluded from analysis due to thrombosis and subsequent occlusion of the graft. Furthermore, three vein grafts in the WT group were classified as dissection and excluded from further analysis, since we regarded this as different pathology (aneurysm formation) than atherosclerosis which would significantly influence our results (**fig. 2B-D**). In addition, autopsy on the two deceased PHD2 WT mice in the follow-up after surgery indicated suspected rupture of graft based on large hemorrhagic area in neck. In total, 5 dissections / suspected ruptures were identified in the PHD2 WT group, compared to none in the cKO group, which was a statistically significant difference (**fig. 2C-D**, p=0.046). As a result, 8 vein grafts from PHD2 WT animals and 10 from PHD2cko were used for morphometric and compositional analysis.

Lumen size was unaffected by PHD2cko and thus comparable between both groups (**fig. 2E-F**). A non-significant decrease in intimal area and a minor, non-significant increase in medial area was observed in the PHD2cko group (**fig. 2G-H**). As a result, the intima / media (I/M) ratio was decreased 35% in the PHD2cko group compared to WT, indicating beneficial vessel wall morphology (**fig. 2I**, p=0.016).

To delineate the mechanism underlying the observed differences in the number of dissections and a potential link to extracellular matrix destabilization, collagen and mesenchymal cell content of the vein grafts was analyzed. Collagen content in the total vessel was increased 80% in the cKO group compared to WT (**fig. 3A-D**, p=0.035). More specifically, there was a 98% significant increase in collagen content in the intima (**fig. 3B**, p=0.041) and a 43% non-significant increase in the media (**fig. 3C**, p=0.15). Mesenchymal cell content of the vessel wall, as assessed by actin alpha 2 (ACTA2) staining, was unaffected by PHD2 deficiency (**fig. 3E-H**).

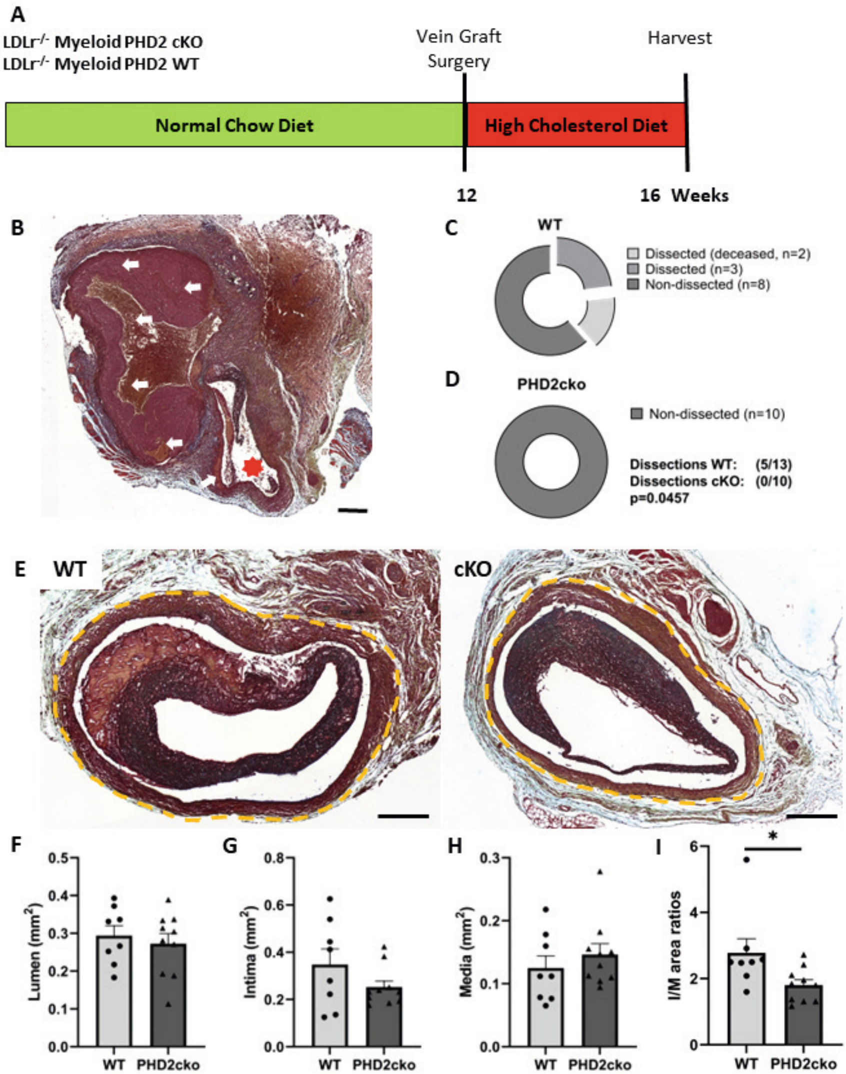

Figure 2. Myeloid PHD2 deficiency decreases vein graft disease and protects from dissection.

(**A**) Schematic overview of experimental design. (**B**) Representative image of dissections, arrows indicate IPH and fibrin deposition, red star indicates lumen of vein graft. Chart-pies of dissections and distribution over (**C**) WT and (**D**) cKO mice. (**E**) Representative images of MOVAT Pentachrome staining of both WT and cKO vein grafts. Morphometric analysis of vein grafts by quantification of: (**F**) lumen, (**G**) intima, (**H**) media and (**I**) intima / media (I/M) ratio. All data are presented as mean ± standard error of the mean (SEM). Scale bars indicate 200μm. *p<0.05. cKO – conditional knock-out; IPH – intraplaque hemorrhage; PHD2 – prolyl hydroxylase 2; WT – wildtype.

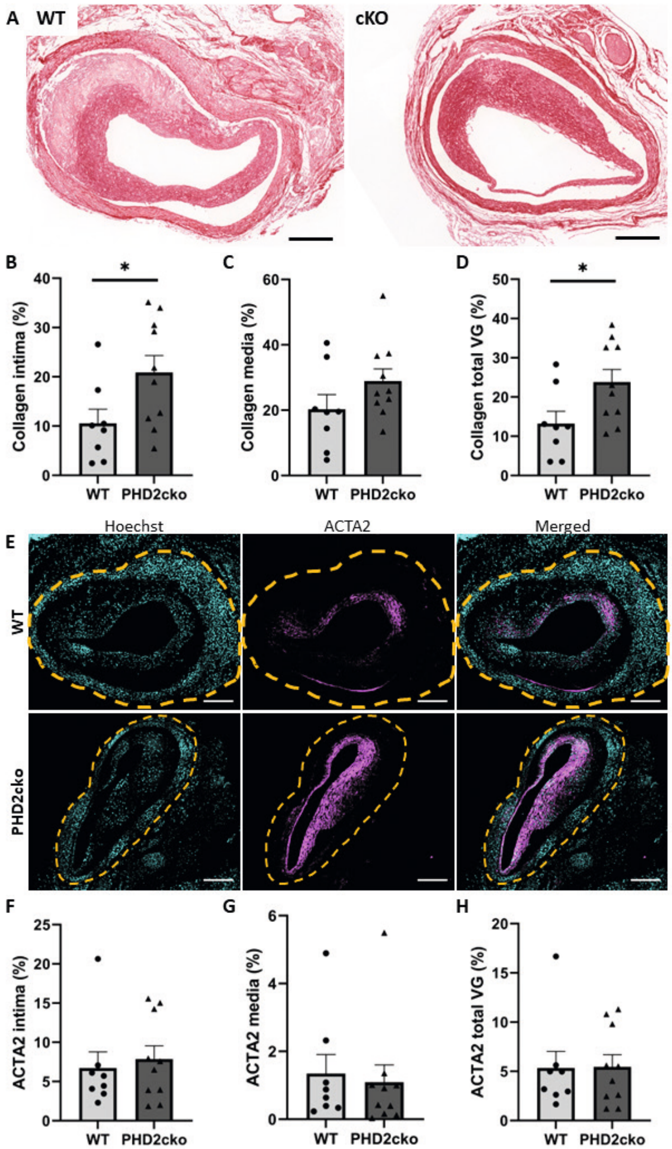

Figure 3. Increased collagen content in the vein graft wall of myeloid PHD2cko mice.

(**A**) Representative images of Sirius Red staining of both WT and cKO vein grafts. Quantification of collagen content of (**B**) intima, (**C**) media and (**D**) total vein graft. (**E**) Representative images of ACTA2 staining. Quantification of ACTA2 content of (**F**) lumen, (**G**) intima and (**H**) total vein graft. All data are presented as mean ± standard error of the mean (SEM). Scale bars indicate 200μm. *$p<0.05$. ACTA2 – actin alpha 2; cKO – conditional knock-out; PHD2cko – prolyl hydroxylase 2 conditional knock-out; WT – wildtype.

Myeloid PHD2cko favorably alters macrophages polarization, whilst reducing systemic but not local monocyte count

In the vein graft, total macrophage presence was unaffected by PHD2cko (**fig. 4A-B**). Furthermore, there was no difference in macrophage presence in both intimal and medial layer (**supplemental fig. 4A-B**). Total inflammatory macrophage number, defined as $MAC3^+$/C-C motif receptor 2^+ ($CCR2^+$) cells, was decreased by 45% (**fig. 4C-D**, p=0.009) in the PHD2cko group compared to control. In the intima, the amount of inflammatory macrophages was reduced by 45% (p=0.017) and in the media by 43% (**supplemental fig. 4C-D**, p=0.04). Systemic CCL2 concentration at post-operative day 28 (POD28) was non-significantly reduced in the PHD2cko group compared to control (**supplemental fig. 3B**, 31±9 vs 9±7, p=0.052). In contrast to the decreased number of inflammatory macrophages, resident, anti-inflammatory macrophage number, defined as $MAC3^+$/cluster of differentiation 206 $(CD206)^+$ cells, was doubled in the vessel wall in the cKO group (**fig. 4E-F**, p=0.042). Inflammatory macrophages are the dominant subtype in this hyperinflammatory murine vein graft model and found in both the intima and media. In contrast, anti-inflammatory macrophages are less present and mostly found in the media (**supplemental fig. 4E-F)**.

To investigate if changes in circulating monocytes were underlying vein graft inflammatory macrophages, we studied systemic leukocyte counts. Total circulating leukocyte count increased significantly in the cKO group compared to control, mainly due to significant increase in B-cells (**supplemental fig. 5A-B**). Systemic monocyte count was significantly reduced in the PHD2cko group compared to control (**fig. 5A**, p=0.017). This effect was derived from a non-significant decrease in number of lymphocyte antigen 6 complex $(Ly6C)^{high}$ monocytes (p=0.14) and a significant decrease in $Ly6C^{low}$ and Ly6C- monocytes (**fig. 5B-D**, p=0.039 & p=0.006). Hence, resident macrophage expansion inside the vein graft may not directly derive from changes in circulating monocytes. Absolute counts of granulocytes were not significantly different between both groups (**supplemental fig. 5C**), despite lysozyme M (lysM) expression in granulocytes.

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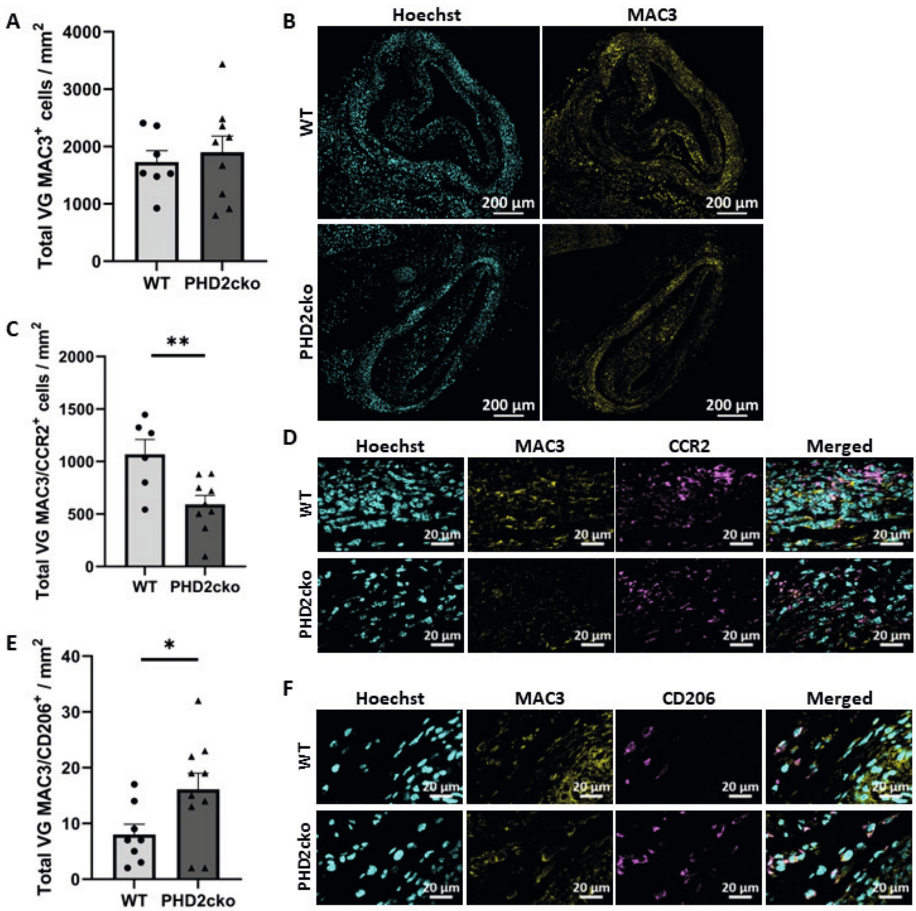

Figure 4. Myeloid PHD2 favourably alters macrophage polarization.

Quantification and representative images of total number (**A,B**), CCR2+ (**C,D**) and CD206+ macrophages (**E,F**). All data are presented as mean ± standard error of the mean (SEM), *p<0.05, **p<0.01. CCR2 – C-C motif chemokine receptor 2; CD206 – cluster of differentiation 206; PHD2 – prolyl hydroxylase 2.

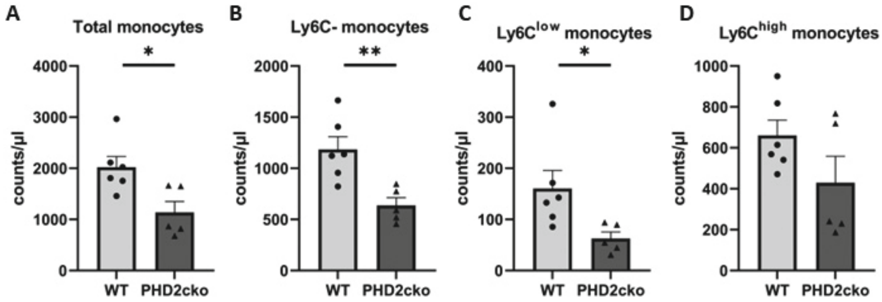

Figure 5. Myeloid PHD2 deficiency reduces systemic monocyte count.

Absolute counts of (**A**) total monocytes, (**B**) Ly6C-, (**C**) $Ly6C^{low}$ and (**D**) $Ly6C^{high}$ monocytes in whole blood. All data are presented as mean ± standard error of the mean (SEM), *$p<0.05$, **$p<0.01$. Ly6C – lymphocyte antigen 6 complex; PHD2 – prolyl hydroxylase 2.

Increased intraplaque angiogenesis and VEGF, but reduced intraplaque hemorrhage in myeloid PHD2cko mice

In addition to the altered macrophage polarization, we investigated the effect of myeloid PHD2cko on VEGF presence and its relation to intraplaque angiogenesis in the vessel wall of the vein grafts. VEGF was located in close proximity to the angiogenic neovessels and in areas with high expression of MAC3 (**fig 6A**). Moreover, a three-fold increase in VEGF-positive area was observed in the PHD2cko group compared to control (**fig. 6B**, p=0.0706). CD31 content of the vein graft was 39% increased in the PHD2cKO group compared to WT (**fig. 6C**, p=0.034). The number of angiogenic neovessels was increased by 66% in the PHD2cko group, indicating increased neovessel density (**fig. 6D**, p=0.014). In contrast, the neovessel size was comparable between both groups (**fig. 6E**, 79±22 vs 78±13, p=0.89). In addition, the endothelial coverage of the lumen was quantified, as a degree of luminal re-endothelialization (since the vein graft procedure results in de-endothelialization of the vessel segment). CD31 coverage of the lumen was not increased upon myeloid PHD2 deficiency (**fig. 6F**, p=0.11).

Angiogenic neovessel maturation, as measured by the coverage of neovessels by ACTA2, was significantly increased in the PHD2cko group compared to control (**fig. 6G-H**, 1.89±0.42 vs 1.33±0.48, p=0.027). Furthermore intraplaque hemorrhage, as defined by extravasated erythrocytes ($Ter119^{+}$ cells) in the lesion, was diminished in the PHD2cko group (**fig. 6G,I**, 1.74±0.47 vs 1.28±0.30, p=0.022), indicating the improved quality of the neovessels also resulted in decreased vascular leakage.

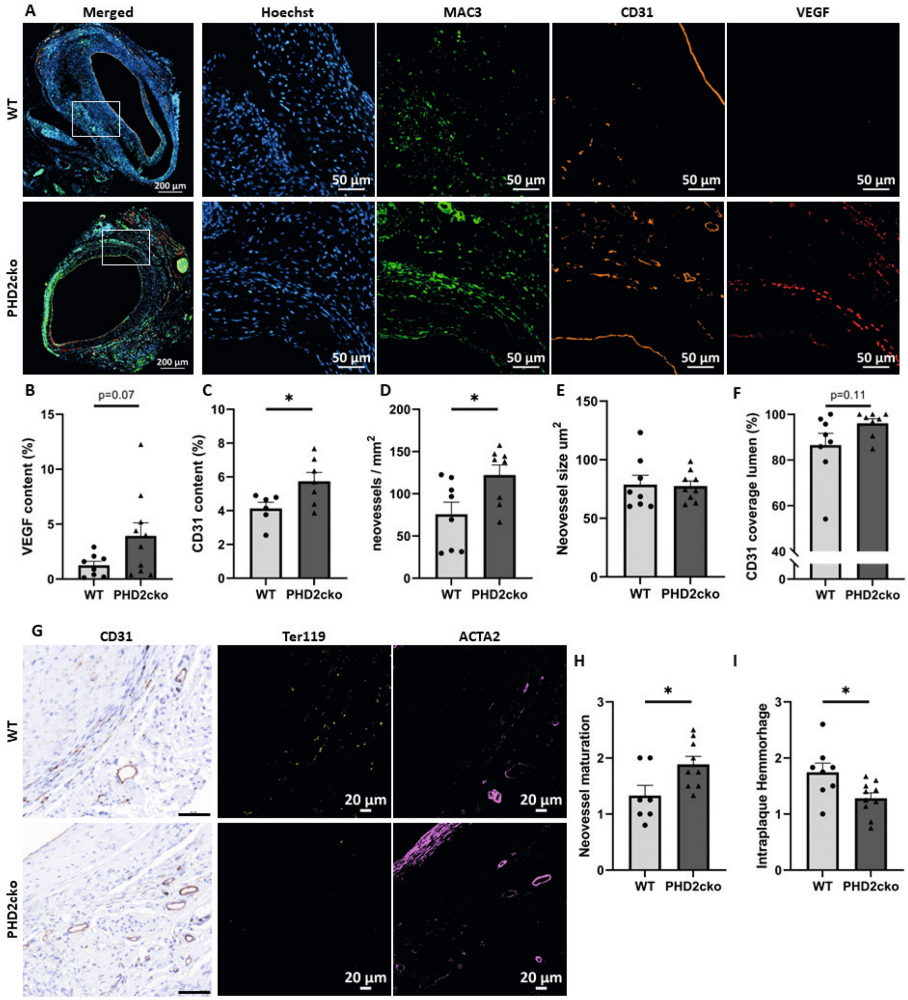

Figure 6. Enhanced and improved intraplaque angiogenesis upon PHD2cko leads to a reduction in intraplaque hemorrhage.

(**A**) Representative images of MAC3, CD31 and VEGF staining and quantification of (**B**) VEGF content, (**C**) CD31 content, (**D**) number of angiogenic neovessels, (**E**) neovessel size and (**F**) CD31 coverage of the lumen. (**G**) Representative images of CD31, ACTA2 and Ter119 staining and quantification of (**H**) neovessel maturation (0 – no maturate neovessels, 3 – fully mature neovessels), (**I**) intraplaque hemorrhage (0 – no extravasated erythrocytes ($Ter119^+$), 3 – severe intraplaque hemorrhage). All data are presented as mean ± standard error of the mean (SEM), scale bar indicates 50 µm. *p<0.05. ACTA2 – actin alpha 2; CD31 – cluster of differentiation 31; PHD2cko – prolyl hydroxylase 2 conditional knock-out; VEGF – vascular endothelial growth factor.

DISCUSSION

In this study, we demonstrate that myeloid PHD2cko improves vascular remodeling and ameliorates lesion stability by enhancing intraplaque angiogenesis and improving angiogenic neovessel maturation leading to reduced intraplaque hemorrhage. Moreover, dissections – a hallmark of unstable lesions characterized by blood-filled gaps within the intimal hyperplasia[25] – were not observed in the PHD2cko group, whilst collagen content was increased compared to WT mice. Additionally, in the myeloid PHD2cko mice, macrophages in the vein graft wall exhibit a polarization from an inflammatory towards an anti-inflammatory phenotype.

Previously, we reported increased HIF-1α signaling in myeloid PHD2 macrophages *in vitro*.[19] In this study, we further characterized these macrophages and observe an increase in VEGF production, elucidating a distinct angiogenic phenotype. Furthermore, a slight increase in VEGF excretion was observed upon increased concentration of LPS in the PHD2cko, but not WT macrophages. This indicates an inhibitory role of PHD2 on LPS-induced VEGF-production, potentially deriving from crosstalk between intracellular inflammatory and hypoxic signaling pathways. PHD2 has been reported to attenuate NF-κB activity[26], for example, whilst NF-κB itself is activated by LPS[27] and regulates VEGF expression in human macrophages.[28] Moreover, hypoxic signaling has been known to induce VEGF production in multiple cell types[29,30], including myeloid cells.[31] Deletion of myeloid PHD2, evoking exaggerated "pseudo"hypoxia-signaling, increased expression of VEGF.[32] Functionally, it has been demonstrated that production of VEGF by transcriptional activation of HIF-1α in monocytes promotes angiogenesis *in vivo*.[33] Additionally, general PHD2 deficiency has been shown to induce angiogenesis through VEGF.[34] *In vivo*, we observe a three-fold increase in VEGF content in the vein grafts of the myeloid PHD2cko group compared to control. Furthermore, the VEGF was predominantly localized near the angiogenic neovessels and also near macrophages, indicating a paracrine effect of these macrophages on the endothelial cells in the vein graft wall.

Myeloid PHD2 deficiency increased intraplaque angiogenesis density, without affecting their diameter. Interestingly, neovessel maturation was increased in the PHD2cko group, yielding improved neovessel stability resulting in a decrease in IPH. It was previously shown that interfering in the angiogenic signaling cascade by bFGF blockade improved neovessel maturation in vein grafts, whilst inhibiting intraplaque angiogenesis without affecting lesion size.[7] Additionally, we have shown that improving maturation of neovessels seems more important for plaque stability rather than reducing intraplaque angiogenesis, using a VEGFR2 blocking antibody.[21] In contrast, inducing intraplaque angiogenesis to potentially overcome plaque hypoxia and restore cellular function by perivascular VEGF-therapy resulted in a non-significant decrease in plaque size.[21] Moreover, here we demonstrate that stimulating and concomitantly improving intraplaque angiogenesis, including a healthy and mature neovessel composition, improves plaque stability. In the oncology

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field, decreased PHD2 activity correlated with increases in tumor angiogenesis.[35] Furthermore, PHD2 haploinsufficiency improved vessel function and maturation, thus inducing endothelial normalization.[22] These effects were mainly dependent on ECs, whilst cell-intrinsic responses to PHD2 alterations differ and affect outcomes.[36] In our previous study, myeloid PHD2cko did not affect microvessel density in the adventitia of atherosclerotic plaques of the aortic root and brachiocephalic artery.[19] No intraplaque vessels were detected as expected in murine naive atherosclerosis, in contrast to the vein graft model of accelerated atherosclerosis used in this manuscript which exhibits pronounced angiogenesis. In the naïve model of atherosclerosis, we demonstrated that myeloid PHD2cko resulted in increased atherogenesis and reduction in macrophage content of the plaque due to increased apoptosis.[19]

In the vein graft model, a difference in macrophage polarization rather than a difference in macrophage content was observed. More specifically, there is a marked reduction in inflammatory macrophages ($CCR2^+$) in the vein graft wall. These inflammatory macrophages represent the vast majority of macrophages, contributing to plaque destabilization by secretion of pro-inflammatory cytokines and matrix-degrading enzymes.[25,37] These macrophages are associated with plaque-progression, in both murine and human lesions.[38] At POD28, the systemic concentration of CCL2, a chemokine that attracts monocytes and activates CCR2[39], was reduced in the PHD2cko animals compared to WT. This systemic reduction could explain the local decrease in $CCR2^+$ macrophages. We have previously found that blocking CCL2-CCR2 signaling by local, perivascular application (after surgery) of lentiviral short-hairpin RNA targeted against CCR2 reduced vein graft thickening at POD28[40], demonstrating beneficial effects of reduced CCR2 expression in the vein graft.

In contrast to pro-inflammatory macrophages, anti-inflammatory macrophages are associated with plaque stability and regression and were increased in PHD2cko vein grafts. These anti-inflammatory macrophages are mostly found at the adventitial side of blood vessels and have also been described to have improved angiogenic capacity *in vivo*.[38,41] This is in line with the general idea that neovessels arise from the vasa vasorum, c.q. the adventitia, and corroborates our finding that VEGF was mainly located near the neovessels and macrophages on the adventitial side of the vein graft. We observe a systemic increase in circulating leukocyte counts at POD28, mainly due to a significant increase of B-cells, and a decrease in circulating monocyte count in the PHD2cko group due to an unknown cause. This, however, does not appear to affect the vein graft, since no differences were observed in total macrophage content of the vein graft wall. Furthermore, the systemic decrease in $Ly6C^{low}$ monocytes, that have been described to differentiate into anti-inflammatory macrophages[42], in the PHD2cko group is in contrast with our increase in anti-inflammatory macrophages in the vein graft in the same group. Overall, this indicates that resident CD206+ macrophage expansion ensues local changes in the vessel wall rather than monocyte-derived infiltration.

The anti-inflammatory macrophage phenotype is not only associated with tissue repair but also with fibrosis.[43] Myeloid PHD2cko was shown to induce fibrosis by enhancing fibroblast collagen secretion in a paracrine manner *in vitro*, without affecting macrophage matrixmetalloprotease (MMP) activity or smooth muscle cell migration and proliferation. Overall, myeloid PHD2 deficiency led to an increase in collagen content *in vivo*, without altering vascular smooth muscle cell function and density.[19] In our current study, we also do not observe a difference in mesenchymal cell content of the vein graft, whereas a significant increase in collagen content was observed in PHD2cko vein grafts. A decrease in collagen compromises lesion stability and was previously found to associate with vein graft dissections.[25] Interestingly, vein graft dissections were only observed in the WT group, indicating improved vessel wall stability upon myeloid PHD2 deficiency due to an increase in collagen content.

Overall, we can conclude that myeloid PHD2cko ameliorates vein graft lesion stability by inducing intraplaque angiogenesis through increased VEGF excretion, in turn improving intraplaque angiogenesis, consequently reducing intraplaque hemorrhage. Moreover, increased collagen content prevents vein graft dissections and macrophage polarization is favourably altered towards an anti-inflammatory phenotype. This study sheds new light on intraplaque angiogenesis and its relation to plaque hypoxia and atherogenesis and amplifies experimental evidence which demonstrates that intraplaque angiogenesis and intraplaque hemorrhage are not intrinsically linked. Moreover, it illustrates for the first time that stimulating, rather than inhibiting, intraplaque angiogenesis whilst simultaneously also improving angiogenic neovessel integrity to reduce intraplaque hemorrhage, can improve lesion stability.

Author contributions

TJS - data curation and formal analysis, visualization, methodology, writing original manuscript;
RJHAT - data curation and formal analysis, visualization, editing manuscript;
AdJ - methodology, review manuscript;
JBGdB - data curation and formal analysis; review manuscript;
HABP - methodology, data curation and formal analysis; review manuscript;
RvdL - data curation and formal analysis; review manuscript;
RH - data curation and formal analysis; review manuscript;
MW - data curation and formal analysis; review manuscript;
LIWS - data curation and formal analysis; review manuscript;
PHAQ - methodology, editing and reviewing manuscript;
JCS - conceptualization, funding acquisition, methodology, supervision, editing and reviewing manuscript;
MRdV - conceptualization, funding acquisition, methodology, supervision, editing and reviewing manuscript.

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Data availability

Data are available from the corresponding author upon reasonable request

Conflict of interest

The authors declare to have no conflict of interest. All authors have read and agreed to the publication of the manuscript

Ethical Approval

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Centre and Maastricht University Medical Centre

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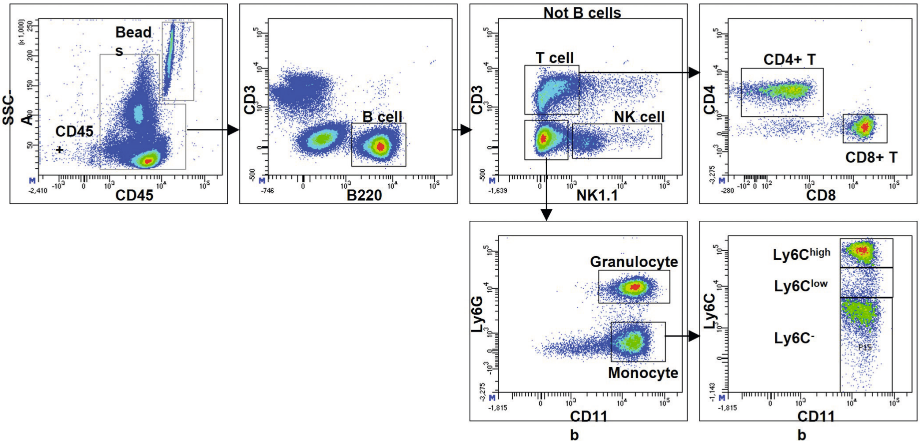
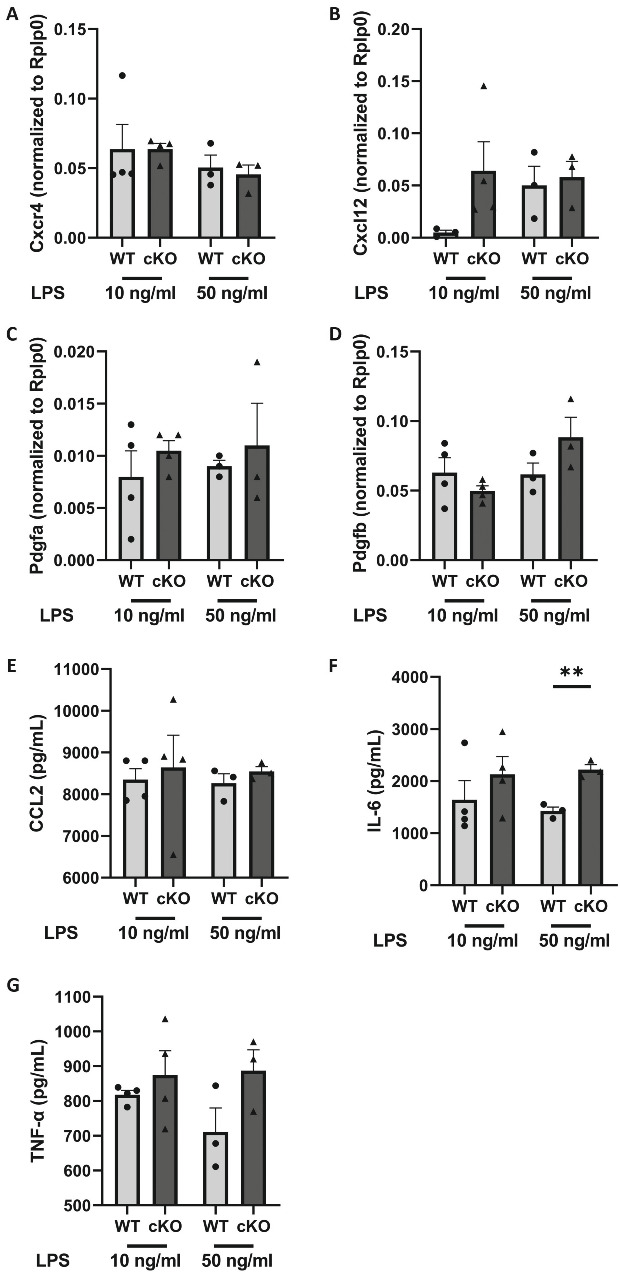
SUPPLEMENTAL FIGURES



Supplemental figure 1. Flow cytometry gating strategy to assess counts of leukocyte subtypes in blood.

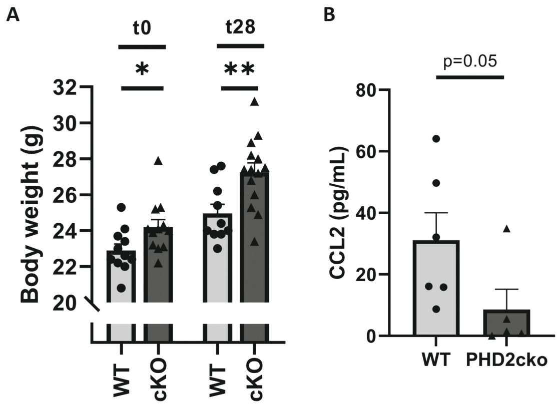
CD – cluster of differentiation; Ly6C – lymphocyte antigen 6 complex; Ly6G – lymphocyte antigen 6 complex locus G.



Supplemental figure 2. Bone marrow-derived macrophages were cultured in vitro and stimulated with different concentrations LPS for 24 hours.

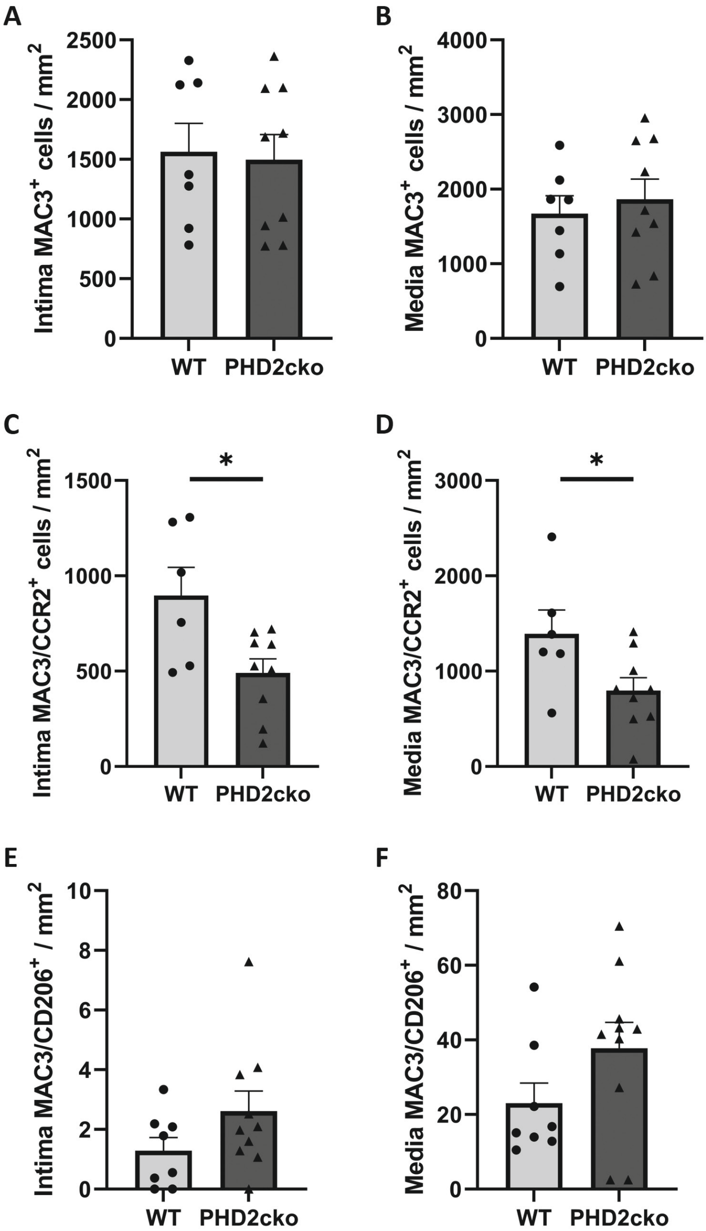
Normalized expression of (**A**) *Cxcr4*, (**B**) *Cxcl12*, (**C**) *Pdgfa* and (**D**) *Pdgfb*. Concentration of (**E**) CCL2, (F) IL-6 and (**G**) TNF-α in supernatant. All data are presented as mean ± standard error of the mean

(SEM). **p<0.01. CCL2 – C-C motif chemokine ligand 2; IL-6 – interleukin 6; LPS – lipopolysaccharide; TNF – tissue necrosis factor.



Supplemental figure 3.

(**A**) Body weight of the mice pre-operatively (t0) and at sacrifice (t28). (**B**) Concentration of CCL2 in plasma at t28. All data are presented as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01. CCL2 – C-C motif chemokine ligand 2.



Supplemental figure 4. Myeloid PHD2 deficiency favourably alters macrophage polarization in the vessel wall.

Quantification of (**A-B**) total, (**C-D**) CCR2$^+$ and (**E-F**) CD206$^+$ macrophages in the intima and media. All data are presented as mean ± standard error of the mean (SEM), *p<0.05. CCR2 – C-C motif chemokine receptor 2; CCL2 – C-C chemokine ligand 2; CD206 – cluster of differentiation 206; PHD2 – prolyl

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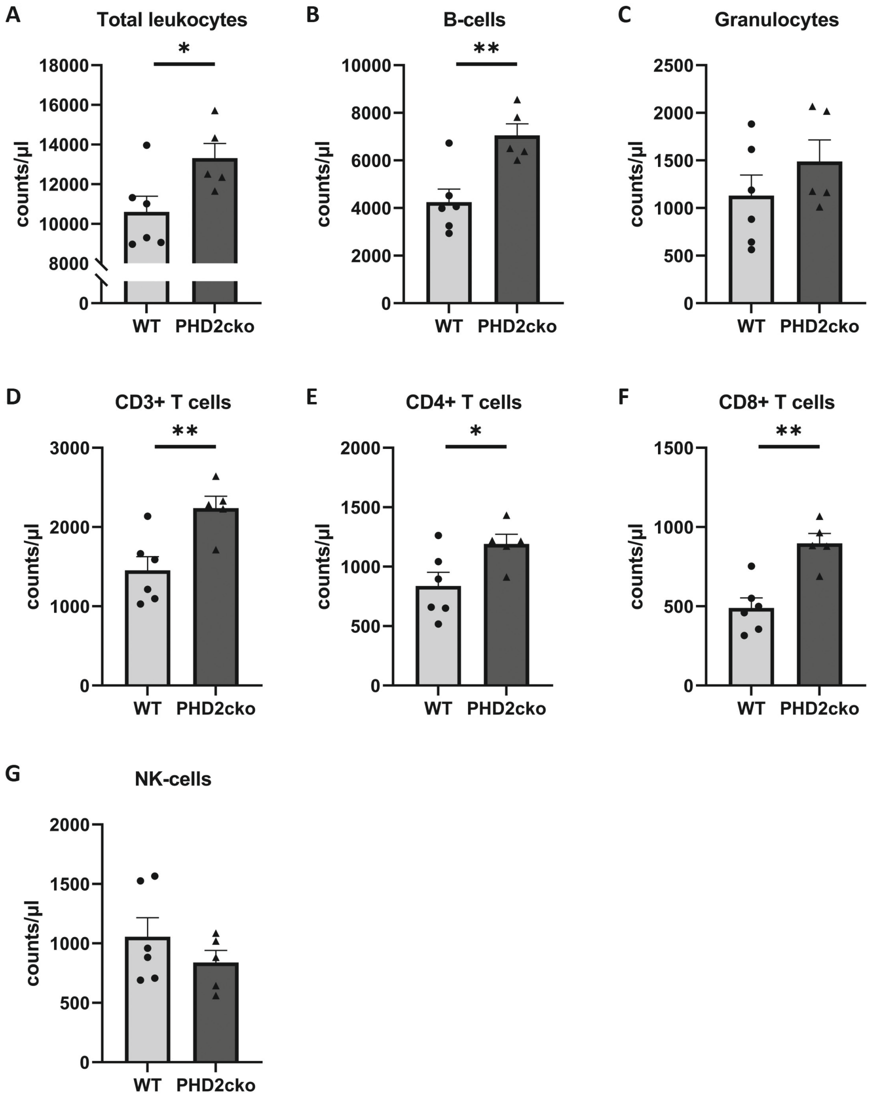
hyroxylase 2.



Supplemental figure 5.

Absolute counts of (**A**) total leukocytes, (**B**) B-cells, (**C**) Granulocytes, (**D**) CD3+ T cells, (**E**) CD4+ T cells, (**F**) CD8+ T cells and (**G**) NK-cells in whole blood. All data are presented as mean ± standard error of the mean (SEM), *p<0.05, **p<0.01. CD – cluster of differentiation.

Supplemental table 1. Primer sequences

Vegfa-MMU-FW	GCACATAGGAGAGATGAGCTTCC
Vegfa-MMU-RV	CTCCGCTCTGAACAAGGCT
Cxcr4-MMU-FW	AGGAAACTGCTGGCTGAAAAGG
Cxcr4-MMU-RV	GGAATTGAAACACCACCATCCA
Cxcl12-MMU-FW	GTCTAAGCAGCGATGGGTTC
Cxcl12-MMU-RV	GAATAAGAAAGCACACGCTGC
Pdgfa-MMU-FW	TTAACCATGTGCCCGAGAAG
Pdgfa-MMU-RV	ATCAGGAAGTTGGCCGATG
Pdgfb-MMU-FW	CGGTCCAGGTGAGAAAGATTG
Pdgfb-MMU-RV	CGTCTTGGCTCGCTGCTC
Rplp0-MMU-FW	GTGATGCCCAGGGAAGACAG
Rplp0-MMU-RV	TCTGCTCCCACAATGAAGCA