Targeting intraplaque angiogenesis : imaging and therapeutic interventions

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Chapter 6.

PCmAb decreases Intraplaque Angiogenesis and Intraplaque Hemorrhage

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ABSTRACT

Background: Phosphorylcholine (PC) is one of the main oxLDL epitopes and plays a central role in atherosclerosis, in particular with its atherogenic and proinflammatory effects. PC can be cleared by natural IgM antibodies and low levels of these natural antibodies have been associated with human vein graft (VG) failure. Although PC antibodies are recognized for their anti-inflammatory properties, its effect on intraplaque angiogenesis (IPA) and intraplaque hemorrhage (IPH), interdependent processes contributing to plaque rupture, are unknown.

Therefore, we studied the effects of a new IgG phosphorylcholine antibody (PCmAB) in advanced atherosclerotic lesions of the hypercholesterolemic ApoE3*Leiden mouse VG model.

Results and Conclusions: PCmAB was an effective treatment to improve plaque stability in advanced atherosclerotic murine lesions. PCmAb decreased pathological intimal thickening (by 22%) and increased lumen area (by 32%). Moreover, PCmAB treated lesions had higher collagen content (by 18%) and decreased macrophages presence (by 32%).

Furthermore, PCmAB improved IPA and IPH. PCmAB treated lesions presented decreased neovessels (by 34%), enhanced neovessel maturity (by 31%) and decreased erythrocytes extravasation. These findings were further supported *in vitro*, by decreased endothelial cell (EC) metabolic activity, EC migration and neovessel sprouting in the PCmAB treated groups.

Within areas of IPH in the lesion, $CD163^{+}$ macrophages secret VEGFA, inducing a vicious cycle of angiogenesis and inflammation. PCmAB treated lesions presented

less $CD163^+$ macrophages (by 23%) in the vessel. *In vitro*, we observed that PCmAB inhibited CD163 expression and VEGFA secretion in human macrophages.

Therefore, PCmAB holds a promise as a new therapeutic approach to target cardiovascular diseases.

INTRODUCTION

Inflammation, intraplaque angiogenesis (IPA) and intraplaque haemorrhage (IPH) are interdependent processes contributing to the development of atherosclerotic plaques and ultimately rupture of the plaque.[1-3] Reduced oxygen availability or hypoxia in the plaque is a direct effect of increased lesion size and active inflammatory cells in the plaque. Triggered by hypoxia, endothelial cells (EC) proliferate and migrate from the adventitia and form neovessels that grow into the lesion to overcome the oxygen demand.[1] However, these neovessels are frequently immature and highly susceptible to leakage, and are therefore the main source of IPH.[4] IPH, defined as the extravasation of blood, is a source for hemoglobin (Hb) and other erythrocyte membrane components, such as free cholesterol and phospholipids to the plaques.[4, 5] Intake of Hb by macrophages drives upregulation of the CD163 scavenger receptor and leads to a distinct macrophage phenotype, M(CD163$^+$).[6] These macrophages produce and secrete high levels of VEGFA via the HIF1a pathway, thereby promoting a vicious cycle of angiogenesis, IPH and inflammation.[6] Moreover since Hb is a strong oxidizer, due to its high iron content, it increases the presence of oxidized phospholipids.[7,8]

During oxidation of phosphatidylcholine lipid (the most abundant phospholipid in oxLDL[9] and cell membranes[10]), phosphorylcholine (PC) headgroups are exposed. These PC epitopes, recognized as DAMPs[11], trigger complex immunoinflammatory responses, induce toxic oxidative stress, apoptosis, EC activation[12] and dysfunction[13]. Moreover, these PC epitopes also mediate the oxLDL uptake by macrophage scavenger receptors. PC contributes via all these processes to atherosclerosis.

Interestingly, PC epitopes can be cleared by IgM autoantibodies against PC, which are naturally produced and released by B cells.[14, 15] These natural antibodies have been shown to control oxidative stress, inhibit macrophages oxLDL uptake thereby preventing foam cells formation.[16-19] In ApoE$^{-/-}$ mice, immunization with anti-PC IgM reduced vein graft (VG) size and plaque inflammation.[19] Moreover, low levels of anti-PC IgM antibodies were associated with VG failure in a large human cohort.[20]

A newly constructed anti-PC IgG has shown to decrease inflammation in naïve atherosclerosis in mice.[21] Therefore, we hypothesized that the new IgG humanized monoclonal antibody against PC (PCmAB) might modulate and normalize IPA and IPH as anti-PC IgM modulates the progression of vascular inflammation, stabilizing atherosclerotic lesions.

We previously show that hypercholesterolemic ApoE3*Leiden VG lesions highly resemble the human atherosclerotic unstable plaques with pathological intimal thickening, severe inflammation, leaky neovessels and haemorrhage.[22, 23]

Here, we studied the role of PCmAB on inflammation, IPA, and IPH in the ApoE3*Leiden VG lesions. Moreover, we investigated the isolated effect of PCmAB in in vitro angiogenesis assays and CD163^{+} macrophage cultures.

MATERIAL AND METHODS

Animals

All animal experiments were performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Male ApoE3*Leiden mice (bred in our own colony), 10-16 weeks old, were fed with a

diet containing 1% cholesterol and 0.05% cholate (AB diets) to induce hypercholesterolemia for three weeks prior to surgery until sacrifice. All animals received food and water ad libitum. Mice were randomized based on their plasma cholesterol levels (Roche Diagnostics, kit 1489437) and body weight (supplemental figure 1).

Vein Graft Surgery

After three weeks on diet, the mice underwent the vein graft surgery, by means of a donor caval vein interposition in the carotid artery of recipient mice, as described before[24]. Mice were anesthetized intraperitoneally with 5 mg/kg of midazolam (Roche Diagnostics), 0.5 mg/kg of dexmedetomidine (Orion Corporation) and 0.05 mg/kg of fentanyl (Janssen Pharmaceutical). After the surgery, the anesthesia was antagonized with 2.5 mg/kg of atipamezol (Orion Corporation) and 0.5 mg/kg of fluminasenil (0.5 mg/kg, Fresenius Kabi). 0.1 mg/kg of buprenorphine (MSD Animal Health) was given for pain relieve. 28 days after the surgery, animals were sacrificed via exsanguination after deep anesthesia (described above) followed by 3 minutes of *in vivo* perfusion-fixation with PBS and 4% formaldehyde (100496, Sigma-Aldrich). The vein grafts were harvested and fixed in 4% formaldehyde.

Treatment

Mice were treated with intraperitoneal injections of a humanized IgG_1 phosphorylcholine monoclonal antibody (5 mg/kg PCmAB, Athera Biotechnologies, n=15) at day 7,14 and 21. As a negative control sterile 0.9% NaCl (Fresenius Kabis, n=15) was used.

Histology and Immunostainings in Vein Grafts

Vein graft samples were embedded in paraffin and sequential cross sections (5 µm thick) were taken from the entire length of the cuffed artery. For each mouse, six (100 µm spaced) cross-sections were used for analysis.

To assess vessel morphometry, vein graft cross-sections were stained with Masson Trichrome (Hematoxylin, Biebrich Scarlet-Acid Fuchsine and Aniline Blue). Using Qwin software (Leica), the following parameters were analyzed: area within the border of the adventitia (*Vessel Area*), area within the media, and area within the luminal border (*Lumen Area*). From this, *Vessel Wall Area* (subtraction of the *Lumen Area* from the *Vessel Area*) and *Intimal Hyperplasia* (subtraction of the *Lumen Area* from the media area) were calculated. Because lumen can accompanied vessel enlargement, *Lumen Area* is expressed as a percentage of *Vessel Area.*

To assess vessel morphology: the presence of *Dissections, Fibrin, Foam cells, Chondrocytes* and *Calcification* were scored in the Masson Trichrome stained sections. No presence was scored as 0, low number as 1, intermediate number as 2, high number as 3. The relative amount of collagen, expressed as a percentage of the *Vessel Wall Area* (*% Collagen)* was quantified in Sirius Red stained sections. To further specify vessel histology: the relative content of vascular smooth muscle cells *(% VSMCs*) and *macrophages (% Macrophages)*, was analyzed by immunohistochemistry for αSMA (1A4, Dako) and Mac-3 (553322, BD Pharmingen), respectively. Additionally, the relative expression of cadherin adhesion molecules, such as *% VCAM-1* (ab134047, Abcam), *% ICAM-1* (ab25375, Abcam), and monocyte chemokines, such as *% MCP-1* (sc-1784, Santa

Cruz) was assessed by immunohistochemistry. Immuno-positive areas were quantified with ImageJ software, and normalized for *Vessel Wall Area*.

To assess IPA and IPH, a triple immunofluorescence staining was used, including CD31 (sc-1506-r, Santa Cruz) to detect neovessels, αSMA to evaluate vessel maturation (pericyte coverage), and the erythrocyte marker TER119 (116202, Biolegend) to rate endothelium leakage. $CD31^+$ neovessels were manually counted (*% Neovessels*) and the percentage of neovessel $CD31^+\alpha SMA^-$ was quantified as *% Immature Neovessels*. *Intraplaque Hemorrhage* was regionally assessed using a scoring system accounting for the presence and the number of erythrocytes outside the neovessels. No presence was scored as 0, low number of erythrocytes outside the neovessels (1–10) was score as 1, intermediate number (11-30) as 2, high number (>30) as 3.

For each antibody, isotype-matched antibodies were used as negative controls. Pictures were acquired with the Pannoramic SCAN II (3DHistech).

Cell Culture

For the isolation of human umbilical vein endothelial cells (HUVEC) anonymous umbilical cords were obtained in accordance with guidelines set out by the 'Code for Proper Secondary Use of Human Tissue' of the Dutch Federation of Biomedical Scientific Societies (Federa), and conform to the principles outlined in the Declaration of Helsinki. HUVEC were isolated and cultured as described by Welten et al.[25] In brief, the vein in the umbilical cords was flushed with warm PBS and incubated with 0.75 mg/mL collagenase type II (LS004177, Worthington Biochemical Corporation) for 20 minutes at 37°C. Detached EC were washed out of the vessel and left to grow in complete medium [EBM-2 medium (00190860,

Lonza) supplemented with EGM BulletKit (CC-3124, Lonza) and 2% of FBS (10082139, ThermoFisher Scientific)] at 37°C in a 5% CO_2 humidified incubator. Culture medium was refreshed every 2-3 days. Cells were passed using trypsin-EDTA (T4049, Sigma-Aldrich) at 90-100% confluency. HUVEC were used up to passage three for proliferation and migration assays, and up to passage seven for western blot.

THP1 cells (88081201, Merck) were seeded at a density of 10^6 cells per ml in 6-well tissue culture plates and incubated with 100 nM of phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) for 24 hours in complete medium (RPMI with 10% Fetal Calf Serum) for differentiation.

MTT Assay

Cell metabolic activity as a marker for cell proliferation was measured by the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M5655, Sigma-Aldrich). HUVEC were seeded in 96-wells plate in complete medium and grown until 80% confluency. To cause cell cycle arrest, cells were incubated for 24 hours in EBM-2 medium supplemented with 0.2% FBS. PCmAB was added in a concentration of 10 µg/ml and 100 µg/ml. After 18 hours, cells were incubated with MTT for 4 hours. A supernatant fraction was replaced by 0.01N HCL-isopropanol (258148 and 563935, Sigma-Aldrich) and absorbance was measured at 570 nm by Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments).

Migration Assay

For migration assays, HUVEC were seeded in 12-wells plate in complete medium and grown until 80% confluence. To cause cell cycle arrest, cells were incubated

in EBM-2 medium supplemented with 0.2% FBS and 24 hours later, a scratch-wound was made. PCmAB was added in a concentration range of 10 µg/ml and 100 µg/ml. In the migration assay, HUVEC were stimulated with 5 µg/ml of oxLDL (L34357, ThermoFisher Scientific) to mimic PC presence. Three locations along the scratch-wound were marked per well and scratch-wound closure at these sites was imaged at time 0 and 16 hours by using Axiovert 40c Inverted & Phase Contrast Microscope (451207, Carl Zeiss). Average scratch-wound closure after 16 hours was calculated by measuring cell coverage at 16 hours vs 0 hours using ImageJ.

Aortic Ring Sprouting Assay

The aortic ring assay was performed as described previously.[26-28] Three ApoE3*Leiden mice, 4-8 weeks old, were anesthetized and the aorta was dissected. Each aorta was cut in 1 mm rings, and serum-starved in Gibco™ Opti-MEM™ GlutaMAX (51985034, ThermoFisherScientific) overnight at 37°C and 5% CO_2. On the next day, each ring was mounted in a well of a 96-well plate in 70 µl of 1.0 mg/ml acid-solubilized collagen type-I (11179179001, Roche Diagnostics) in DMEM (12634010, ThermoFisher Scientific). After collagen polymerization, Gibco™ Opti-MEM™ GlutaMAX supplemented with 2.5% FCS and 30 ng/ml VEGF (293-VE, R&D systems) was added with PCmAB (10 µg/ml and 100 µg/ml). The rings were cultured for 7 days and photographed by using Axiovert 40c microscope. The number of sprouts were counted manually. For immunohistochemistry, rings were formalin-fixed and permeabilized with 0.2% Triton X-100 (11332481001, Merck). Rings were stained with αSMA, CD31 and VE-Cadherin (AF1002, R&D Systems). Extended focus pictures were made with the Pannoramic SCAN II and quantified with Image J.

Protein Expression Analysis

Differentiated THP-1 cells were incubated in HH enrich media (0.1 mg/ml of Hb:Hp (H0267 and SRP6507, Sigma Aldrich) in complete medium) over 6 days. At day 7, 10 µg/ml and 100 µg/ml of PCmAB was added to the medium and incubated overnight.

Cells were scraped and homogenized in modified RIPA buffer containing sodium-orthovanadate and protease inhibitors. Proteins were separated by SDS-PAGE (4–15%) and transferred to nitrocellulose. Blots were incubated with antibodies against CD163 (93498, Cell Signaling). A peroxidase conjugated secondary antibody was used (31462, 31400, ThermoFisher Scientific). Proteins of interest were imaged with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34580, ThermoFisher Scientific) and the ChemiDoc™ Touch Imaging using System (1708370, Bio-Rad Laboratories). β-actin (ab8220, Abcam) was used as internal control and blots were quantified with Image J.

Cell supernatant was also collected and VEGFA was measured by a sandwich ELISA (DYC5079-2, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical Analysis

All data is presented as mean ± standard error of the mean (SEM). Normality was examined using the Shapiro-Wilk normality test. Overall comparisons between groups were performed using 1-way ANOVA or 2-way ANOVA on parametric data and a Kruskal-Wallis test for nonparametric data using the statistics software GraphPad Prism 8.02. P-values less than 0.05 were regarded as statistically significant.

RESULTS

PCmAB decreases vein graft thickening and increases lumen area

PCmAB treatment did not affect bodyweight or cholesterol levels (supplemental figure 1). In both groups (CTRL: n=3, PCmAB: n=4) were excluded from further analysis due to fully occluded vein grafts as a result of thrombosis. PCmAB effects on vein graft morphometry and vessel wall remodeling were assessed using the Masson's Trichrome staining (Figure 1A).

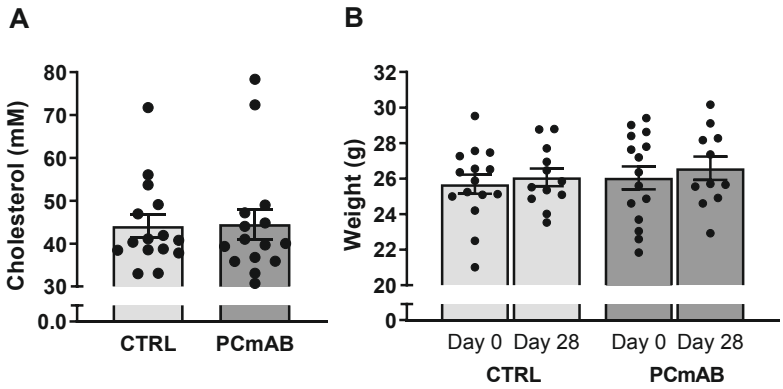

Figure 1S. Cholesterol **(A)** levels and Weight **(B)** of hypercholesteremic ApoE3*L mice treated with 0.9% NaCl sterile solution (n=12) and 5 mg/kg of PCmAB (n=11).

Passive immunization with PCmAB did not affect *Vessel Area* or lumen area or negative remodelling (Figure 1B) but decreased the *Vessel Wall Area* by 25% (p=0.0246, Figure 1C), which resulted in a 22% decrease in *Intimal Hyperplasia* (p=0.0398) in comparison to the CTRL group (Figure 1D). Moreover, the *Lumen Area* was significantly increased (by 32%) by PCmAB treatment in comparison with the CTRL group (p=0.0236, Figure 1E).

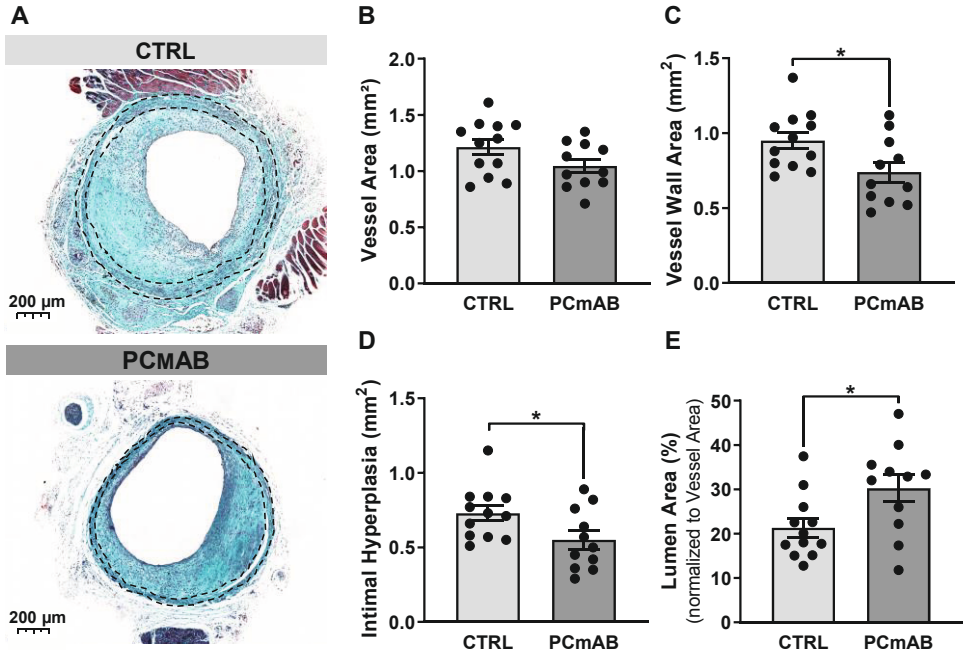

Figure 1. PCmAB decreases intimal hyperplasia and increases lumen area in vein graft atherosclerosis. Masson Trichrome staining representative vein grafts cross-sections **(A)** of hypercholesteraemic ApoE3*L mice treated with 0.9% NaCl sterile solution (n=12) and 5 mg/kg of PCmAB (n=11). Quantitative measurements of *Vessel Area* **(B)**, *Vessel Wall Area* **(C),** Intimal Hyperplasia **(D)** and Lumen Area **(E).** Data presented as mean ± SEM. *$p \leq 0.05$ by t-test.

PCmAB improves lesion stability by increasing collagen content

To assess PCmAB effects on plaque stability, we analysed stable and unstable plaque features[22, 29], such as %*VSMC, % Collagen, Fibrin, Foam Cells, Chondrocytes* and *Calcifications* (Figure 2). Although, the *% VSMC*, the main producers of collagen, did not differ between the two groups, the *% Collagen* was increased by PCmAB treatment (by 18%) in comparison to the CTRL group (p=0.0404, Figure 2A,B). Furthermore, the presence and severity of *Dissections, Fibrin, Foam Cells, Chondrocytes* and *Calcifications* did not significantly change between the groups (Figure 2D). Nevertheless, a trend towards reduction was observed in *Foam Cell* score.

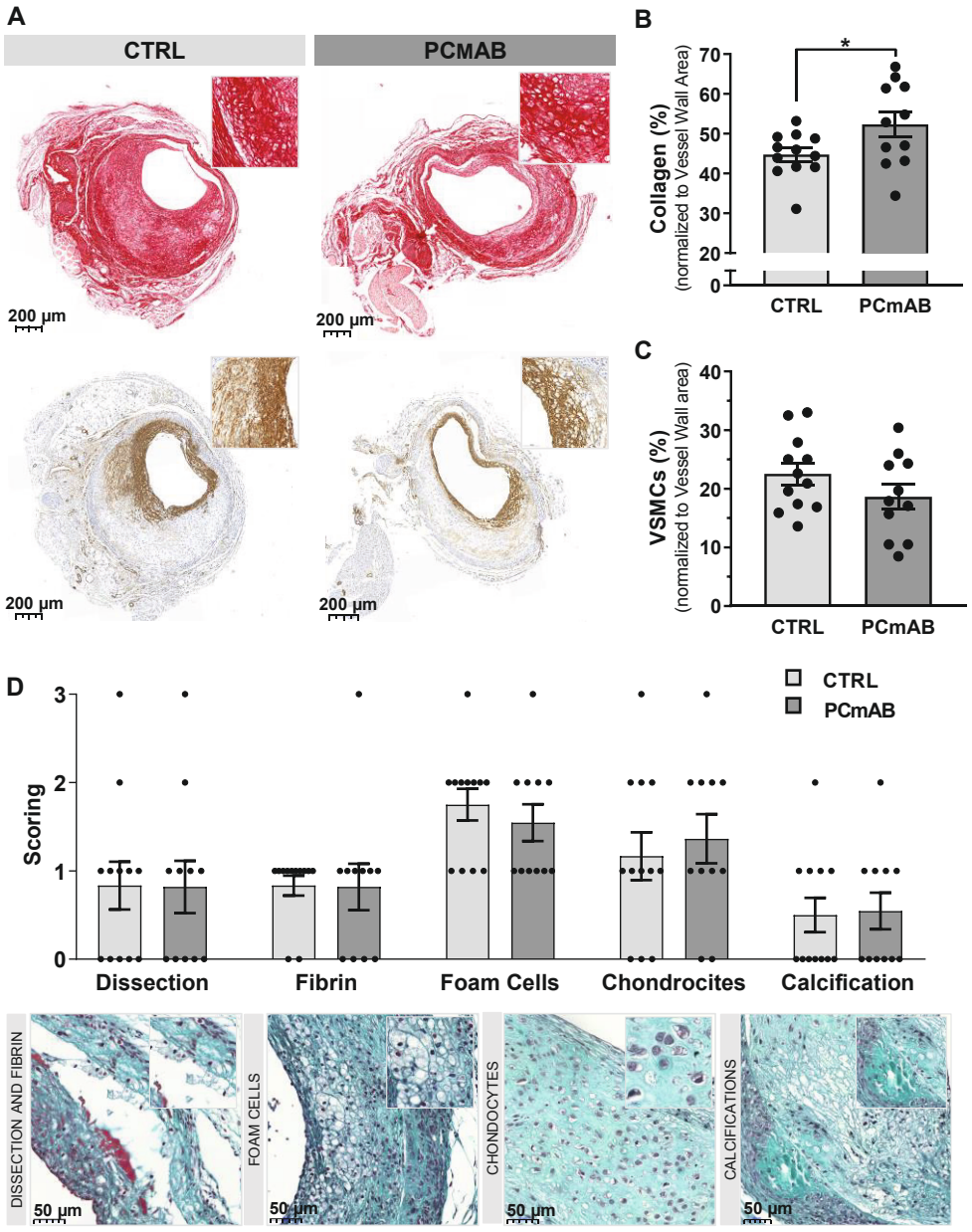

Figure 2. PCmAB improves plaque stability by increasing collagen content in vein graft atherosclerosis. Representative vein grafts cross sections (A) of Sirus Red Staining and αSMA of CTRL (n=12) and PCmAB group (n=11). Quantitative measurements of % Collagen **(A)** and % VSMCs **(C)**. Data presented as mean ± SEM. *p≤0.05, **p≤0.01 by t-test.

PCmAB decreases inflammation, ICAM-1 and VCAM-1 expression

To investigate PCmAB effects on inflammation, we determined the relative presence of macrophages in the vessel wall (*% Macrophages*). PCmAB treatment significantly decreased (by 31%) the *% Macrophages* in comparison to the CTRL group (p=0.0333, Figure 3A and B).

Next, we quantified the expression of adhesion molecules in the vein graft lesions such as ICAM-1 and VCAM-1, as well as MCP1, the most potent chemoattracting chemokine for monocytes. PCmAB treatment reduced ICAM-1 (by 29%, p=0.0104) and VCAM-1 (by 36%, p=0.0073) expression in comparison with the CTRL group (Figure 3C,D,E). However, the % MCP1 expressing cells did not vary between the two groups (Figure 3C and D).

PCmAB decreases intraplaque angiogenesis and intraplaque hemorrhage

To evaluate PCmAB effects on IPA, we measured the density of the neovessels in the vein grafts expressed as *% Neovessels* as well as the neovessels that lacked pericyte coverage *(% Immature Neovessels)* and we evaluated *Intraplaque Haemorrhage* in the VG lesions by scoring extravasated erythrocytes in a triple staining (Figure 4A).

Quantification of the *% Neovessels* revealed a 34% decrease in the PCmAB group in comparison with the CTRL group (p=0.0006, Figure 4B).

Quantification of *% Immature vessels* showed a 31% decrease in PCmAB-treated group in comparison with the CTRL group (p=0.0042, Figure 4C).

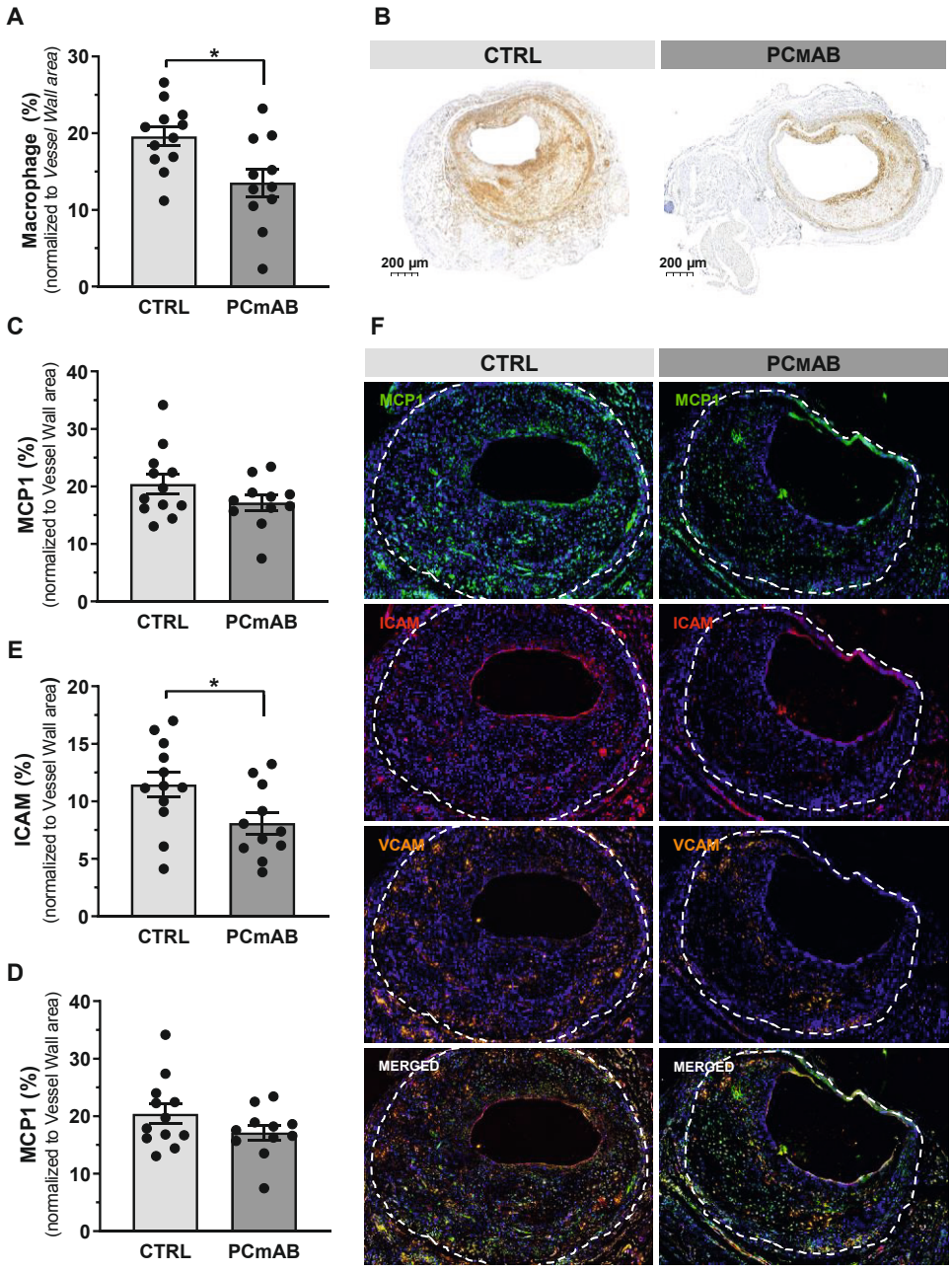

Figure 3. PCmAB improves plaque inflammation by decreasing macrophage content and VCAM and ICAM expression. Quantification of % Macrophages **(A)**, and VCAM-1 **(C)**, ICAM-1 **(D)** and MCP1 **(E)** expression in the CTRL (n=12) and PCmAB group (n=11). Respective representative VG sections in **(B)** and **(F)** Data presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by t-test.

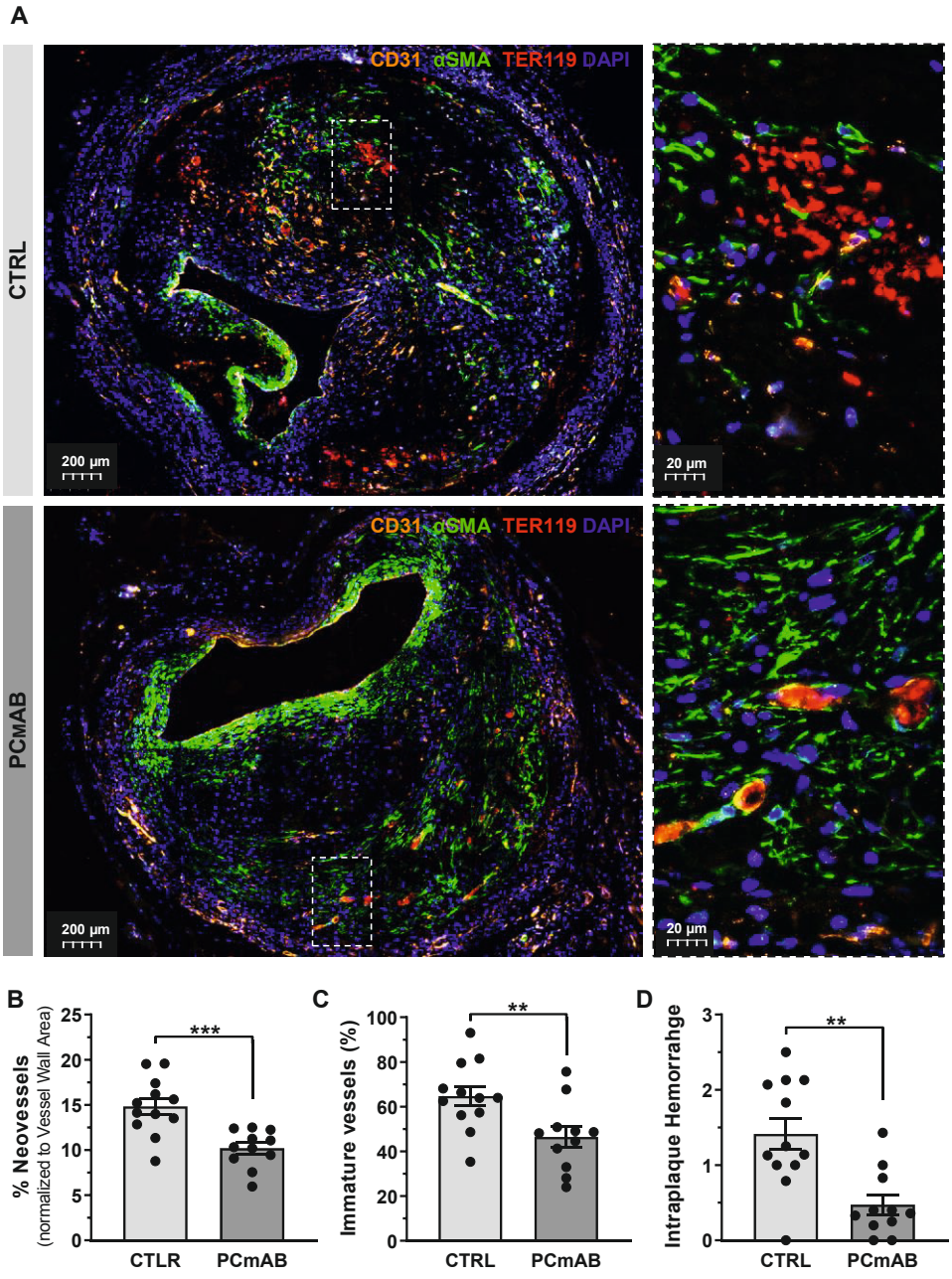

Figure 4. PCmAB reduces plaque neovessels density, increases vessel maturity, reducing intraplaque haemorrhage. Quantitative measurements of % Neovessels **(A)** and % Immature Neovessels **(B)** and Intraplaque Hemorrhage **(C)** scoring**.** Representative vein grafts cross sections of CD31 (orange), αSMA (green), TER119 (red) and DAPI (blue) staining of CTRL and PCmAB group **(E)**. Data presented as mean ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ by T-test.

PCmAB treatment decreased *Intraplaque Haemorrhage* presence and severity as scored in Figure 4D. In the CTRL group, 92% (11/12) of mice presented extravasated erythrocytes, while in the PCmAB group was 46% (5 /11). Moreover, in the CTRL group, 54% *Intraplaque Haemorrhage* severity was scored as ~1 in 54% (6/11) of the mice, as ~2 in 36% (4/11) and as ~3 in one mouse. In the PCmAB group, *Intraplaque Hemorrhage* severity was scored as ~1 in 90% (4/5) of mice presented and as ~2 in one mouse.

PCmAB decreases EC metabolic activity and migration and sprouts formation

Because the observed changes in plaque size and inflammation can reduce oxygen demand and consequently decrease IPA, we studied the PCmAB effects on EC behaviour and neovessel sprouting *in vitro*.

HUVEC metabolic activity in the MTT assay was reduced by 22% by 10 μg/ml of PCmAB ($p=0.0875$) and by 28% by 100 μg/ml of PCmAB ($p=0.0415$), as shown in Figure 5A, whereas HUVEC migration (Figure 5B and B1) or the ability of wound closure was decreased by 54% and 33% when treated with 10 μg/ml ($p=0.0395$) and 100 μg/ml of PCmAB ($p=0.155$). In the presence of oxLDL. HUVEC migration increased by 52% when compared to CTRL group. Further treatment with PCmAB decreased HUVEC migration dose-dependently by 31% (10 μg/ml, $p=0.0439$) and 73% (100 μg/ml, $p=0.0001$) when compared with oxLDL-CTRL group. In the aortic ring assay (Figure 5C and C1), the number of sprouts was decreased dose-dependently by PCmAB in comparison with CTRL group, with a 50% decrease for the 100 μg/ml treated segments.

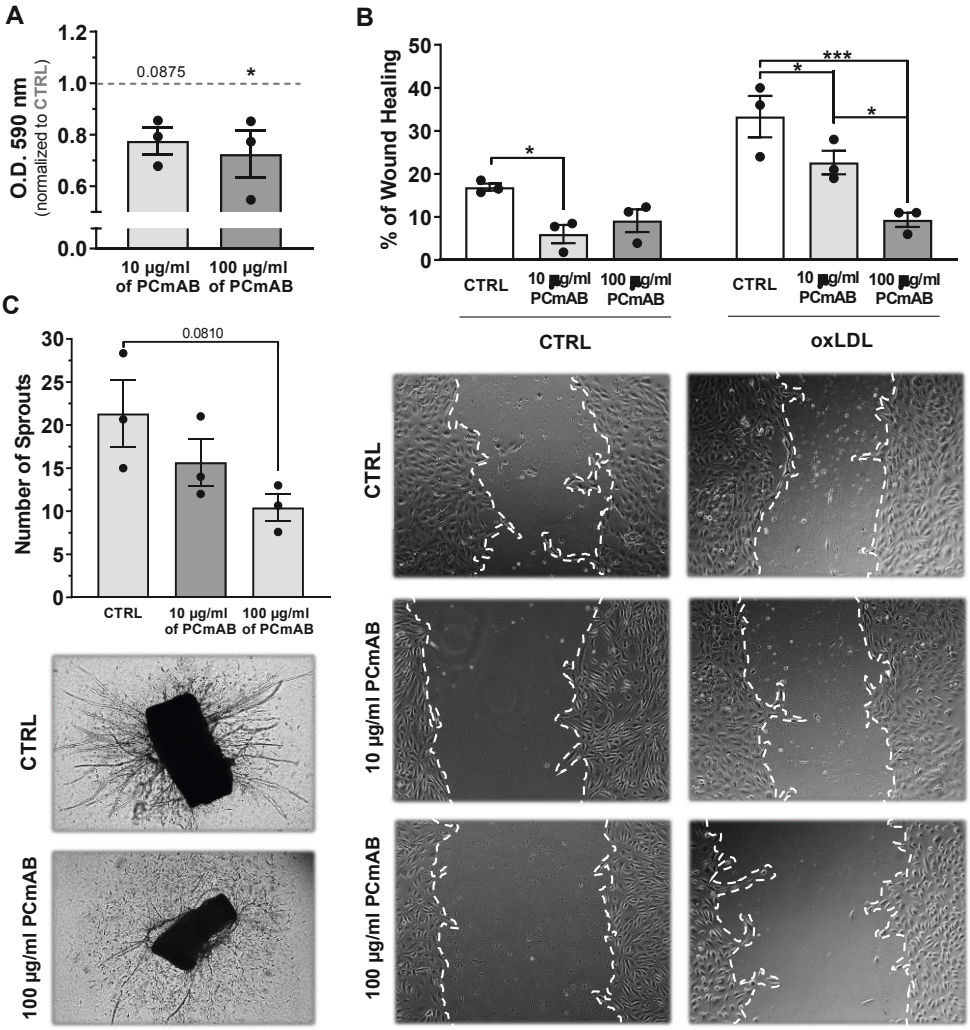

Figure 5. PCmAB reduces EC metabolic activity and migration *in vitro* and neovessel sprouting *ex vivo*. Quantification of PCmAB effects on the MMT assay **(A)**, on the migration assay **(B)** and on the aortic ring assay **(C)**. Representative images of the scratches **(B)** treated with increasing doses of PCmAB and with and without 5 µg/ml oxLDL, 16 hours after scratching. Representative images of the aortic rings **(C)** treated with VEGF and PCmAB.

(A) Data normalized to CTRL group (indicated as 1 by a dashed red line in the graph) and presented as mean ± SEM (n=3). **P*<0.05, ***P*<0.01; by 1-way ANOVA (***** (in red) are significances versus control). **(B)** Data presented as mean ± SEM (n=3). **P*<0.05, ****P*<0.001; by 2-way ANOVA. **(C)** Data presented as mean ± SEM (n=3) by 1-way ANOVA.

PCmAB targets M(CD163) macrophages *in vivo* and *in vitro* by decreasing CD163 expression

It has been shown that $CD163^+$ macrophages not only promote leucocyte infiltration but also induce angiogenesis and vessel permeability by secreting VEGFA.[6] Therefore, we also determined the *%M(CD163)* macrophages in the VG lesions. *%M(CD163)* was reduced by 23% in the PCmAB group in comparison to the CTRL group (p=0.0014, Figure 6A,B).

Because PCmAB decreased the amount of extravasated erythrocytes in VG lesion (Figure 4D), we reproduced M($CD163^+$) *in vitro* to studied the effect of PCmAB on the upregulation of CD163 receptor in macrophages. Unstimulated THP1 cells already express CD163 receptor and treatment with PCmAB did not change CD163 expression (Figure 6.CD). Stimulation with HH-enriched media increased CD163 expression by 86% (p=0.0226) in comparison with the CTRL group. Under this condition treatment with PCmAB decreased CD163 expression by 50% (10 µg/ml, p=0.0130) and 44% (100 µg/ml, p=0.0314) in comparison with the HH group.

M($CD163^+$) are active secretors of VEGFA, therefore we quantified VEGFA protein levels in macrophage medium. Stimulation with HH-enriched medium show a trend increased in VEGFA levels by 68% in comparison with the CTRL group. Treatment with both PCmAB concentrations decreased VEGFA levels in comparison with HH group (15% and 20%, respectively).

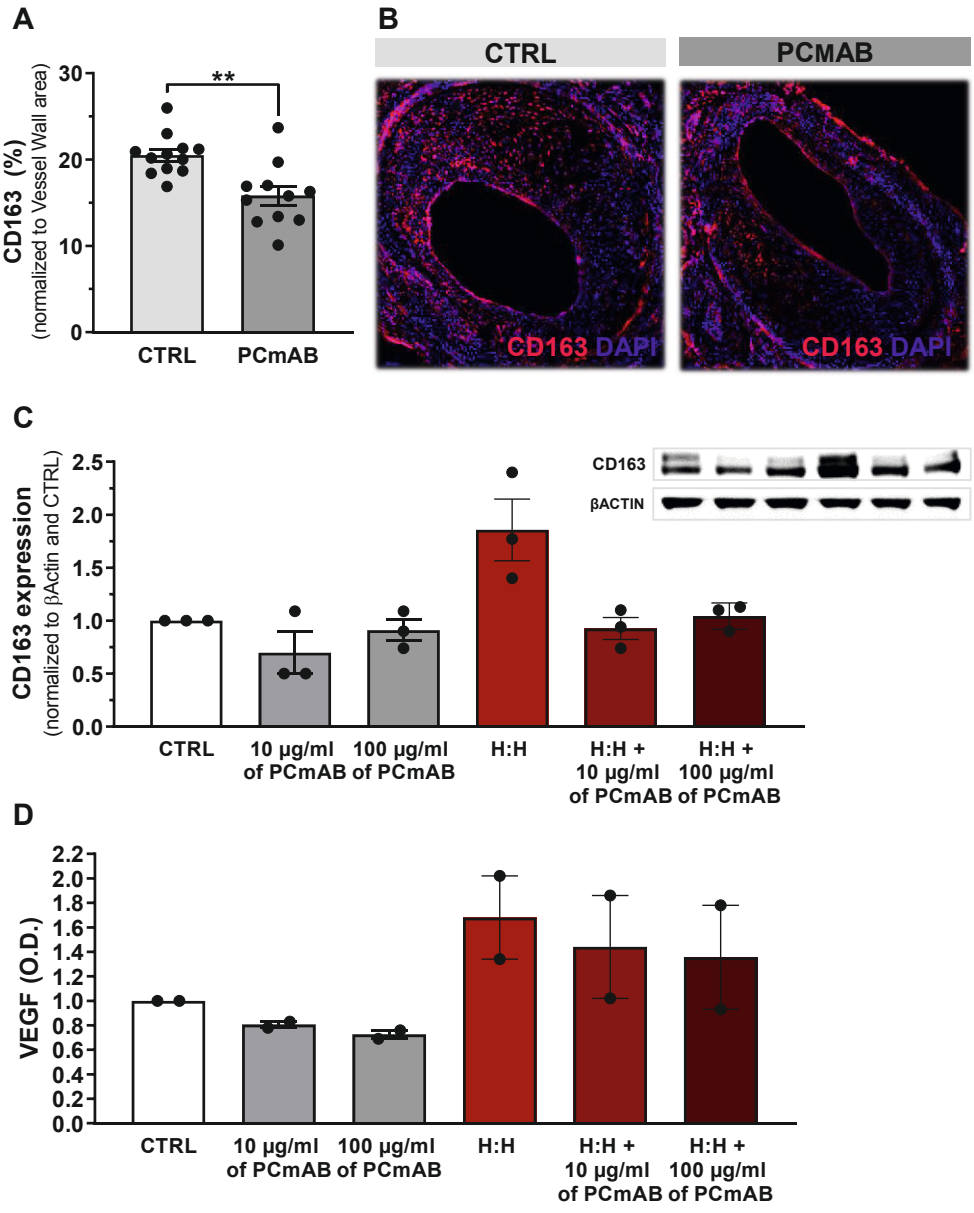

Figure 6. PCmAb targets M(Hb) macrophages *in vivo* and *in vitro* by decreasing CD163 expression. Quantification of CD163 expression in VG lesions **(A)** and representative cross sections of CD163 (red) and DAPI (blue) staining of CTRL and PCmAB group **(B)**. Quantification of CD163 expression in THP-1 cells treated with increasing doses of PCmAB and with and without Hb:Hp **(C)**.Quantification of VEFG levels in THP-1 cell supernatant **(E)**. Data presented as mean ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ by t-test and by 1-way ANOVA.

DISCUSSION

In the present study, we provide evidence that in the hypercholesterolemia ApoE3*Leiden vein graft model PCmAB has strong effects 1) on inflammation by reducing macrophage content, 2) on IPA by decreasing the neovessel density and by improving their maturity, and 3) on IPH by decreasing erythrocytes extravasation. The anti-angiogenic capacity of PCmAB, was observed by a decrease in EC proliferation (or metabolic activity in the MTT assay), EC migration and neovessel sprouting. Moreover, the oxLDL induced HUVEC migration was also reduced in presence of PCmAB demonstrating that PCmAB, by neutralizing oxLDL-PC epitopes, reduces EC angiogenic behavior. Interestingly, PCmAB seems also to have a positive effect on IPH. PCmAB improved vessel maturation (by increasing pericyte coverage), which probably decreased the presence of erythrocytes in plaque.

We also evaluated PCmAB effects on plaque morphometry and morphology. Although the size of the vessel was not affected by passive immunization with PCmAB, vessel wall thickening (intima + media) was decreased in a beneficial way upon treatment. Interestingly in human cohorts, increases in intima media thickening were significantly less common in subjects with higher baseline levels of natural anti-PC antibodies.[30] Moreover, the size of the intima layer also changed when treated with PCmAB, displayed as less intimal hyperplasia. Lumen area was increased in PCmAB group, which is the most clinical relevant parameter since this directly improves blood flow.

Additionally, PCmAB treated plaques present a more stable plaque phenotype as demonstrated by increased levels of collagen and decreased macrophage content. Also, a trend towards reduction in amount of foam cells was observed with PCmAB

treatment. These findings are in accordance to previous studies that reported that anti-PC antibodies bind to PC epitopes on oxidized phospholipids, inhibiting inflammatory signaling[31] and blocking uptake of oxLDL and foam cell formation.[31-33]

Oxidized phospholipids are known to trigger ECs to undergo into inflammatory activation.[34] Therefore, we assessed the expression of adhesion molecules such as ICAM-1 and VCAM-1, and the chemokine MCP-1, which represent important triggers to attract monocytes in early (expressed on the lumen surface and in VSMCs) and late lesion development (expresses on neovessels endothelium). We showed that PCmAB treatment decreases VCAM-1 and ICAM-1 levels in the vessel wall, which can be a direct cause for the lower macrophage content in the plaque. Moreover, since there is a direct relationship between entry of monocytes/macrophages and leakage of neovessels, a reduction in the amount of macrophages may be a direct result of reduced IPA in the plaque by PCmAB.

Furthermore, PCmAB decreases the presence of M(CD163^{+}) in VG lesions significantly. When we tested the effect of PCmAB on cultured macrophages stimulated with Hb:Hp complexes, PCmAB significantly decreased CD163 expression. This suggests that PC epitopes may also be involved in the CD163 scavenger activity, as it happens for other scavenger receptors, such as CD36.[35, 36]

According to Guo et al., CD163 macrophages perpetuate IPA and IPH through the secretion of VEGFA.[6] In our *in vitro* setup, stimulation with Hb:Hp complexes increased macrophage VEGFA secretion levels which could be prevented by treatment with PCmAB.

Taken together, our findings reveal that PCmAB improves plaque stability by decreasing lesion size, inflammation, IPA and IPH. Added to that, PCmAB increases vessel maturation by improving pericyte coverage and decreases M(CD163) presence. In conclusion, our findings explain the beneficial PCmAB effects on advanced atherosclerotic lesions.

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