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Tetrahydrobiopterin supplementation reduces atherosclerosis and vascular inflammation in Apolipoprotein E-knockout mice

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Abstract

Tetrahydrobiopterin (BH4) supplementation improves endothelial function in models of vascular disease by maintaining endothelial nitric oxide synthase coupling and nitric oxide bioavailability. However, the cellular mechanisms through which enhanced endothelial function leads to reduced atherosclerosis remain unclear. We have used a pharmaceutical BH4 formulation to investigate the effects of BH4 supplementation on atherosclerosis progression in ApoE-KO mice.

Single oral dose pharmacokinetic studies revealed rapid BH4 uptake into plasma and organs. Plasma BH4 levels returned to baseline by 8 hours after oral dosing, but remained markedly increased in aorta at 24 hours. Daily oral BH4 supplementation in 8 week old chow-fed ApoE-KO mice for 8 or 12 weeks had no effect on plasma lipids or hemodynamic parameters, but significantly reduced aortic root atherosclerosis, compared with placebo treated animals. BH4 supplementation significantly reduced VCAM-1 RNA levels on aortic endothelial cells, markedly reduced the infiltration of T cells, macrophages and monocytes into plaques *and* reduced T cell infiltration in the adjacent adventitia, but importantly had no effect on circulating leukocytes. GTP-cyclohydrolase 1 transgenic mice, with a specific increase in endothelial BH4 levels, exhibited a similar reduction in vascular immune cell infiltration compared with BH4 deficient controls, suggesting that BH4 reduces vascular inflammation through endothelial cell signalling.

BH4 supplementation reduces vascular immune cell infiltration in atherosclerosis and may therefore be a rational therapeutic approach to reduce the progression of atherosclerosis.

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Introduction

Atherosclerosis and its complications such as coronary artery disease (CAD) remain major causes of global morbidity and mortality. Atherosclerosis initiates through the accumulation of lipids in the arterial intima, provoking a chronic inflammatory response in the arterial wall.[1] Early atherosclerotic plaques are characterised by local accumulations of lipid-laden macrophages, or 'foam cells'. As plaques develop, there is further leukocyte recruitment with monocytes –the macrophage progenitor– being recruited, driving further foam cell accumulation. With increasing size plaques also become more complex in nature, characterised by the recruitment of additional leukocyte subtypes such as T-lymphocytes. Whilst cells of the adaptive immune response can act in an atheroprotective fashion,[2-6] the net effect of immune activation is pro-atherogenic in the ApoE-/- mouse model.[7]

Oxidative stress and endothelial dysfunction are hallmarks of cardiovascular disease states. Nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), is a key mediator of vascular homeostasis.[8] NO bioavailability is reduced early in vascular disease states such as hypercholesterolemia,[9] diabetes[10,11] and hypertension,[12] and throughout the progression of atherosclerosis;[13] this is a result of both reduced NO synthesis and increased NO consumption by reactive oxygen species. The balance between NO and superoxide production by eNOS appears to be determined by the availability of its essential cofactor tetrahydrobiopterin (BH4). The molecular and cellular mechanisms through which BH4 exerts its effects on endothelial NOS activity are now well-established. When intracellular BH4 levels are limiting, oxygen reduction becomes enzymatically 'uncoupled' from L-arginine oxidation, and eNOS generates superoxide rather than NO, contributing to vascular oxidative stress and endothelial dysfunction.[14,15] Clinical studies using acute pharmacological BH4 supplementation have reported an improvement of NO-mediated endothelial function in patients with vascular disease states such as hypercholesterolemia,[16] type II diabetes[17] and overt coronary atherosclerosis.[13] In addition, supplementation with BH4 or its precursor sepiapterin in animal models of vascular disease such as diabetes,[18,19] hypertension[10,20] and hypercholesterolemia[9] improves endothelial function. Work from our own group revealed preservation of nitric oxide-mediated endothelial function in diabetes[21] and atherosclerosis[22] by targeted transgenic overexpression of GTP-cyclohydrolase I (GCH), the rate-limiting enzyme in BH4 synthesis, pointing towards the therapeutic potential of BH4 supplementation. Further studies from our group indicated that BH4 may be an important regulator of inflammation and vascular remodeling.[23] ApoE-KO/GCH-Tg mice exhibited reduced vein graft atherosclerosis with a marked reduction in vein graft macrophage content compared to ApoE-KO controls.

Recently, beneficial effects of exogenous rather than transgenic BH4 supplementation on the progression of atherosclerosis in ApoE-KO mice have been reported, through mechanisms related to improved eNOS coupling and endothelial function.[24] However, this study did not determine the downstream cellular mechanisms leading to reduced atherosclerosis. We designed the present study to address the hypothesis that improved endothelial function resulting from exogenous BH4 supplementation leads to reduced atherosclerosis through effects on vascular inflammation.

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Methods

Animals

All animal procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Experimental ApoE-KO mice were originally sourced from Jackson Laboratories (Bar Harbour ME, USA). The mice were maintained in temperature-controlled (20°C to 22°C) cages with 12-hour light-dark cycle, and had free access to sterilised water and standard rodent chow (Rodent diet BK002P, B&K Ltd, Hull, UK). ApoE-KO/Hph and ApoE-KO/Hph/GCH-Tg were weaned onto a high-fat diet (no. 100244, Dyets Inc, Bethlehem, PA; USA, consisting of 21% fat and 0.15% Cholesterol) and used at age 15 weeks.

BH4 administration

To assess the pharmacokinetic properties of the BH4 compound (KUVAN® [sapropterin dihydrochloride], BIOMARIN® Pharmaceutical Inc., Novato CA, USA), 12-weeks old mice received a single oral dose of either BH4 at a range of concentrations (10, 50 or 500mg/kg) or control therapy by gavage, and were sacrificed at 5 different time points in the course of 24 hours after drug administration. For chronic oral BH4-supplementation, BH4 or control therapy were administered to ApoE-KO male mice by oral gavage from the age of 8 weeks for 8 or 12 weeks. Mice were weighed daily during treatment, and observed for any changes in health or behaviour. Animals were sacrificed at 16 or 20-weeks of age, 20-24 hours after the final daily dose. Plasma and tissues were harvested and snap frozen as described below. All experiments were performed using a placebo control, which contained all chemical components of the manufacturer's BH4 formulation in the correct proportions, except for the active BH4. BH4 or placebo was dissolved in sterile water immediately before gavage.

Blood pressure and pulse measurements in conscious mice

Mouse heart rate and systolic, diastolic and mean arterial blood pressure were measured using an automated computerised tail-cuff system (Visitech BP2000, Visitech Systems Inc, Apex, NC, USA). All recordings were performed between 0800 and 1200 hours, 24 hours after the last drug administration.

Mouse blood and organ harvest

Blood was collected from animals under terminal anaesthesia into heparinised syringes, and immediately snap-frozen. Plasma samples for biopterin detection were supplemented with a final concentration of 1 mM dithioerythritol to prevent BH4 oxidation and snap-frozen. The animal was then perfused with 10 ml phosphate buffered saline, tissues of interest subsequently harvested and snap-frozen. Samples were stored at -80°C.

Lipid and lipoprotein analysis

Biochemical analyses of plasma lipids were performed on heparinised blood plasma using a Cobas Mira Plus automated analyzer (Roche, Switzerland).

Biopterin detection by HPLC

BH4, BH2, and biopterin levels in cell lysates were determined by HPLC followed by electrochemical and fluorescent detection, as described previously.[25,26]

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Histology and immunohistochemistry of aortic root sections

For the quantification of atherosclerotic lesion sizes in Paraffin-embedded aortic root sections, a Masson-Goldner staining was performed according to the manufacturer's instructions (Merck, Germany). The average value for atherosclerotic lesion size was calculated from 3 sections. Starting from the first section showing all 3 complete aortic cusps, each of the next 2 sections was taken 144 µm distally to the previous one. Lesion size was quantified with ImagePro Plus software (MediaCybernetics, MD, USA). The infiltration of macrophages/monocytes into aortic lesions was analysed as absolute positive staining area using an anti-Mac-3 (Rat anti-mouse Mac-3; BD Pharmingen 550292) immunostaining. The level of fibrosis within atherosclerotic lesions was quantified with an immunostaining against the extracellular matrix component Collagen Type IV (Rabbit anti-mouse Collagen Type IV; Chemicon International AB756P). Smooth muscle cells were visualised by an alpha-smooth muscle cell staining (Cy3-conjugated mouse monoclonal anti-alpha smooth muscle actin; Sigma C6198). T-cell infiltration into atherosclerotic plaques and surrounding adventitia was analysed as absolute positive staining area by anti-CD3 (Rabbit polyclonal anti-human, cross-reacts with mouse; Dako A0452) immunostaining. Absolute Mac-3, Collagen Type IV, alpha-Smooth Muscle Actin and CD3 positive areas were quantified using a Leica DMRBE fluorescence microscope (Leica Microsystems, Germany) connected to a Media Cybernetics Coolsnap Pro color camera in combination with ImagePro Plus software. GraphPad Prism software (GraphPad Software Inc, CA, USA) was used to generate graphs in which total stained areas are expressed in mm^2.

Quantitative reverse transcriptase polymerase chain reaction (qRT PCR)

Whole thoracic aortas were obtained from 16 week old $ApoE^{-/-}$ mice treated with either placebo or BH4 for 8 weeks. Thoracic aortas were dissected clean of adherent tissue and snap frozen. Total RNA was obtained by Trizol extraction followed by clean up using the RNeasy system (Qiagen, UK). Reverse transcription was carried out using Quantitect Reverse Transcription kit (Qiagen, UK) on 1 µg total cell RNA. Quantitative PCR was performed with an iCycler IQ real-time detection system (BioRad Laboratories, USA) with primers and probes from the gene expression assays system as supplied by Applied Biosystems, USA, with GAPDH used as a house keeping gene.

Isolation of peripheral blood mononuclear cells

PBMCs were isolated from whole blood and separated from platelets using two consecutive Optiprep© Density Gradients (AxisShield, UK) according to the manufacturer's instructions.

Flow cytometry

To identify individual classes of blood leukocytes, 50 µl of blood was blocked with anti-FcγRII/RIII (BD Pharmingen, UK) and then stained using saturating concentrations of fluorochrome conjugated antibodies: anti-CD11b -(clone M1/70)- PerCP-Cy5.5, anti–Ly-6G (1A8)-FITC, anti–CD4 -(clone L3T4)-PE, anti–CD3 -(clone 145-2C11)-Pacific Blue, anti–CD8a (53-6.7)-FITC (BD Pharmingen, UK) and anti–mouse neutrophils (7/4)-PE (Serotec, UK) using a standard protocol. Erythrocytes were lysed using 'Whole Blood Immunofluorescence Solution' (BD Pharmingen, UK) and samples analysed using a DAKO CyAn flow cytometer (DAKO, UK). Absolute cell counting was performed by adding a known quantity of calibration beads (CaliBrite; BD Biosciences, UK) to a known sample volume as previously described.[27] Data was

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acquired using DAKO Summit software (DAKO, UK) and analysed using FlowJo (Treestart Inc, MI, USA).

Statistical analysis

Each animal in the experimental program was identified with a unique code, enabling all primary data to be analysed blinded to the experimental group. The data are presented as the means ± S.D. Groups were compared using the Mann-Whitney *U* test for nonparametric data or the Student's *t* test for parametric data. When comparing multiple groups, analysis of variance with a Kruskal-Wallis test with Dunn's post test for nonparametric data was used. A value of $P<0.05$ was considered statistically significant.

Results

Aortic tissue retains elevated BH4 levels independently of plasma BH4 concentration

We first analysed the single dose pharmacokinetics and biodistribution of the pharmaceutical BH4 formulation (BIOMARIN® Pharmaceutical Inc., Novato CA, USA) used in this study. Plasma pharmacokinetics revealed rapid uptake of BH4 from the gastrointestinal tract and significantly increased plasma BH4 levels at all doses tested within 5 minutes after gavage (Figure 1A). Peak concentrations in plasma were detected 30 to 60 minutes after gavage, and were increased up to 70-fold (at 500 mg/kg) compared with placebo treated animals. Notably, elevated plasma BH4 levels returned to baseline between 8 and 24 hours after supplementation.

Strikingly, aortic BH4 levels remained elevated at 24 hours after gavage (Figure 1B), even after a single low dose of 10 mg/kg. This finding suggests that BH4 levels can be maintained within aortic tissue for several hours even after plasma BH4 levels have returned to baseline. We did not observe this phenomenon in heart, lung, liver or brain, where tissue BH4 levels had returned to baseline by 24 hours (data not shown).

Chronic oral BH4 supplementation has no effect on hemodynamics or plasma lipids

To assess the pharmacodynamic potential of BH4 supplementation in the context of native murine atherosclerosis, we treated hypercholesterolemic ApoE-KO male mice with chronic oral BH4 supplementation. Daily gavage from 8 weeks of age for up to 12 weeks was well tolerated. No overt toxicity or unexpected adverse events were observed at the 10 mg/kg/day dose but there was a modest reduction in weight gain over 12 weeks at 50 mg/kg/day ($P<0.01$) (Figure 2B) and a greater reduction in weight gain at the 500 mg/kg/day dose ($P<0.01$) (Figure 2B) compared with placebo, indicating adverse effects at higher BH4 concentrations. We observed a 20% mortality during the first 4 weeks of treatment amongst animals in the high dose 500 mg/kg group. Post-mortem examination revealed evidence of gut necrosis consistent with significant acidity of high dose BH4. There were no mortalities in the medium dose (50 mg/kg) or low dose (10 mg/kg) groups. Importantly, we did not observe any effects of the treatment at any dose on either blood pressure or pulse (Figure 2A). In addition, plasma lipid profiles were similar in all groups of mice, with the absolute values within the expected range for ApoE-KO mice fed a standard chow diet (Supplemental Table 1).

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Long-term low dose oral BH4 supplementation retards native aortic root atherosclerosis in ApoE-KO mice

Morphometric analysis revealed that aortic root atherosclerotic plaque developed at the expected rate at 16 and 20 weeks of age in the placebo groups. Treatment with 10 mg/kg/day BH4 resulted in a significant 45% reduction in plaque area after 8 weeks of treatment (0.049 mm^2; $P<0.05$; age 16 weeks) compared with placebo (0.088 mm^2; Figure 3). This reduction in plaque progression persisted after 12 weeks BH4 treatment (0.142 vs. 0.259 mm^2; 45% reduction $P<0.05$; age 20 weeks). Importantly, although there was a trend for a reduction in plaque area in the 50 or 500 mg/kg/day groups versus placebo, this did not reach statistical significance (data not shown).

Low dose BH4 supplementation reduces leukocyte infiltration into aortic root plaques and vascular adventitia concomitant with a reduction in VCAM-1 RNA levels

We now aimed to explore the underlying mechanisms of the observed beneficial effect of BH4 treatment on atherosclerosis progression, by investigating the cellular composition of atherosclerotic lesions in ApoE-KO male mice. Using fluorescence microscopy we observed that 8 weeks daily BH4 treatment significantly reduced the infiltration of $CD3^+$ T cells not only into atherosclerotic plaques (0.001886 vs. 0.006653 mm^2; age 16 weeks; Figure 4A) but strikingly also into the surrounding vascular adventitia (0.007741 vs. 0.020133 mm^2). Overall, we observed a 72% relative reduction ($P<0.05$) of T cell infiltration into plaques and a 62% reduction ($P<0.05$) into adventitia compared with placebo treated littermates (Figure 4A). The infiltration of T cells into the adventitia was predominantly adjacent to areas of plaque. Of note, after 12 weeks BH4 treatment (age 20 weeks) there was no longer any significant reduction in $CD3^+$ T cells compared with placebo in either plaque or adventitia.

Interestingly, a similar effect was observed for the infiltration of $Mac3^+$ macrophages and monocytes into the lesions. Eight weeks daily BH4 treatment lead to a marked 64% ($P=0.003$; age 16 weeks) relative reduction in Mac-3 positive cells in atherosclerotic plaques compared with placebo controls (0.012908 vs. 0.03622 mm^2; Figure 4B).

To further investigate the mechanism underlying the decreased leukocyte infiltration into atherosclerotic areas, we employed qRT PCR technique on thoracic aortas to quantify RNA levels of vascular adhesion molecules VCAM-1 and ICAM-1, both of which have been shown to play a critical role in leukocyte homing to sites of atherosclerotic lesions. Importantly, we measured a significant 45% reduction in VCAM-1 ($P=0.03$), but not ICAM-1 RNA levels after 8 weeks daily BH4 treatment compared to placebo controls (Figure 4C).

In conjunction with the reduction of leukocytes in atherosclerotic plaques, we observed a decrease in smooth muscle cells, a reduction in cholesterol clefts and decreased deposition of Collagen Type IV within the plaque (Supplemental Figure 1). Although all analysed plaque constituents showed a significant reduction after 8 weeks of BH4 supplementation (age 16 weeks) these effects were less marked after 12 weeks of daily BH4 treatment (age 20 weeks). Only the deposition of Collagen Type IV within the plaque remained significantly reduced after 12 weeks of treatment compared with placebo controls (relative 50% reduction; 0.090083 vs. 0.179533 mm^2; $P=0.02$; Supplemental Figure 1).

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Taken together, these data suggest that BH4 supplementation leads to an early, but not sustained, effect on vascular leukocyte infiltration, likely as a result of a down-regulation of the vascular adhesion molecule VCAM-1.

Chronic BH4 supplementation has no systemic effect on lymphoid and monocytic cell numbers

Following our observation that BH4 treatment leads to a down-regulation of VCAM-1 RNA levels in the aortic endothelium and a reduction of vascular immune cell infiltration into atherosclerotic lesions, we next sought to further confirm that this effect was mediated by local changes within the vascular wall rather than systemic immune effects. For this purpose we used flow cytometry to quantify circulating lymphoid and monocytic cell populations in whole mouse blood from 16 week old ApoE-KO mice treated with 10 mg/kg/day BH4 for 8 weeks. We observed no changes in the numbers of circulating $CD3^+$, $CD4^+$, $CD8^+$ T-lymphocytes or monocytes compared with placebo treated and untreated littermates of the same age (Figure 5B). These data suggest that BH4 treatment has no obvious systemic effect on circulating immune cell numbers.

In addition we investigated whether peripheral blood mononuclear cells (PBMCs) were able to actively take up orally administered BH4 from plasma, which would argue for a direct effect of BH4 treatment on circulating immune cells. We administered a single oral dose of 10 mg/kg BH4 or placebo to ApoE-KO mice, isolated PBMCs 60 minutes after the dose and measured intracellular BH4 levels (Figure 5C). No changes in PBMC BH4 levels were detected. These results further confirm that the observed reduction of leukocyte infiltration into atherosclerotic plaques after long-term BH4 supplementation (Figure 4) is likely to be mediated through augmented endothelial cell signalling rather than systemic effects on the immune response or altered responses of immune cells driven by the active uptake of BH4 from the plasma.

Endothelium-specific increase in BH4 levels reduces leukocyte infiltration into atherosclerotic plaques and adventitia

For the experiments discussed above we used ApoE-KO mice on standard chow diet as a model for the pharmacotherapy of native atherosclerosis. Under these conditions, experimental animals developed modest atherosclerosis. To further address the hypothesis that vascular leukocyte cell infiltration is directly mediated by BH4-dependent endothelial cell signalling, we used an experimental mouse model (GCH-Tg) in which BH4 levels were augmented only and specifically within the vascular endothelium, by endothelium-targeted transgenic over-expression of the rate-limiting enzyme in BH4 synthesis, GTP cyclohydrolase-1 (GCH).[21] In order to enhance the relative impact of increased levels of endothelial (rather than systemic) BH4, we crossed this transgenic mouse with Hph-1 mice, which exhibit a genetic systemic reduction in BH4 levels.[28] We further crossed this line with ApoE-KO mice to generate ApoE-KO/Hph/GCH-Tg mice or ApoE-KO/Hph littermate controls, and fed experimental animals a high-fat diet to generate substantial atherosclerosis. Indeed, even in this more extreme model of atherosclerosis, we observed a striking reduction in both intra-plaque and adventitial T cell infiltration as well as a significant reduction in intra-plaque $Mac3^+$ macrophages and monocytes in ApoE-

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KO/Hph/GCH-Tg mice compared with ApoE-KO/Hph controls (Figure 6). These data provide further evidence that vascular leukocyte infiltration in atherosclerosis is determined by BH4 dependent effects within the vascular endothelium.

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Discussion

In this study, we used exogenous BH4 supplementation in ApoE-KO mice with a clinical grade pharmacological formulation of BH4 to address the hypothesis that improved BH4-dependent endothelial function leads to reduced atherosclerosis through effects on vascular inflammation, and report the following major findings. First, after a single oral gavage of low dose BH4 (10 mg/kg), aortic BH4 levels remained elevated at 24 hours after gavage, several hours after plasma BH4 levels returned to baseline. Second, low dose BH4 supplementation for 8 or 12 weeks (age 16 or 20 weeks) had no effect on blood lipids, pulse or blood pressure but resulted in toxicity at concentrations exceeding 50 mg/kg/day. Third, aortic root atherosclerosis progression was reduced by 45% both after 8 and 12 weeks of daily 10 mg/kg BH4 gavage, but not at higher concentrations, compared with placebo treated animals. Importantly, BH4 supplementation for 8 weeks lead to a significant reduction in aortic endothelial VCAM-1 RNA levels. BH4 treatment furthermore markedly reduced the infiltration of T cells, macrophages and monocytes into plaques *and* reduced T cell infiltration in adjacent adventitia after 8 but not 12 weeks of daily treatment compared with placebo. Fourth, chronic BH4 supplementation had no systemic effect on lymphoid or monocytic cell numbers and there was no evidence that peripheral blood mononuclear cells take up BH4 from plasma 60 minutes after a single oral 10 mg/kg BH4 dose. Fifth, the endothelial-specific increase in BH4 levels in ApoE-KO/GCH-Tg mice was sufficient to markedly reduce T cell, macrophage and monocyte infiltration into atherosclerotic lesions and to reduce infiltration of T cells in adjacent adventitia in a mouse background with systemically reduced BH4 levels.

The molecular and cellular mechanisms through which BH4 exerts its effects on endothelial NOS activity are well-established.[14,29] Clinical studies using acute pharmacological BH4 supplementation in patients with a variety of cardiovascular pathologies,[13,16,17] as well as supplementation experiments with BH4 or its precursor sepiapterin in animal models of vascular disease[9,10,18-20] have reported an improvement of NO-mediated endothelial function. A range of *in vitro* approaches, *in vivo* pharmacological experiments, and genetically modified mouse models have established that BH4 supplementation promotes eNOS dimerisation, increases NO synthesis and suppresses superoxide generation, indicating a restoration of eNOS coupling.[30-34] However, the underlying downstream cellular mechanisms through which the effects of augmented BH4-dependent eNOS coupling lead to reduced atherosclerosis were not explored in these studies.

Previous work from our own group revealed that endothelial-specific increase in BH4 levels in GCH-Tg mice significantly reduced aortic root atherosclerotic plaque size in the atherogenic ApoE-KO background (ApoE-KO/GCH-Tg).[22] This study additionally showed, that endothelium-dependent relaxations to the receptor-mediated eNOS agonist acetylcholine (ACh) were significantly impaired in ApoE-KO mice compared with relaxations in ApoE-KO/GCH-Tg mice, while endothelium-dependent relaxations in ApoE-KO/GCH-Tg mice were not significantly impaired in comparison with control C57Bl/6J mice fed a high-fat diet. A subsequent study indicated that BH4 may be an important regulator of inflammation and vascular remodelling, since ApoE-KO/GCH-Tg mice exhibited reduced vein graft atherosclerosis with a marked reduction in vein graft macrophage content compared to ApoE-KO controls.[23] Aortic tissue in ApoE-KO/GCH-Tg mice showed significantly reduced mRNA as well as protein levels

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of MCP-1 (CCL2) but not RANTES (CCL5), with a specific decrease in CCR2-mediated chemotaxis to vascular tissue extracts and plasma samples from ApoE-KO/GCH-Tg animals.[23] These studies suggested that beneficial effects of increased BH4 levels on endothelial dysfunction and vascular injury may be mediated through an effect on endothelial signalling to vascular inflammatory cells. The present study now demonstrates the direct response of T cells, macrophages and monocytes to chronic BH4 supplementation and the associated reduction in atherosclerotic plaque size.

Exogenous rather than transgenic BH4 supplementation was reported by Hattori et al. to have beneficial effects of on the progression of atherosclerosis in ApoE-KO mice.[24] In that study daily supplementation with approximately 10 mg/kg BH4 in the drinking water of ApoE-KO mice on a high-cholesterol diet caused a 25% reduction in mean plaque area compared with untreated ApoE-KO mice. However, the effects on the cellular composition of the plaque leading to reduced atherosclerosis were not determined. Furthermore, since Hattori et al. used BH4 supplementation in drinking water ad libitum, the oxidation state and precise dose of BH4 could not be fully determined. In the present study, we used direct weight-adjusted daily oral gavage of a clinical grade pharmacological BH4 formulation. Our analysis of single oral dose pharmacokinetics and biodistribution using 3 different BH4 concentrations has provided insights into the uptake of BH4 from the GI tract into plasma and organs, dose-dependent peak concentrations and excretion. These experiments have revealed that aortic tissue, but not other organs, appears to retain elevated BH4 levels after a single low BH4 dose independent of the plasma BH4 concentration, and suggests mechanisms relating to active uptake, retention, or recycling within vascular tissue. Importantly, chronic supplementation with BH4 had no effect on hemodynamics but revealed toxicity at concentrations exceeding 50 mg/kg/day. These insights will be valuable in informing dosing regimens in human studies.

We observed a significant reduction in aortic root atherosclerosis progression in ApoE-KO mice after long-term low dose BH4 administration. The marked 45% relative reduction in lesion size we observed after both 8 and 12 weeks BH4 treatment confirms the therapeutic potential of BH4 supplementation in vascular disease. Our detailed analysis of the cellular composition of aortic root plaques and surrounding vascular adventitia has shown that long-term BH4 treatment markedly reduced $CD3^+$ T cell infiltration into atherosclerotic lesions *and* into the surrounding vascular adventitia, as well as the infiltration of Mac-3 positive macrophages/monocytes into the plaque. As we furthermore showed a significant reduction in VCAM-1 RNA levels in the thoracic aorta, the clear reduction in leukocyte infiltration is likely to be a direct consequence of reduced expression of this inducible endothelial adhesion molecule shown to mediate both rolling-type adhesion and firm adhesion of lymphocytes and monocytes to activated endothelial cells.[35] As recently shown by Hag et al., the constitutively expressed ICAM-1 is not upregulated during early stages of atherosclerosis in ApoE-KO mice which may explain the low levels of ICAM-1 RNA detected in our experiments.[36] These data give further evidence to support the observations by Ali et al. that increased endothelial BH4 levels exert their beneficial effects on vascular injury and neointima formation in a vein graft model through decreased vascular inflammation.[23]

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In addition to reduced leukocyte numbers, early plaques in 16 week old animals contained, fewer smooth muscle cells, less cholesterol clefts and reduced deposition of Collagen Type IV after 8 weeks of daily 10 mg/kg BH4 supplementation. However, since collagen type IV was the only plaque constituent which showed a significant reduction after 12 weeks BH4 treatment we hypothesise that BH4 supplementation leads to an early, but not sustained, augmentation of endothelial function and reduction in vascular immune cell infiltration.

We next specifically sought to distinguish the potential endothelial-specific mechanisms by which BH4 supplementation affects vascular inflammation from systemic effects on peripheral immune cells. We first observed that BH4 treatment did not influence total numbers of circulating immune cell populations, nor was there evidence of direct uptake of BH4 by peripheral blood mononuclear cells. Secondly, we revealed that GCH-Tg mice, with a specific increase in endothelial BH4 levels[21, 22] exhibited a marked reduction in vascular immune cell infiltration both within plaques and in adventitia compared with non-transgenic littermates, even in the setting of the ApoE-KO genetic background, the systemically low BH4 levels in the Hph-1 strain, and the use of high-fat diet. These results are consistent with the reported effects of increased endothelial BH4 on endothelial signalling and vascular inflammation.[23]

Our findings now extend previous studies demonstrating BH4-dependent eNOS coupling and enhanced NO bioavailability, to indicate a possible downstream mechanism through which improved endothelial function may result in reduced early vascular leukocyte infiltration and a consequent reduction in atherosclerosis progression. These results, together with our BH4 dose-ranging observations, may assist the design of clinical trials investigating BH4 supplementation therapy in vascular diseases.

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Conflicts of interest statement

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Figure Legends

Figure 1
Single oral dose pharmacokinetics and biodistribution after BH4 gavage. **A**, BH4 levels in plasma and aorta over 24 hours after administration of 10, 50 or 500 mg/kg BH4. **B**, Plasma and aortic BH4 levels 24 hours after single oral dose. Significantly increased BH4 levels after gavage of 10 mg/kg BH4 in aorta remained up to 24 hours, independent of plasma BH4 levels (n=5 per group; *P<0.05).

Figure 2
A, Non-invasive tail-cuff pulse and blood pressure measurements 20-24 hours after last drug dose showed no effects of chronic BH4 treatment on hemodynamics (n=8 per group). **B**, Weight gain of experimental animals over 12 week treatment course showed no overt toxicity at 10 mg/kg/day BH4 but a modest reduction in weight gain at 50 mg/kg/day (P<0.01) and a greater reduction in weight gain at 500 mg/kg/day BH4 (P<0.01) compared with placebo controls, indicating overt toxicity at higher BH4 concentrations (n=12 per group; *P<0.01).

Figure 3
Aortic root plaque area after 8 and 12 weeks of daily 10 mg/kg BH4 or placebo treatment, with representative Masson's trichrome and Haematoxylin stained aortic root sections. After 8 weeks treatment, mean plaque area was 45% lower in BH4 treated ApoE-KO mice (0.049 mm^2) compared with placebo (0.088 mm^2) (*P<0.05). After 12 weeks treatment mean plaque area remained 45% lower in BH4 treated ApoE-KO mice compared with placebo (0.142 vs. 0.259 mm^2; *P<0.05; n=12 per group). Black bar represents 200 µm.

Figure 4
Reduced leukocyte infiltration into aortic root plaques and surrounding adventitia. **A**, Quantification of absolute $CD3^+$ cell area in plaques and adventitia after 8 or 12 weeks of daily 10 mg/kg BH4 or placebo treatment. Representative sections are shown with specific CD3 immuno fluorescence (P - plaque; A - adventitia; M - media). Eight weeks daily BH4 treatment lead to a significant 72% relative reduction in $CD3^+$ T cell infiltration into atherosclerotic plaques (0.001886 vs. 0.006653 mm^2; *P<0.05) and a relative 62% reduction into the surrounding adventitia (0.007741 vs. 0.020133 mm^2; *P<0.05) compared to placebo treated littermates. **B**, Quantification of absolute Mac-3 positive cell area in plaques. Significant 64% relative reduction in Mac-3 positive cells in plaques after 8 weeks daily BH4 treatment compared to placebo controls (0.012908 vs. 0.03622 mm^2; **P=0.003). Representative sections are shown with specific Mac-3 immuno fluorescence (P - plaque; M – media; n.s. - not significant). White bar represents 400 µm. **C,** qRT PCR analysis of aortas from 16 week old ApoE-KO male mice shows significantly reduced VCAM-1 RNA levels (45% relative reduction; *P<0.03) but no differences in ICAM-1 RNA levels after 8 weeks BH4 treatment versus placebo controls.

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Figure 5
Analysis of whole blood and isolated peripheral blood mononuclear cells (PBMCs). **A**, Representative examples of analysis of ApoE-KO blood leukocyte populations after 8 weeks daily treatment with 10 mg/kg BH4 using flow cytometry defining $CD3^+$ T-cells as $SSC^{lo}/CD3^+$, $CD4^+$ T-cells as SSC^{lo}/CD3+/$CD4^+$, $CD8^+$ T-cells as $SSC^{lo}/CD3^+/CD8^+$ and monocytes as $CD11b^+$ $Ly6G^-$ 7/4 HI cells. **B,** BH4 treated animals showed no differences in $CD3^+$, $CD4^+$, $CD8^+$ T-cells or monocytes compared to placebo or untreated control mice, indicating that long-term BH4 treatment does not have a systemic effect on circulating immune cell populations (n=5 to 10). **C**, BH4 detection by electrochemical HPLC in isolated PBMCs 60 minutes after single oral 10 mg/kg BH4 dose or placebo. No increase in PBMC BH4 levels after BH4 administration detected, suggesting that no active uptake of BH4 from plasma occurred (n=5).

Figure 6
Reduced leukocyte infiltration into aortic root plaques and adventitia of ApoE-KO/Hph/GCH-Tg mice. **A**, Quantification of CD3 positive cell area in plaques and adventitia. Representative sections are shown with specific CD3 immuno fluorescence (P - plaque; A - adventitia; M - media). Similar to the pharmacological BH4 supplementation, transgenic endothelial-specific increase in BH4 levels lead to a significant 88% relative reduction in T-cell infiltration into atherosclerotic plaques of ApoE-KO/Hph/GCH-Tg mice (0.000442 vs. 0.003896 mm^2; ***P=0.0003) and a relative 93% reduction into the surrounding adventitia (0.000917 vs. 0.013889 mm^2; ***P<0.0001) compared with ApoE-KO/Hph controls (n=10). **B**, Quantification of Mac-3 positive cell area in plaques. Significant 44% relative reduction in Mac-3 positive cells in plaques after 8 weeks daily BH4 treatment compared to placebo controls (0.086041 vs. 0.155988 mm^2; **P=0.0047) (n=10). Representative sections are shown with specific Mac-3 immuno fluorescence (P - plaque; M - media). White bar represents 400 µm.

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Figure 1

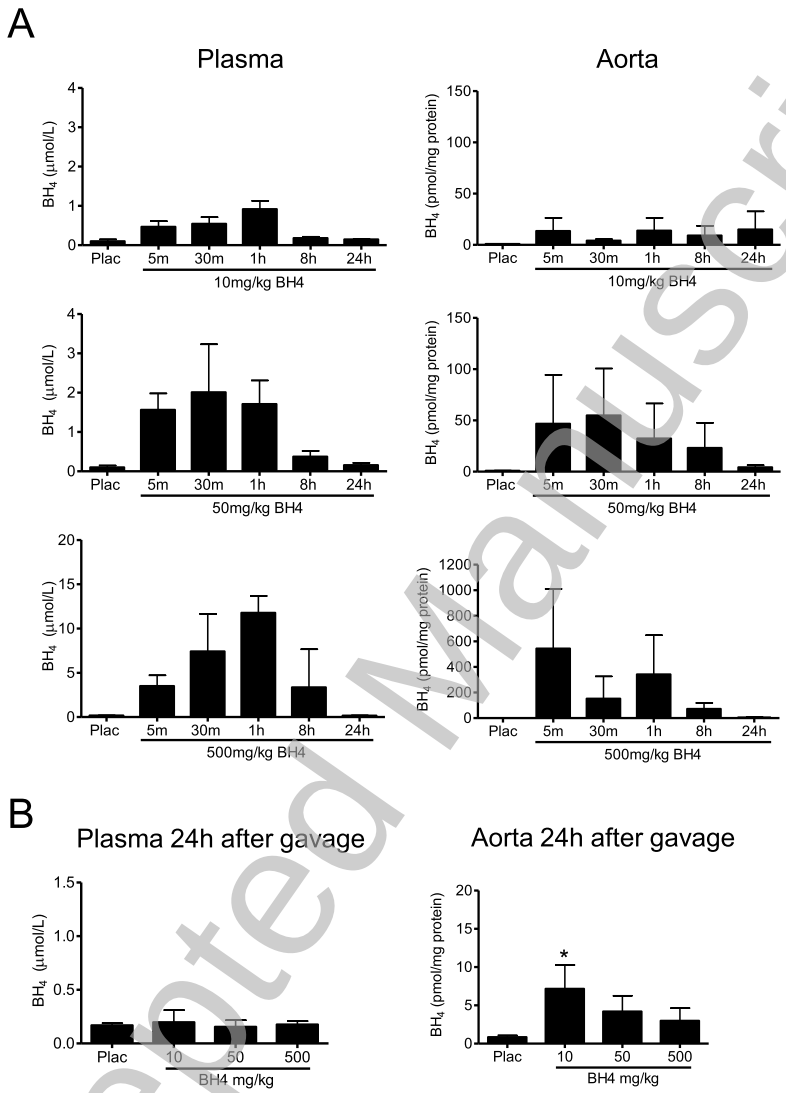

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Figure 2

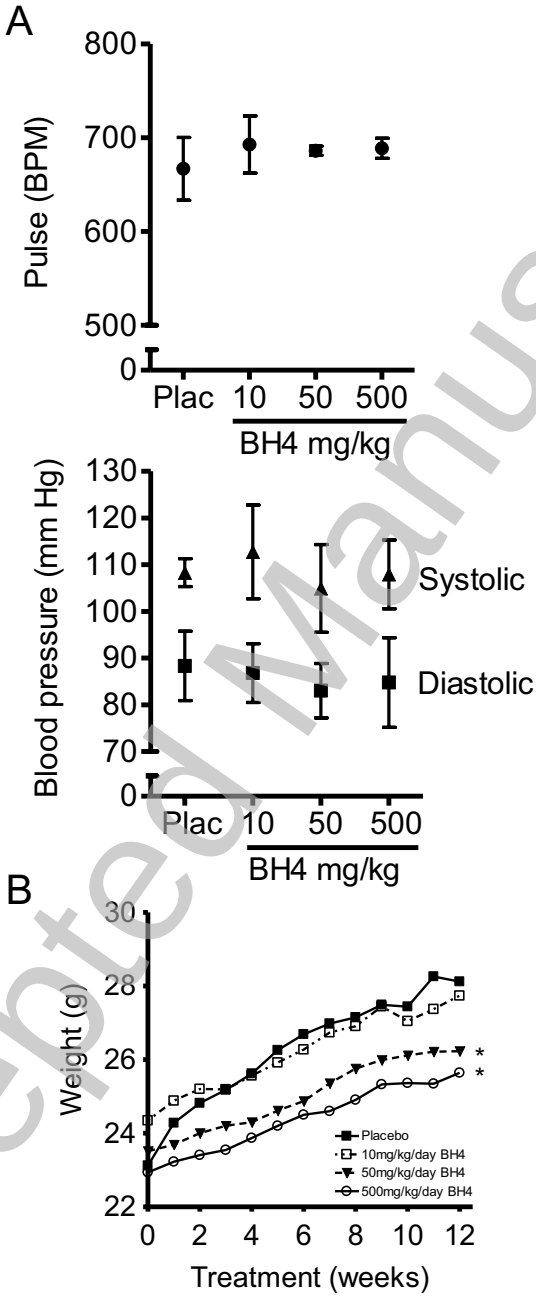

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Figure 3

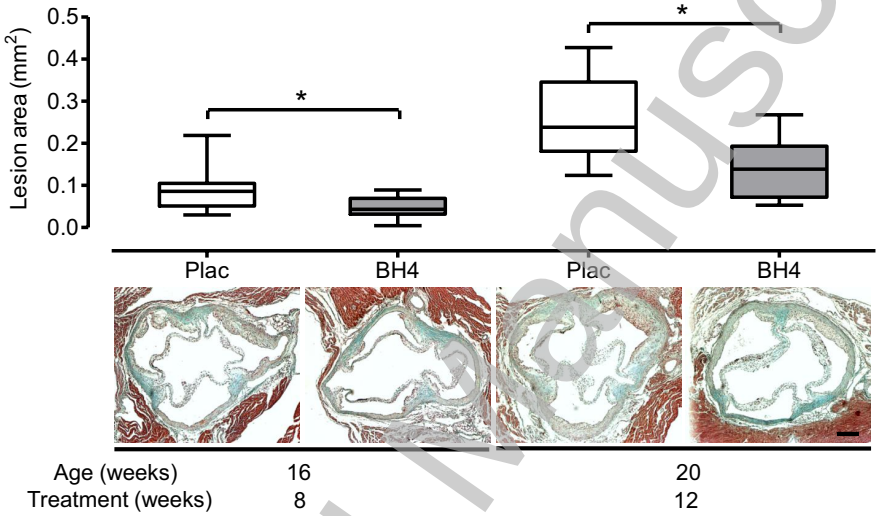

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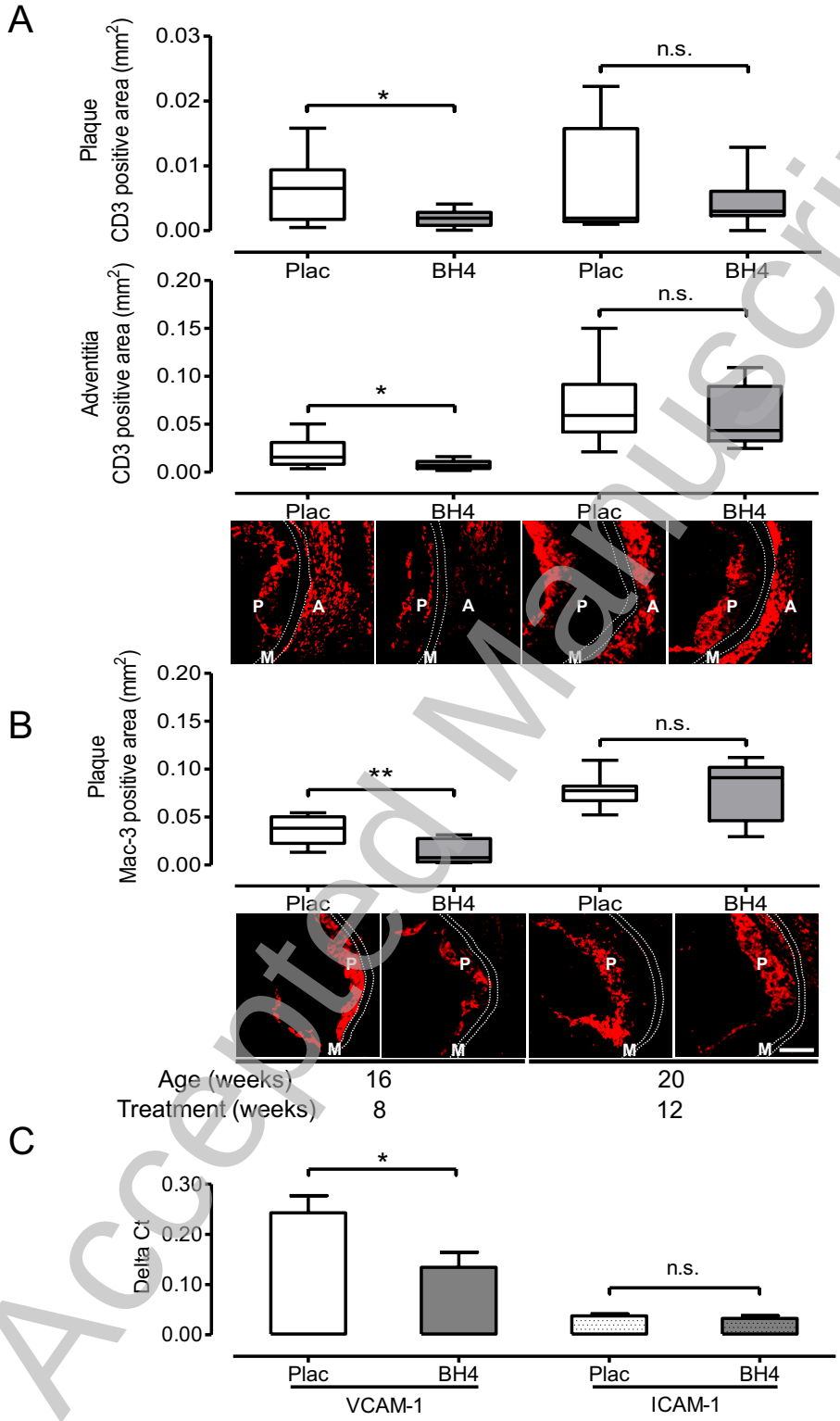
Figure 4



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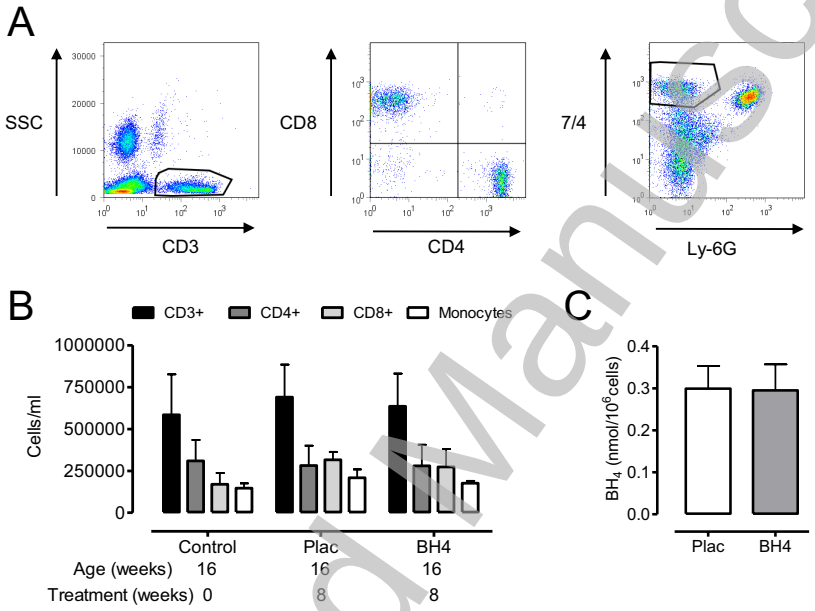

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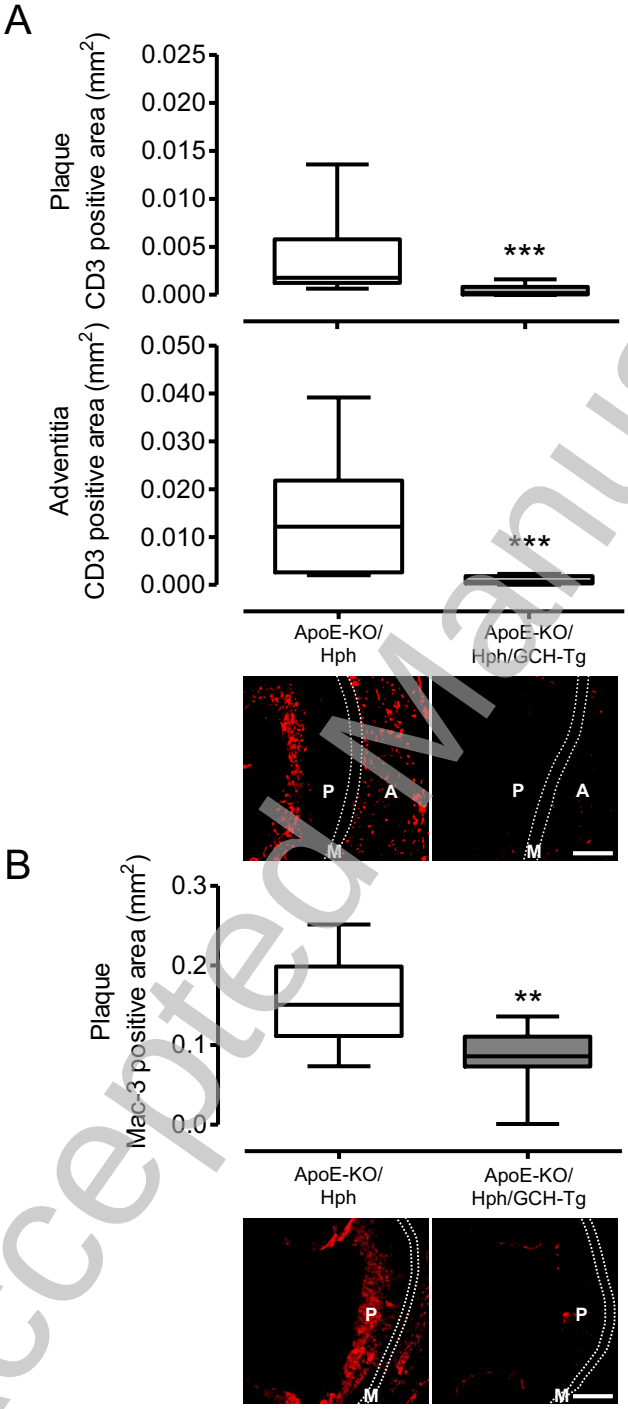

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