European Journal of Pharmacology 880 (2020) 173186

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Melanocortin 3 receptor activation with [D-Trp8]-γ-MSH suppresses inflammation in apolipoprotein E deficient mice

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ARTICLE INFO

Keywords:
Atherosclerosis
Inflammation
Melanocortin
Macrophage
$ApoE^{-/-}$ mice
[D-Trp8]-γ-MSH

ABSTRACT

The melanocortin MC_1 and MC_3 receptors elicit anti-inflammatory actions in leukocytes and activation of these receptors has been shown to alleviate arterial inflammation in experimental atherosclerosis. Thus, we aimed to investigate whether selective targeting of melanocortin MC_3 receptor protects against atherosclerosis. Apolipoprotein E deficient ($ApoE^{-/-}$) mice were fed high-fat diet for 12 weeks and randomly assigned to receive either vehicle (n = 11) or the selective melanocortin MC_3 receptor agonist [D-Trp(8)]-gamma-melanocyte-stimulating hormone ([D-Trp8]-γ-MSH; 15 μg/day, n = 10) for the last 4 weeks. Lesion size as well as macrophage and collagen content in the aortic root plaques were determined. Furthermore, leukocyte counts in the blood and aorta and cytokine mRNA expression levels in the spleen, liver and aorta were quantified. No effect was observed in the body weight development or plasma cholesterol level between the two treatment groups. However, [D-Trp8]-γ-MSH treatment significantly reduced plasma levels of chemokine (C–C motif) ligands 2, 4 and 5. Likewise, cytokine and adhesion molecule expression levels were reduced in the spleen and liver of γ-MSH-treated mice, but not substantially in the aorta. In line with these findings, [D-Trp8]-γ-MSH treatment reduced leukocyte counts in the blood and aorta. Despite reduced inflammation, [D-Trp8]-γ-MSH did not change lesion size, macrophage content or collagen deposition of aortic root plaques. In conclusion, the findings indicate that selective activation of melanocortin MC_3 receptor by [D-Trp8]-γ-MSH suppresses systemic and local inflammation and thereby also limits leukocyte accumulation in the aorta. However, the treatment was ineffective in reducing atherosclerotic plaque size.

1. Introduction

The melanocortins consist of α-, β- and γ-melanocyte-stimulating hormones and adrenocorticotropic hormone. Melanocortins are recognized as central regulators of energy homeostasis but they also mediate significant anti-inflammatory actions in many cell types including leukocytes (Brzoska et al., 2008; Catania et al., 2010, 2004). The diverse physiological functions of melanocortins are mediated by 5 different melanocortin receptors (MC_1 - MC_5). They are G-protein-coupled receptors that have distinct expression profiles and the melanocortin peptides bind to these receptors with varying degrees of affinity. Melanocortin MC_2 and MC_3 receptors are unique in this regard because the former can only be activated by adrenocorticotropic hormone, while γ-melanocyte-stimulating hormone (γ-MSH) is the only endogenous peptide known to have a high affinity for melanocortin MC_3 receptor (Brzoska et al., 2008; Catania et al., 2004). The anti-inflammatory actions of melanocortins are mainly attributable to melanocortin MC_1 and MC_3 receptors, which are widely expressed in the cells of the immune system. Thus, activation of these receptors has also shown to provide protection against multiple inflammatory disorders such as rheumatoid arthritis and colitis (Catania et al., 2010). We and others have reported that activating these receptors alleviates inflammation also in the vasculature (Catania et al., 2004; Getting et al., 1999b; Ichiyama et al., 1999; Manna and Aggarwal, 1998; Rinne et al., 2017, 2014).

The anti-inflammatory potency of melanocortins could be therapeutically extended to atherosclerosis which is characterized by chronic arterial inflammation. Accumulation of low-density lipoprotein

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https://doi.org/10.1016/j.ejphar.2020.173186
Received 25 November 2019; Received in revised form 29 April 2020; Accepted 9 May 2020
Available online 13 May 2020
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(LDL) particles and oxidized LDL in arteries drives local inflammation by activating endothelial cells to express adhesion molecules and chemokines that attract leukocytes, primarily monocytes, to the scene. Monocyte-derived macrophages accumulate in the lesions, engulf LDL particles and transform into foam cells. Ultimately, foam cells undergo apoptosis due to cholesterol overload, which promotes the formation of necrotic core (Seimon and Tabas, 2009). This, in addition to thinning of the fibrous cap, gives rise to rupture-prone vulnerable plaques. Thus, there are multiple inflammatory pathways involved in the pathogenesis of atherosclerosis that could be favorably modulated by melanocortins and melanocortin receptors to halt the disease progression. In fact, we have previously observed that nonselective melanocortin receptor activation by pharmacological or genetic approaches suppresses inflammation of atherosclerotic plaques (Nuutinen et al., 2018; Rinne et al., 2014). These studies do not, however, allow evaluating the exact contribution of individual receptor subtypes in the possible atheroprotective effect.

Given that the anti-inflammatory effects are strongly linked to melanocortin MC_3 receptor (Catania et al., 2004; Getting et al., 2003), we set out to investigate whether selective targeting of melanocortin MC_3 receptor could alleviate vascular inflammation and limit the progression of atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Furthermore, by selectively targeting melanocortin MC_3 receptor, it could be possible to avoid the unwanted pigmentary effects that are linked to melanocortin MC_1 receptor stimulation. The study was conducted using [D-Trp8]-γ-MSH that has high selectivity for melanocortin MC_3 receptor and has been shown to evoke strong anti-inflammatory effects in murine models of acute and chronic inflammation (Getting et al., 2006b, 1999a; Getting and Perretti, 2001; Leoni et al., 2008).

2. Material and methods

2.1. Mice and study design

Eight-week-old female apolipoprotein-E deficient ($ApoE^{-/-}$) mice were placed on a cholesterol-rich high-fat diet (HFD) (RD Western Diet, D12079B, Research Diets Inc, NJ, USA) for 12 weeks to enhance atherosclerosis. After 8 weeks on the HFD, mice were randomly assigned to receive daily intraperitoneal injections of either vehicle (phosphate-buffered saline; n = 11) or the selective melanocortin MC_3 receptor agonist [D-Trp8]-γ-MSH (γ-MSH; 15 μg/mouse/day; n = 10) for 4 weeks. After the drug intervention, mice were euthanized *via* CO_2 asphyxiation and whole blood was obtained *via* cardiac puncture. After perfusion of the arterial tree with phosphate-buffered saline, the aorta, spleen and liver were collected for further analyses. Mice were group-housed on a 12 h light/dark cycle and the experiments were approved by the local ethics committee (Animal Experiment Board in Finland, License Number: ESAVI/6280/04.10.07/2016) and conducted in accordance with the institutional and national guidelines for the care and use of laboratory animals.

2.2. Plasma cholesterol and cytokine analysis

Plasma was obtained from EDTA-anticoagulated whole blood after centrifugation. Plasma total cholesterol and triglyceride concentrations were determined using enzymatic colorimetric assays (CHOD-PAP and GPO-PAP, mti Diagnostics, Idstein, Germany) according to the manufacturer's protocols. Furthermore, plasma high-density lipoprotein (HDL) and LDL/very low-density lipoprotein (VLDL) fractions were separated using LDL/VLDL precipitation buffer (Abcam, Cambridge, United Kingdom) and cholesterol concentrations in the resulting fractions were measured. Pro-inflammatory cytokines and chemokines in the plasma were quantified with ProcartaPlex™ Multiplex Immunoassays (Chemokine 20-Plex Mouse Panel 1, catalogue number: EPX200-26090-901 & High Sensitivity 5-Plex Mouse Panel, catalogue number: EPXS050-22199-901, eBioscience).

2.3. Quantitative real-time PCR

Total RNA from the aorta, spleen and liver was extracted (Qiagen, Venlo, Netherlands) and reverse-transcribed (PrimeScript RT reagent kit, Takara Clontech) into cDNA. Quantitative real-time PCR (qPCR) was performed with SYBR Green protocols (Kapa Biosystems, MA, USA) on an Applied Biosystems 7300 Real-Time PCR system. Samples were run in duplicate. Primer sequences are given in Supplemental Table I. Target gene expression was normalized against the geometric mean of two house-keeping genes (S29 and β-actin) using the comparative ΔCt method and is presented as relative transcript levels ($2^{-\Delta\Delta Ct}$) (Rinne et al., 2018b).

2.4. Flow cytometry

Samples from the aorta, spleen and whole blood were prepared for flow cytometry to quantify total leukocytes and leukocyte subsets as previously described (Rinne et al., 2018b). Aortic samples were digested with an enzymatic cocktail (Collagenase I, 450 U/ml; Collagenase XI, 250 U/ml; Hyaluronidase, 120 U/ml; DNase I, 120 U/ml; Sigma Aldrich) for 60 min at 37 °C. Thereafter, single-cell suspensions were prepared by filtering the aortic tissue lysate through a 50-μm cell strainer (BD Biosciences). Aortic lysate as well as blood and splenic cells were stained for 30 min at 4 °C with fluorochrome-conjugated antibodies against CD11b (clone M1/70, BioLegend), CD11c (clone N418, Biolegend), CD31 (clone MEC13.3, BD Biosciences), CD45.2 (clone 30-F11, BD Biosciences), CD54/ICAM-1 (clone 3E2, BD Biosciences), CD106/VCAM-1 (clone MVCAM.A, BD Biosciences), CD115 (clone AFS98, BioLegend), Ly6C (clone AL-21, BD Biosciences) and Ly6G (clone 1A8, BD Biosciences). Viable cells were differentiated from dead cells using fixable viability dye (Zombie Aqua, Biolegend) at room temperature for 15 min prior to leukocyte staining. Data were acquired on an LSR Fortessa (BD Biosciences) and the results were analyzed with FlowJo software (FlowJo, LLC, Ashland, USA).

2.5. Tissue sectioning, histology and immunohistochemistry

Aortic root, spleen and liver samples were embedded in paraffin after fixing in 10% formalin overnight and then cut in 4 μm-thick serial sections. Atherosclerotic plaque area, the size of necrotic core and plaque collagen content were determined at the level of the aortic sinus in hematoxylin and eosin (H&E) and Masson's trichrome stained sections. For immunohistochemistry, sections were incubated in 10 mM sodium citrate buffer (pH 6) for 20 min in a pressure cooker for antigen retrieval. Thereafter, sections were blocked in 1% H_2O_2 for 20 min and then in 10% normal horse serum. Samples were incubated overnight with Mac-2 (1:300, Abcam), melanocortin MC_3 receptor (1:200, Abcam) or alpha smooth muscle actin (α-SMA, 1:200, Sigma-Aldrich, St. Louis, MO, USA) primary antibodies followed by horseradish peroxidase-conjugated secondary antibody incubation and detection with diaminobenzidine (ABC kit, Vector Labs, Burlingame, USA) to estimate macrophage- and smooth muscle cell-positive areas, respectively. Sections were counterstained with hematoxylin (CarlRoth), coverslipped and then scanned with a digital slide scanner (Pannoramic 250 or Pannoramic Midi, 3DHISTECH Kft, Budapest, Hungary). For double immunofluorescence, sections were incubated with antibodies against melanocortin MC_3 receptor and Mac-2 (Cedarlane Labs, Burlington, ON, Canada) or CD31 (Novus Biologicals, Littleton, CO, USA) followed by detection with fluorochrome-conjugated secondary antibodies (Alexa Fluor 647 and Alexa Fluor 488, Jackson ImmunoResearch, West Grove, USA). ImageJ software (NIH, Bethesda, MD, USA) was used to analyze images.

2.6. Cell culture

Primary mouse endothelial cells were isolated from freshly

harvested thoracic aortae as previously described (Rinne et al., 2015). Cells were cultured in a 1:1 mix of DMEM-F12 and RPMI (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), endothelial cell growth supplement (R&D Systems, Minneapolis, MN, USA) and 100 U/100 µg/ml penicillin-streptomycin (Gibco Life Technologies, NY, USA) on Geltrex-coated (Gibco) plates. When 80% confluency was reached, cells were treated with [D-Trp8]-γ-MSH for 1–24 h and harvested in Qiazol (Qiagen) for RNA extraction and quantitative PCR analyses. To obtain bone marrow-derived macrophages, femurs and tibiae of *ApoE*$^{-/-}$ mice were flushed and bone marrow cell were suspended in IMDM medium (Gibco) supplemented with 10% FBS, penicillin/streptomycin and 20 ng/ml M-CSF (PeproTech, NJ, USA). Cells were plated on bacteriological dishes and allowed to differentiate for 6–7 days. Thereafter, cells were seeded on 24-well plates, treated with [D-Trp8]-γ-MSH for 1–24 h and harvested for RNA extraction.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism 7 software (La Jolla, CA, USA) and unpaired Student's t-test or one-way ANOVA followed by Bonferroni *post hoc* tests. Data that did not pass the D'Agostino and Pearson omnibus normality test were analyzed using the Mann-Whitney *U* test or Kurskal-Wallis test. Possible outliers in the data sets were detected using the robust regression and outlier removal (ROUT) method at Q-level of 1%. All data are presented as mean ± standard error of the mean (S.E.M.). A two-tailed *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Melanocortin MC_3 receptor is expressed in atherosclerotic plaques of ApoE$^{-/-}$ mice

Given the wide expression of melanocortin MC_3 receptor in leukocytes, we first aimed to investigate whether leukocyte-rich atherosclerotic plaques also express melanocortin MC_3 receptor. First, using immunohistochemistry, we found that aortic root sections from HFD-fed *ApoE*$^{-/-}$ mice show subtle but clear expression of melanocortin MC_3 receptor, which localized in the luminal side of atherosclerotic plaques (Fig. 1A). Immunofluorescence staining further revealed that melanocortin MC_3 receptor mainly co-localizes with the monocyte and macrophage marker Mac-2 in the lesions (Fig. 1B). Furthermore, low levels of melanocortin MC_3 receptor was found in the aortic endothelium as evidenced by co-localization with the endothelial cell marker CD31 (Fig. 1C). We also studied melanocortin MC_3 receptor expression in the spleen and liver, which have a central role in lipid metabolism and atherosclerosis-related immunity. Melanocortin MC_3 receptor was widely present in the spleen and localized to large extent in Mac2-positive cells (Supplemental Fig. IA), while in the liver, the expression was relatively scarce (Supplemental Fig. IB). This data provided evidence that melanocortin MC_3 receptor is present in atherosclerotic plaques and other disease-related tissues such as the spleen and liver, and prompted us to investigate whether it could be targeted to mitigate the progression of atherosclerosis. To this end, *ApoE*$^{-/-}$ mice were placed on HFD for 12 weeks. During the last 4 weeks on HFD, mice were treated with either vehicle or the selective melanocortin MC_3 receptor agonist [D-Trp8]-γ-MSH (γ-MSH, 15 µg/mouse/day).

Since melanocortin MC_3 receptor is also implicated in the central regulation of energy balance (Marks et al., 2006), we closely monitored body weight and food intake of *ApoE*$^{-/-}$ mice during the drug intervention. However, with the selected dose and dosing interval, γ-MSH treatment did not affect body weight or food intake in these mice (Table 1). Likewise, there was no significant effect of drug treatment on the liver, spleen or retroperitoneal/gonadal white adipose tissue weight (Table 1). Plasma total cholesterol, HDL cholesterol, LDL/VLDL cholesterol and triglyceride concentrations were also comparable between the treatment groups (Table 1).

3.2. Selective activation of melanocortin MC_3 receptor suppressed systemic inflammation in atherosclerotic ApoE$^{-/-}$ mice

To explore whether γ-MSH treatment evoked anti-inflammatory effects in *ApoE*$^{-/-}$ mice, we first analyzed the level of different cytokines and chemokines in the plasma. We observed significant reductions in the plasma levels of pro-inflammatory cytokines in γ-MSH-treated mice. Specifically, γ-MSH lowered the plasma concentrations of chemokine (C–C motif) ligands 2, 4 and 5 (CCL2, CCL4 and CCL5) (Fig. 2A), which are well-defined chemoattractants for leukocytes and promote atherosclerosis. Daily administration of γ-MSH also dampened the circulating interleukin 4 (IL-4) level (Fig. 2A), which is generally considered as a signature marker of anti-inflammatory Th2 lymphocytes and M2-type macrophages but it also possesses proinflammatory and proatherogenic properties (Kleemann et al., 2008; Okoye and Wilson, 2011). In line with these results, flow cytometry analysis revealed reduced leukocyte counts in the blood of γ-MSH-treated mice (Fig. 2B). Total leukocyte count was lower in these mice, which was mainly attributable to reduced lymphocyte count (Fig. 2C). Furthermore, neutrophil count was reduced by γ-MSH treatment, while no difference was noted in classical Ly6C^{high} monocytes (Fig. 2D). Leukocytes and their subsets were also quantified in the spleen and bone marrow (Supplemental Fig. II). Similarly to the changes in the blood, total leukocyte and lymphocyte counts were reduced in the spleen of γ-MSH-treated mice (Supplemental Fig. IIB). Flow cytometric analysis of bone marrow showed no difference in leukocyte counts between the treatment groups (Supplemental Fig. IIA), suggesting that myelopoiesis was unaffected by the treatment.

3.3. γ-MSH treatment reduced leukocyte counts in the aorta of ApoE$^{-/-}$ mice and down-regulated hepatic and splenic mRNA expression of pro-inflammatory markers

Flow cytometry of aortic lysates (descending aorta) further revealed that γ-MSH treatment was effective in attenuating leukocyte accumulation in the whole aorta (Fig. 3A and B). γ-MSH treatment seemed to evenly affect lymphocyte and myeloid cell accumulation since both leukocyte subsets were reduced in these mice (Fig. 3B). Crucial to the inflammation in the blood vessels are the cellular adhesion proteins such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) that mediate the adhesion of leukocytes to the vascular endothelium and promote their accumulation at the sites of inflammation. To investigate the role of endothelial adhesion proteins in the observed effects, aortic cell lysates were further gated for CD31^{+} cells and quantified for VCAM-1 and ICAM-1 expression (Fig. 3A). However, γ-MSH intervention had no effect on the expression of these adhesion molecules in the aortic endothelial cells (Fig. 3C). Supporting this finding, qPCR analysis of samples from the aortic arch showed no effect on *VCAM-1* or *ICAM-1* expression in γ-MSH-treated mice (Fig. 3D). Likewise, other adhesion molecules such as platelet endothelial cell adhesion molecule (*PECAM-1*) and P-selectin (*CD62P*) were unaffected by the drug intervention (Fig. 3D). The possible direct effects of γ-MSH on the endothelium were further studied in primary endothelial cells that were isolated from mouse aortae and treated with different durations (1–24 h) and concentrations (0.01–10 µM) of γ-MSH. No clear-cut and consistent effects were observed on *VCAM-1*, *ICAM-1* or *CCL2* expression (Supplemental Fig. III). However, it was of note that γ-MSH up-regulated *CCL5* expression in a concentration-dependent manner (Supplemental Fig. III).

We also examined the effect of γ-MSH on markers of atherosclerotic plaque stability in the aortic tissue lysates by qPCR analysis. The mRNA levels of different collagen isoforms, matrix metalloprotease 9 (*MMP9*)

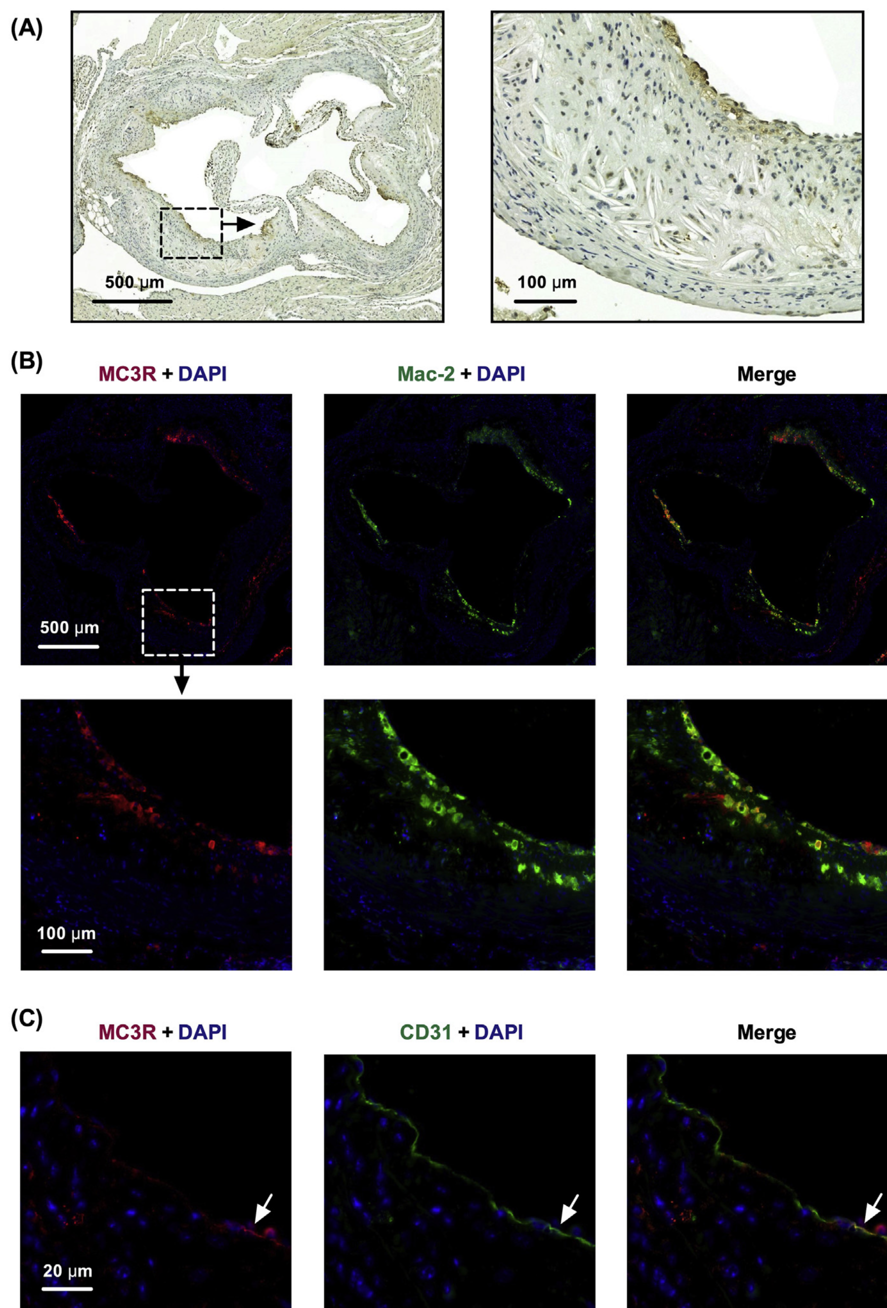

Fig. 1. Melanocortin MC_3 expression on mouse atherosclerotic plaque. (A) Immunohistochemical staining of melanocortin MC_3 receptor in aortic root section of HFD-fed (12 weeks) $ApoE^{-/-}$ mouse. Scale bars, 500 μm (left panel) and 100 μm (right panel). (B) Immunofluorescence staining of melanocortin MC_3 receptor (MC3-R, red) and Mac-2 (green) in aortic root section of HFD-fed (12 weeks) $ApoE^{-/-}$ mouse. Scale bars, 500 μm (top panel) and 100 μm (bottom panel). (C) Immunofluorescence staining of melanocortin MC_3 receptor (MC3-R, red) and CD31 (green) in aortic root section of HFD-fed (12 weeks) $ApoE^{-/-}$ mouse. Scale bars, 20 μm.

or *α-SMA* were not significantly different between the treatment groups (Fig. 3D). Interestingly, the treatment appeared to promote the expression of M2-type macrophage markers in the aorta as evidenced by further gene expression analyses (Fig. 3E). Changes in the expression of different macrophage markers did not reach statistical significance but in general, γ-MSH treatment seemed to down-regulate M1-type markers like *CCL5*, C–C chemokine receptor type 1 (*CCR1*) and tumor necrosis factor alpha (*TNF-α*) ($P = 0.28$, 0.06 and 0.12, respectively), while M2-type markers such as chitinase-like 3 (*Ym1*), C-type mannose receptor 1 (*CD206*) and interleukin-1 receptor antagonist (*IL-1Ra*) ($P = 0.08$, 0.06 and 0.06, respectively) were up-regulated in the aorta of γ-MSH-treated mice. To investigate the direct effects of γ-MSH on macrophages, bone marrow cells from $ApoE^{-/-}$ mice were differentiated into macrophages and treated with γ-MSH in a similar way as aortic endothelial cells. In line with the qPCR findings in the aorta, γ-MSH down-regulated the M1-type markers interleukin-1β (*IL-1β*), *CCL5* and *CCR1* in bone marrow-derived macrophages with the strongest effect appearing 3 h after applying the treatment (Supplemental Fig. IV, A-E). M2-type markers were not strongly affected, except for Ym-1, which was up-regulated after 24-h treatment (Supplemental Fig. IV, F-H). Screening for the concentration-dependency of the effects also revealed that the maximal response on the M1-type markers such as *IL-1β* was achieved at 0.1 μM concentration, while treatment with the highest concentration (10 μM) led to marked blunting of the responses or even increased the expression of the pro-inflammatory markers such as *TNF-α* (Supplemental Fig. IV, I-L).

We proceeded to examine the effect of γ-MSH administration on the hepatic and splenic gene expression profile of inflammatory markers. Corroborating the findings of reduced systemic inflammation, we found that γ-MSH down-regulated the mRNA expression of key modulators of inflammation in the liver and spleen (Fig. 4A and B). Particularly, melanocortin MC_3 receptor activation with γ-MSH reduced *TNF-α* and transforming growth factor beta (*TGF-β*) expression in both tissues and additionally, *CCL5* expression in the spleen. γ-MSH treatment also

Table 1
Body and tissue weights and plasma lipid profile in vehicle- and γ-MSH-treated *ApoE*$^{-/-}$ mice.

	Vehicle	γ-MSH	*P*-value
	(n = 11)	(n = 10)	
Body weight (g) before treatment	27.5 ± 0.6	28.5 ± 0.4	0.19
Body weight (g) after treatment	28.5 ± 0.6	29.6 ± 0.6	0.20
Change (g) in body weight	0.96 ± 0.35	0.12 ± 0.36	0.75
Food intake (g/day)	2.43 ± 0.14	2.53 ± 0.11	0.63
Liver (g)	1.35 ± 0.08	1.27 ± 0.08	0.47
Spleen (g)	0.11 ± 0.01	0.13 ± 0.02	0.46
Gonadal WAT (g)	0.57 ± 0.04	0.65 ± 0.05	0.19
Retroperitoneal WAT (g)	0.38 ± 0.03	0.44 ± 0.05	0.35
Plasma total cholesterol (mM)	13.1 ± 0.8	14.4 ± 0.8	0.29
Plasma HDL cholesterol (mM)	0.44 ± 0.07	0.47 ± 0.07	0.77
Plasma LDL/VLDL cholesterol (mM)	12.7 ± 0.8	13.9 0.8	0.28
Plasma triglycerides (mM)	1.23 ± 0.10	1.36 ± 0.11	0.42

Value represented as mean ± S.E.M. γ-MSH indicates [D-Trp8]-γ-MSH; WAT, white adipose tissue.

down-regulated consistently different adhesion molecules in the liver and spleen of *ApoE*$^{-/-}$ mice (Fig. 4C and D). Collectively, pharmacological targeting of melanocortin MC_3 receptor in atherosclerotic *ApoE*$^{-/-}$ mice reduced signs of systemic and local inflammation, but the effects, as judged by qPCR analyses, were less profound in the aorta compared to the liver and spleen.

3.4. Targeting of melanocortin MC_3 receptor does not reduce plaque size or modulate plaque composition in atherosclerotic Apoe$^{-/-}$ mice

Despite reduced inflammation, γ-MSH treatment did not mitigate the development of atherosclerosis. Plaque size in the aortic root sections was comparable between the treatment groups (Fig. 5A and B). Characterization of plaque composition did not either reveal any therapeutic effects for γ-MSH (Fig. 5). More specifically, quantification of acellular areas in the aortic root plaques to estimate necrotic core size showed no treatment effect (Fig. 5C). Furthermore, plaque collagen content or α-SMA-positive area of aortic root plaques was unchanged (Fig. 5D and E), which is in line with results from the qPCR analysis of aortic samples. Finally, analysis of plaque macrophage content did not either reveal any change in favor of γ-MSH treatment (Fig. 5F).

4. Discussion

The melanocortin system has emerged as an interesting target with therapeutic potential in various diseases. In recent years, melanocortin peptides have been proven to be effective, for example, in the management of obesity, porphyria and sexual dysfunction (Falls and Zhang,

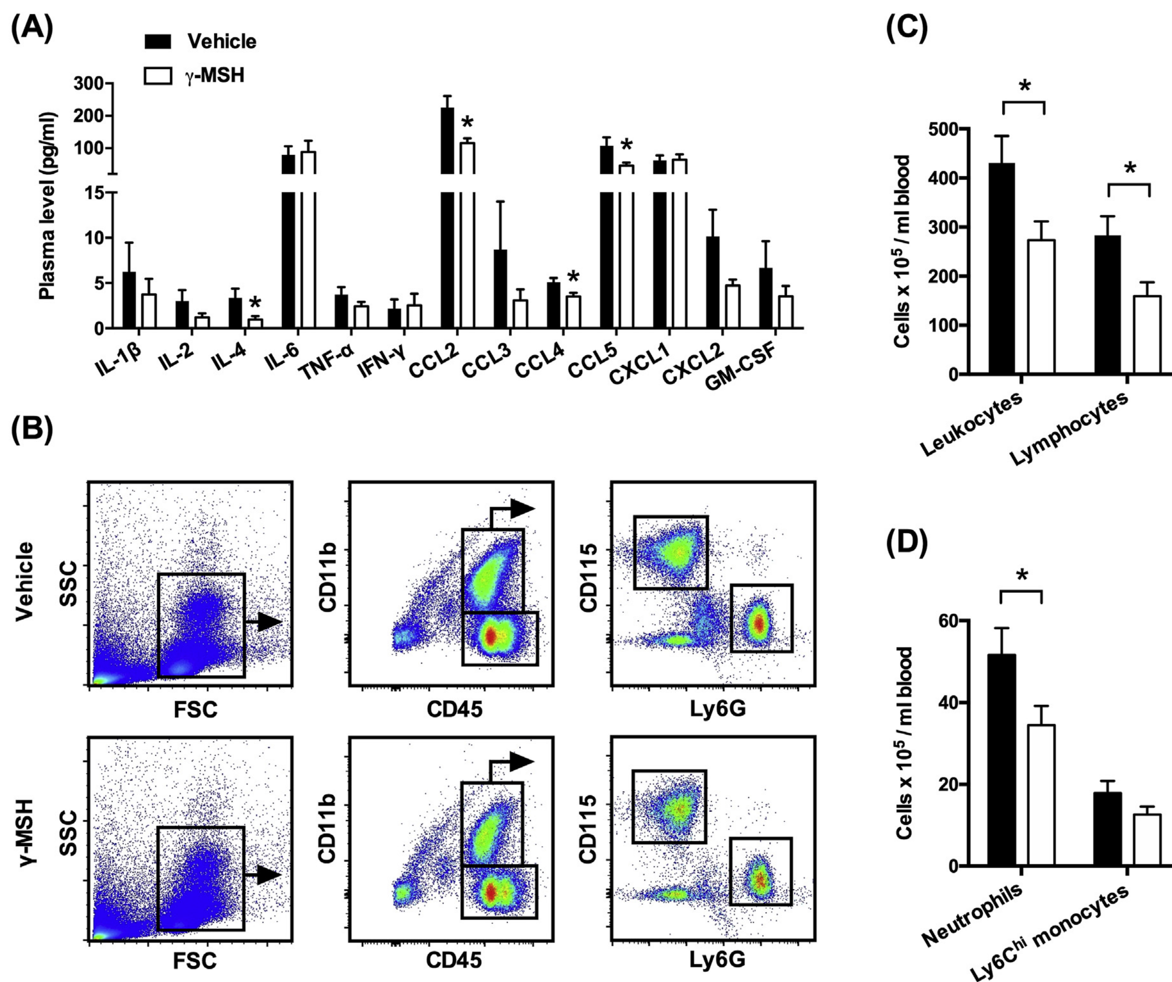

Fig. 2. γ-MSH treatment reduced plasma cytokine levels and blood leukocyte counts. (A) Effect of γ-MSH on the pro-inflammatory cytokine levels in the plasma of *ApoE*$^{-/-}$ mice. (B) Representative dot plots for the gating of blood total leukocytes ($CD45^+$), lymphocytes ($CD45^+$, $CD11b^-$), neutrophils ($CD45^+$, $CD11b^+$, $Ly6G^+$) and monocytes ($CD45^+$, $CD11b^+$, $CD115^+$) in vehicle and γ-MSH-treated mice. (C and D) Quantification of total leukocyte, lymphocyte, neutrophil and $Ly6C^{high}$ monocyte ($CD45^+$, $CD11b^+$, $CD115^+$, $Ly6C^{high}$) counts (cells per ml of blood) in the blood of *ApoE*$^{-/-}$ mice. Data are mean ± S.E.M., n = 11 (vehicle) and 10 (γ-MSH). *P < 0.05 Vs vehicle. SSC-A indicates side scatter area; FSC-A, forward scatter area.

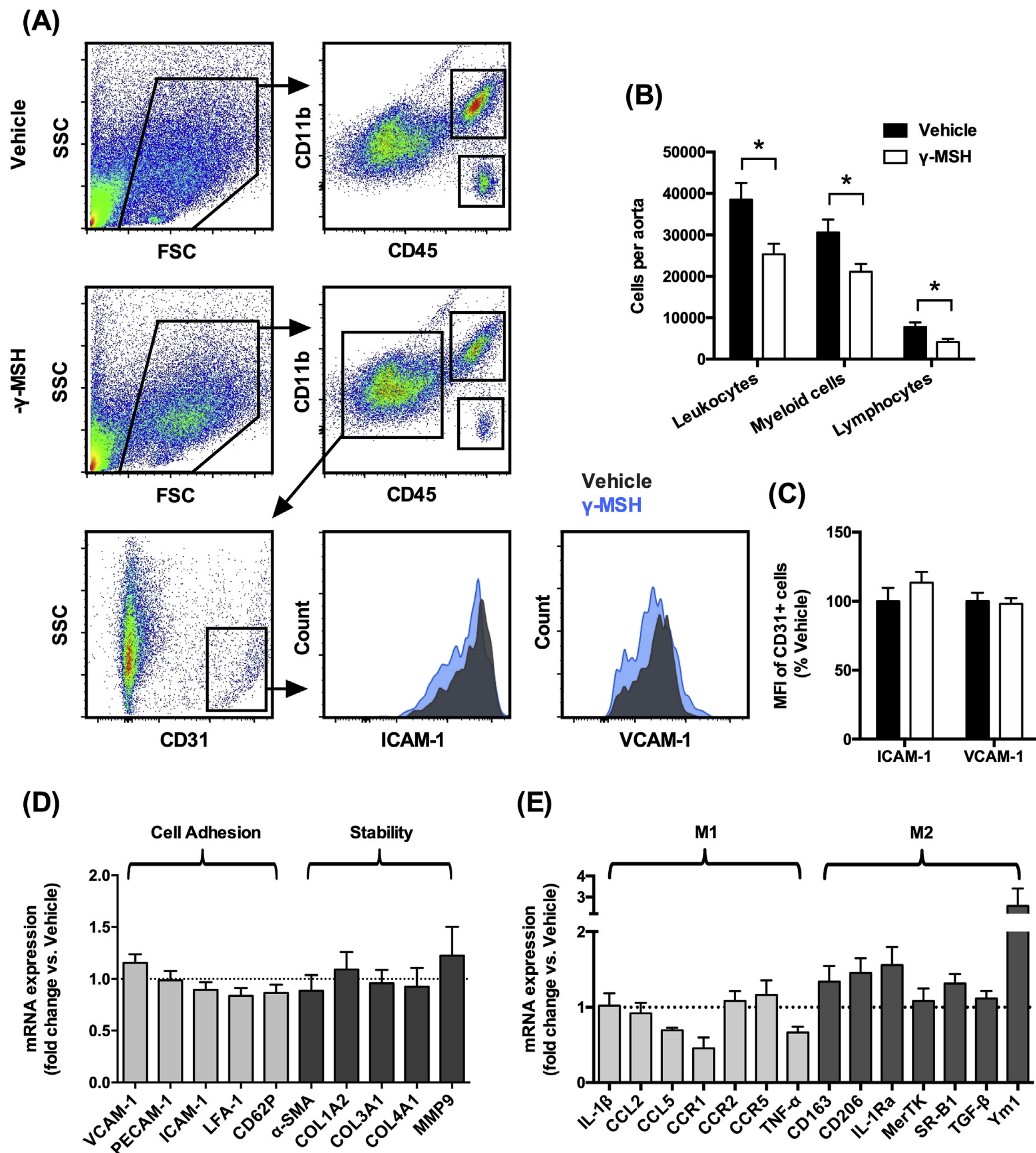

Fig. 3. Reduced leukocyte counts in the aorta of γ-MSH-treated mice. Representative flow cytometry results for the gating (A) and quantification of (B) total leukocytes ($CD45^+$), myeloid cells ($CD45^+$, $CD11b^+$) and lymphocytes ($CD45^+$, $CD11b^-$) in aortic lysates (descending aorta) of vehicle- and γ-MSH-treated mice. Gating of aortic endothelial cells ($CD45^-$, $CD31^+$) and histograms for VCAM-1 and ICAM-1 staining are also presented. (C) Mean fluorescence intensity (MFI, expressed as percentage of vehicle) of VCAM-1 and ICAM-1 in the gated $CD31^+$ cells in the aorta. (D and E) Quantitative real-time PCR (qPCR) analysis of *cell adhesion molecules, plaque stability markers* and M1-and M2-type macrophage markers in the aorta of vehicle- and γ-MSH treated mice. Data are mean ± S.E.M., n = 11 (vehicle) and 10 (γ-MSH). *$P < 0.05$ Vs vehicle. SSC-A indicates side scatter area; FSC-A, forward scatter area.

2019; Langendonk et al., 2015; Clayton et al., 2016). As we and others have reported, melanocortins and their receptors mediate anti-inflammatory actions in multiple organs including the aorta (Rinne et al., 2018b, 2018c; Nuutinen et al., 2018). In this study, we demonstrate therapeutic effects of targeting melanocortin MC_3 receptor with the selective agonist γ-MSH in atherosclerotic $ApoE^{-/-}$ mouse model. We present 3 major findings from this study: (1) melanocortin MC_3 receptor is expressed in the atherosclerotic plaques of $ApoE^{-/-}$ mice; (2) the activation of melanocortin MC_3 receptor subtype with γ-MSH suppressed systemic inflammation and reduced leukocyte counts in the aorta and blood; and (3) down-regulated the expression of pro-inflammatory cytokines in the spleen and liver of $ApoE^{-/-}$ mice. However, γ-MSH treatment did not modify plaque size or composition.

Melanocortins modulate the immune system and elicit anti-inflammatory actions in multiple cell types including leukocytes and endothelial cells (Catania et al., 2010, 2004). Endogenous γ-MSH is a post-translational product of proopiomelanocortin with relatively high affinity for melanocortin MC_3 receptor. It is well-established that proopiomelanocortin is not only transcribed in the central nervous system but also in peripheral cells such as leukocytes, where it is also processed into biologically active melanocortin peptides including adrenocorticotropic hormone and α-melanocyte-stimulating hormone

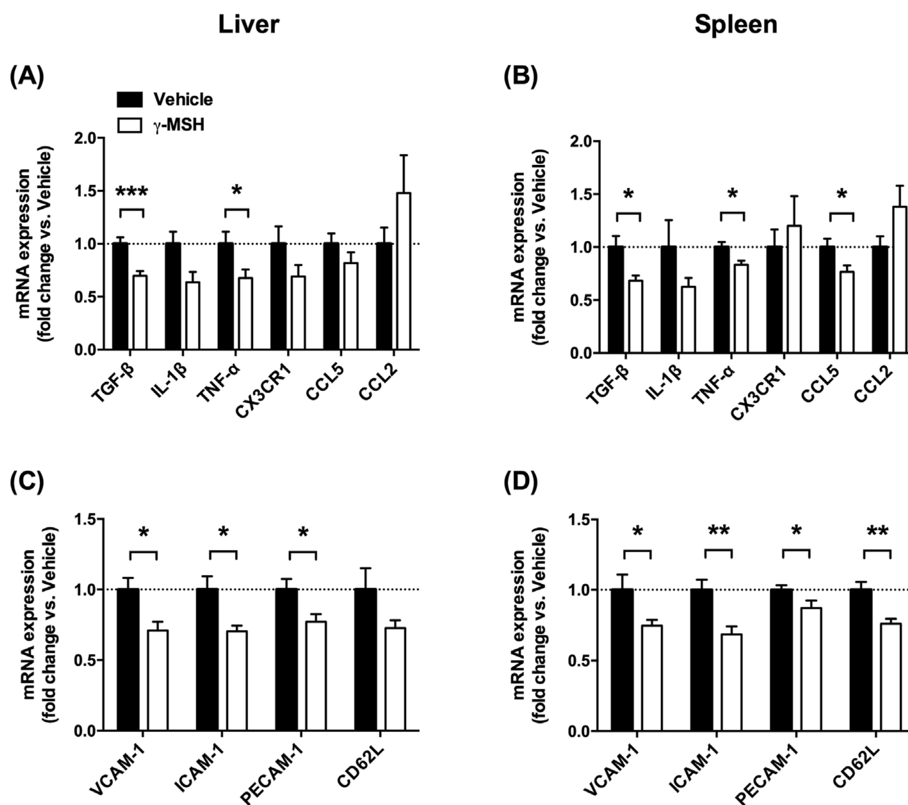

Fig. 4. γ-MSH treatment down-regulated the hepatic and splenic expression of pro-inflammatory cytokines and adhesion molecules. (A and B) qPCR analysis of inflammatory cytokine (*IL-1β*, *CCL2*, *CCL5*, *TNF-α*, *TGF-β* and *CX3CR1*) transcript expression in the spleen and liver of $ApoE^{-/-}$ mice. (C and D) Expression of cell adhesion molecules (*VCAM-1*, *PECAM-1*, *ICAM-1* and *CD62-L*) in the spleen and liver of $ApoE^{-/-}$ mice. Data are mean ± S.E.M., n = 11 (vehicle) and 10 (γ-MSH). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ Vs vehicle.

(Blalock, 1999). However, it is unclear whether leukocytes also produce and secrete γ-MSH. Importantly, it is widely reported that melanocortin MC_3 receptor subtype expression occurs in various leukocyte subpopulations, allowing γ-MSH to activate this receptor *via* endocrine signaling. In humans, melanocortin MC_3 receptor mRNA is expressed in peripheral blood leukocytes with the highest transcript levels occurring in $CD4^+$ T helper cells and lower levels in monocytes, NK cells and granulocytes (Andersen et al., 2005; Cooper et al., 2005). In mice, it is uncertain how widely melanocortin MC_3 receptor is present in different immune cells but macrophages, at least, express functional melanocortin MC_3 receptor. Activation of melanocortin MC_3 receptor in peritoneal macrophages by [D-Trp8]-γ-MSH inhibited the release of pro-inflammatory cytokines, an effect that was completely absent in macrophages derived from melanocortin MC_3 receptor deficient mice (Getting et al., 2006a, 1999b; 1999a; Getting and Perretti, 2001). Immunohistochemical analyses have also revealed melanocortin MC_3 receptor expression in neutrophils and mononuclear cells in mice (Leoni et al., 2008), but the physiological role of melanocortin MC_3 receptor in these cells remains unclear. Supporting the presence of melanocortin MC_3 receptor in murine macrophages, we found melanocortin MC_3 receptor expression on atherosclerotic plaque macrophages in $ApoE^{-/-}$ mice. Given the dominant role of macrophages as effector cells in the progression of atherosclerosis, we postulated that selectively activating melanocortin MC_3 receptor in plaque macrophages could suppress arterial inflammation and thereby also limit atherosclerosis.

In the present study, $ApoE^{-/-}$ mice were first fed an HFD for 8 weeks and then randomized to the treatment arms. The study was thus designed to test the effects of [D-Trp8]-γ-MSH in established atherosclerosis, which reflects better the clinical situation and provides more translational value compared to a preventive intervention that would have been started before prominent plaque formation. With this treatment strategy, we observed that the activation of melanocortin MC_3 receptor dampened systemic inflammation and favorably modulated leukocyte counts in the blood and aorta. Specifically, γ-MSH agonist treatment reduced the circulating levels of the pro-inflammatory chemokines CCL2, CCL4 and CCL5, which attract monocytes and other leukocytes to inflamed atherosclerotic plaques during the pathogenesis of the disease (Tacke et al., 2007). For example, CCL2 is highly atherogenic due to its ability to recruit monocytes to the atherosclerotic plaque via its cognate receptor CCR2. Genetic deletion of *CCL2* or *CCR2* protects against atherosclerosis in mice (Boring et al., 1998; Dawson et al., 1999), while elevated CCL2 levels are associated with a higher risk of atherosclerosis and myocardial infarction in humans (McDermott et al., 2005). In good agreement with the observed reductions in the atherogenic chemokine levels, γ-MSH-treated mice displayed lower leukocyte counts in the blood and aorta. On the other hand, the expression of adhesion molecules such as VCAM-1 and ICAM-1, which mediate leukocyte adhesion to the vascular endothelium, was unaltered in aortic endothelial cells of γ-MSH-treated mice. Unexpectedly, we found that γ-MSH treatment upregulated *CCL5* in cultured endothelial cells. It is known that CCL5, by binding to its target receptors CCR1 and CCR5, regulates $Ly6C^{high}$ monocyte recruitment to atherosclerotic lesions particularly in advanced stages of the disease (Zernecke et al., 2008). Thus, γ-MSH-mediated induction of *CCL5* expression in aortic endothelial cells might counterbalance the anti-inflammatory effects in other cell types such as macrophages. Aside from findings in the aorta and cultured endothelial cells, γ-MSH intervention significantly down-regulated adhesion molecule expression in the liver and spleen of $ApoE^{-/-}$ mice, suggesting tissue-specific effects for γ-MSH and melanocortin MC_3 receptor in this regard.

Collectively, the present findings of [D-Trp8]-γ-MSH-induced anti-inflammatory effects in atherosclerotic $ApoE^{-/-}$ mice are well in line with previous studies using the same compound in different disease models. In crystal-induced peritonitis, [D-Trp8]-γ-MSH inhibited the accumulation of neutrophils and reduced pro-inflammatory cytkine levels in the peritoneal lavage fluid (Getting et al., 2006a; Leoni et al., 2008). These anti-inflammatory effects were completely absent in melanocortin MC_3 receptor knockout mice and were also reversible by the administration of a melanocortin MC_3 receptor antagonist. Additionally, the anti-inflammatory efficacy of [D-Trp8]-γ-MSH was fully

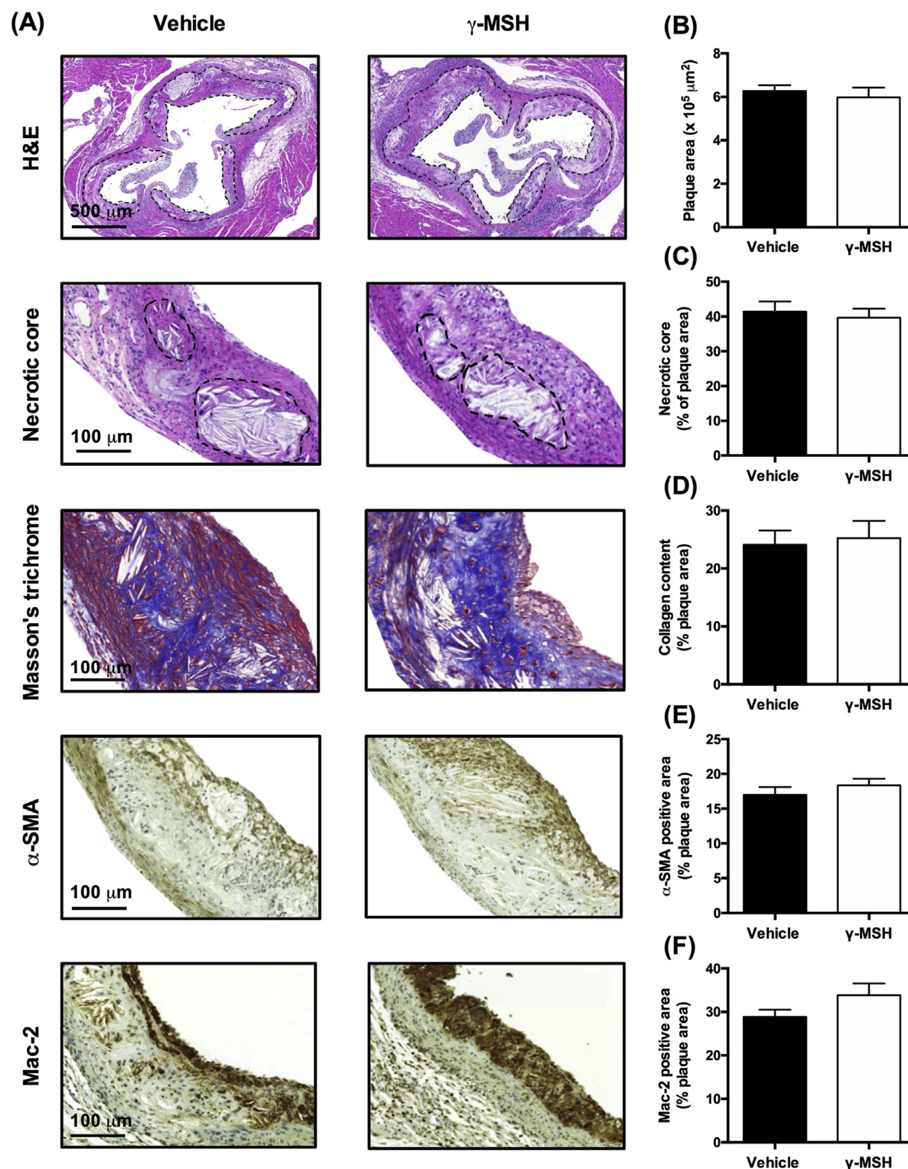

Fig. 5. Plaque size and composition in γ-MSH-treated $ApoE^{-/-}$ mice. (A) Representative images of hematoxylin and eosin (H&E) and Masson's trichrome -stained aortic root sections for the analysis of plaque size, necrotic core size and plaque collagen content. Representative images of immunohistochemistry staining for α-SMA and Mac-2 are also included in the panel. (B–F) Quantification of plaque size and necrotic core area as well as plaque collagen, smooth muscle cell (α-SMA) and macrophage (Mac-2) content (expressed as percentage of total plaque area) in the aortic root sections of vehicle- and γ-MSH-treated $ApoE^{-/-}$ mice. Data are mean ± S.E.M., n = 11 (vehicle) and 10 (γ-MSH).

preserved in mice lacking functional melanocortin MC_1 receptor (Getting et al., 2006b), indicating that the compound is specific for melanocortin MC_3 receptor and is not considerably activating melanocortin MC_1 receptor. Anti-inflammatory effects of [D-Trp8]-γ-MSH have been also reported models of microvascular inflammation and cerebral injury induced by ischemia-reperfusion (Leoni et al., 2008; Holloway et al., 2015). Most of these studies have used a fixed dose of [D-Trp8]-γ-MSH (10 μg per mouse) and the first *in vivo* study also indicated dose-responsiveness that was peaking around this dose level (Getting et al., 2006a). These studies guided our dose selection for the intervention in $ApoE^{-/-}$ mice. We, however, chose to titrate the dose level to 15 μg per mouse, since mice are considerably heavier after several weeks on HFD. Applying this dose regimen in HFD-fed $ApoE^{-/-}$ mice consolidated earlier findings that [D-Trp8]-γ-MSH is effective in reducing systemic and local inflammation.

Despite showing anti-inflammatory efficacy, [D-Trp8]-γ-MSH treatment did not significantly impact plaque size or composition in $ApoE^{-/-}$ mice. A comparable observation was, however, made in our earlier study in atherosclerotic LDL receptor knockout ($Ldlr^{-/-}$) mice, which were treated with the nonselective melanocortin agonist melanotan II for 4 weeks (Rinne et al., 2014). Melanotan II treatment did not reduce plaque size or lesional macrophage accumulation but it attenuated plaque inflammation and promoted M2-type polarization of plaque macrophages. In the present study, [D-Trp8]-γ-MSH tended to up-regulate M2-type macrophage markers and to down-regulate some of the pro-inflammatory M1-type markers such as *CCL5* and *TNF-α* in the aorta of $ApoE^{-/-}$ mice. Similar responses were also observed in primary macrophages that were treated with γ-MSH *in vitro*. In contrast to these pharmacological approaches, transgenic overexpression of α- and γ- MSH in $Ldlr^{-/-}$ mice was shown to limit atherosclerotic plaque formation (Nuutinen et al., 2018). These findings raise the question of whether the selected dosage regimen was suboptimal in restricting atherosclerotic plaque development. Increased dosing frequency (*e.g.* twice daily) or longer treatment duration (> 4 weeks) might more effectively halt plaque progression. On the other hand, feeding $ApoE^{-/-}$ mice HFD for 12 weeks is a robust atherosclerosis model that challenges the investigational new drug in the best possible way. Other studies, testing anti-inflammatory compounds, have demonstrated reduced atherosclerosis after preventive treatment but when starting the intervention in established disease, plaque development was unaffected (Rinne et al., 2018a; Riopel et al., 2019). Importantly, the finding of increased CCL5 expression in γ-MSH-treated endothelial cells might, at least in part, explain the lack of anti-atherosclerotic effect in the present study. Taken together, [D-Trp8]-γ-MSH evoked clear-cut anti-

inflammatory effects in atherosclerotic $ApoE^{-/-}$ mice, which did not translate into therapeutic benefits in terms of plaque development. Selective targeting of melanocortin MC_3 receptor appears to suppress inflammation in a comparable way to nonselective melanocortin activation but this kind of treatment approach needs to be refined in the setting of atherosclerosis, *e.g.* by intensifying the dosing or by finding ways to more selectively target melanocortin MC_3 receptor in macrophages.

In conclusion, the present results demonstrate that the anti-inflammatory effects of melanocortin MC_3 receptor activation occur also in a chronic inflammatory disease such as atherosclerosis. Pharmacological activation of melanocortin MC_3 receptor activation with [D-Trp8]-γ-MSH exerted a systemic and local anti-inflammatory effect in $ApoE^{-/-}$ mice, which resulted in reduced leukocyte counts in the blood and aorta but did not change lesion size or composition. Future studies will be therefore instrumental to clarify whether melanocortin MC_3 receptor evoked anti-inflammatory effects could protect against atherosclerosis.

CRediT authorship contribution statement

James J. Kadiri: Investigation, Formal analysis, Data curation, Writing - original draft. **Keshav Thapa:** Investigation. **Katja Kaipio:** Investigation. **Minying Cai:** Resources, Writing - review & editing. **Victor J. Hruby:** Resources, Writing - review & editing. **Petteri Rinne:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

None.

Acknowledgments

We thank Sanna Bastman, Hanna Haukkala and Elina Kahra for their excellent technical support. Erica Nyman and Marja-Riitta Kajaala (Histology unit, Turku Center for Disease Modelling) are acknowledged for processing histology samples.

This work was financially supported by the Drug Research Doctoral Programme (University of Turku), The Finnish Foundation for Cardiovascular Research, Turku University Foundation, the Academy of Finland (grant numbers: 274852 and 320136) and the National Institutes of Health (grant number: NIH GM108040).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173186.

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