Cannabinoid Receptor 2 Signaling Does Not Modulate Atherogenesis in Mice

Florian Willecke1, Katharina Zeschky1*, Alexandra Ortiz Rodriguez1, Christian Colberg1, Volker Auwa¨rter2, Stefan Kneisel2, Melanie Hutter2, Andrey Lozhkin1, Natalie Hoppe1, Dennis Wolf1, Constantin von zur Mühlen1, Martin Moser1, Ingo Hilgendorf1, Christoph Bode1, Andreas Zirlik1*

1 Department of Cardiology, University of Freiburg, Freiburg, Germany, 2 Forensic Toxicology, Institute of Forensic Medicine, University Medical Center Freiburg, Freiburg, Germany

Abstract

Background: Strong evidence supports a protective role of the cannabinoid receptor 2 (CB2) in inflammation and atherosclerosis. However, direct proof of its involvement in lesion formation is lacking. Therefore, the present study aimed to characterize the role of the CB2 receptor in Murine atherogenesis.

Methods and Findings: Low density lipoprotein receptor-deficient (LDLR−/−) mice subjected to intraperitoneal injections of the selective CB2 receptor agonist JWH-133 or vehicle three times per week consumed high cholesterol diet (HCD) for 16 weeks. Surprisingly, intimal lesion size did not differ between both groups in sections of the aortic roots and arches, suggesting that CB2 activation does not modulate atherogenesis in vivo. Plaque content of lipids, macrophages, smooth muscle cells, T cells, and collagen were also similar between both groups. Moreover, CB2−/−/LDLR−/− mice developed lesions of similar size containing more macrophages and lipids but similar amounts of smooth muscle cells and collagen fibers compared with CB2+/+LDLR−/− controls. While JWH-133 treatment reduced intraperitoneal macrophage accumulation in thioglycollate-illicited peritonitis, neither genetic deficiency nor pharmacologic activation of the CB2 receptor altered inflammatory cytokine expression in vivo or inflammatory cell adhesion in the flow chamber in vitro.

Conclusion: Our study demonstrates that both activation and deletion of the CB2 receptor do not relevantly modulate atherogenesis in mice. Our data do not challenge the multiple reports involving CB2 in other inflammatory processes. However, in the context of atherosclerosis, CB2 does not appear to be a suitable therapeutic target for reduction of the atherosclerotic plaque.

Introduction

Atherosclerosis is a chronic inflammatory disease and represents the primary cause of heart disease and stroke worldwide [1]. While the inflammatory nature of atherosclerosis has been uncovered for sometime already, genuine anti-inflammatory treatment options are still lacking. Drugs with pleiotropic anti-inflammatory properties, such as statins, are cornerstones of current state-of-the-art therapy, while great efforts are made to find new agents primarily designed to abate the inflammatory and immunologic mechanisms promoting atherosclerosis and its complications. A growing body of evidence suggests that the cannabinoid system plays a critical role in the pathogenesis of inflammation and recent reports also implicated it with the pathobiology of atherosclerosis [2,3,4]. The endocannabinoid system comprises two membrane receptors, CB1 and CB2, their endogenous ligands, such as anandamide (arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), and several enzymes required for their biosynthesis and inactivation [5,6]. The receptor CB1 is primarily expressed in the central nervous systems (CNS), but also in peripheral tissues and on immune cells [7]. Selective blockade of the CB1 receptor inhibits atherogenesis in LDL receptor (LDLR)-deficient mice [8].

The CB2 receptor is predominantly expressed in immune and hematopoetic cells but also in adipose tissue [9], brain [10], myocardium [11], and endothelial cells [12]. Numerous studies have demonstrated anti-inflammatory effects of CB2 receptor activation in different diseases and pathological conditions, including cerebral injury [13,14], inflammatory pain [15], and myocardial injury [16]. Most notably, CB2 receptor activation has also been suggested to modulate atherosclerosis [17]. In this latter study, Steffens and colleagues showed that oral administration of low doses of Δ9-tetrahydrocannabinol (THC, 1 mg/kg per day) significantly reduced plaque progression in apolipoprotein E (ApoE) knockout mice. They also observed CB2 receptor-expressing immune cells in Murine and human atherosclerotic
plaques and reduced macrophage content in atherosclerotic lesions of THC-treated mice. Since these effects were reversed by a selective CB2 but not CB1 receptor antagonist, the authors hypothesized the involvement of CB2 receptors on immune cells in atherogenesis [17]. Another recent study also showed amelioration of atherosclerosis in ApoE-deficient mice after treatment with a CB2/CB1 receptor agonist and the authors postulated a CB2 receptor-dependent effect [3]. However, up to now no in vivo study evaluated the direct contribution of CB2 receptor signaling in the context of atherosclerosis. There is a need for such studies to ultimately evaluate whether CB2-targeted therapies may be suitable to fight atherosclerosis. Therefore, the aim of this study was to investigate the influence of the CB2 receptor on atherogenesis in low density lipoprotein receptor (LDLR)-deficient mice.

**Methods**

**In vivo study**

CB2-deficient mice and LDLR-deficient mice, both on a pure C57/BL6 background, were obtained from Jackson Laboratories. Mice were crossbred to generate CB2+/-/LDLR+/- mice. Genotyping of each mouse used polymerase chain reaction and employed the following primers: LDLR, 5'-CCA TAT gGA TCC CCA gTC TT-3' (common primer), 5'-gCC ATg gAT ACA CTC ACT gC-3' (wild-type primer) and 5'-AAAT CCA TCT TGt gTA ATg gCC gAT C-3' (mutant primer); CB2, 5'-gAC Tag AgC TTT gTA ggT Agg C-3' (common primer), 5'- ggA gTT CAA CCC CAT gAA gga gTA-3' (wild-type primer) and 5'- gAT gAA TCC gTC gTG TAA gTC T-3' (mutant primer). Six-week-old male CB2+/-/LDLR+/- and CB2-/-/LDLR-/- mice consumed a high-cholesterol diet (HCD) for 16 weeks (Sniff modified after Research Diets D12108 containing 21% total fat and 1.235% cholesterol, Soest, Germany). In parallel, JWH-133 (diluted in a water-soluble solution with Tocrisolve, from Tocris, Madison, WI) was administered intraperitoneally to mice of both genotypes at a concentration of 5 mg/kg body weight three times a week for 16 weeks. Subsequently, mice were euthanized, hearts and aortas were removed, and tissue was prepared and analyzed histologically as described previously [18,19,20]. Total cholesterol and triglyceride concentrations of JWH-133 or vehicle, followed by TNFα (20 ng/ml) for 24 hours. After incubation, murine endothelial cells were lysed and used for Western Blotting. Apoptosis and cytotoxicity was evaluated by Apo-ONE® and CytoTox-One™ Assay according to the instructions of the manufacturer (Promega, Madison, WI).

**Enzyme-linked immuno-absorbent assay (ELISA)**

Mouse MCP-1 was quantified in the supernatants of cell cultures using commercially available ELISA Kits (R&D DuoSet, Minneapolis, MN) according to the manufacturer’s instructions.

**Western blotting**

Murine endothelial cells were stimulated with TNFα (20 ng/ml) for 24 hours. After incubation, murine endothelial cells were lysed, separated by SDS-PAGE under reducing conditions, and blotted to polyvinylidene difluoride membranes as described previously [21]. An anti-mouse ICAM-1 antibody (Santa Cruz, Santa Cruz, CA) was used as primary antibody, followed by an anti-peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson Laboratories, West Grove, PA) as secondary antibody.

**Cytokine challenge and cytometric bead assay**

To induce inflammation, mice were subjected to intraperitoneal injection of TNFα (200 ng/ml) as indicated. Blood was collected by cardiac puncture and serum separation. For analysis of inflammatory markers in mice the cytometric bead assay for Murine inflammation detecting IL-6, MCP-1, IFNγ, IL-10, and IL-12p70 was used according to manufacturer’s instructions (BD Biosciences, San Diego, CA) optimized for higher sensitivity. Results were analyzed using the corresponding FCAP software (BD Franklin Lakes, NJ). The lower detection limits were in the range of 5–10 pg/ml.

**Dynamic adhesion assays**

Dynamic adhesion assays in the flow chamber were performed as described previously [19]. Murine endothelial cells were grown in 35 mm dishes (Costar, Bethesda, MD) and were subjected to the flow chamber. In brief, the Glycotech flow chamber (Gaithersburg, MD) was assembled with the dish as the bottom of the resulting parallel flow chamber. The chamber and tubes were filled with PBS without serum prior to the experiment. Subsequently, Murine leukocytes were applied with a syringe pump (Harvard apparatus PHD2000, Holliston, MA) with flow rates of 0.04 dyne/cm² (venous flow; a total of 10 min). Adherent cells were quantified under the microscope.

**Flow cytometry**

Flow cytometry was performed as previously described [22]. Cells were pre-incubated with mouse Fc-Block (αCD16/32, e Bioscience, San Diego, CA). Antibodies included CD11b-FITC, CD115-PE, Ly6C/G (Gr1)-APC, CD4-Alexa488, CD8-PE, CD3-APC, CD20-PE, ICAM-1-FITC (all from e Bioscience, San Diego, CA), ICAM-2-FITC, and PECAM-1-PE (PharMingen, San Diego, CA). The mean fluorescence indices (MFI) were quantified employing the FlowJo software (Tree Star Inc, Ashland, OR).

**Murine peritonitis**

CB2+/-/LDLR+/- and CB2-/-/LDLR-/- mice were treated intraperitoneally with 4% thiglycollate. After 4 and 72 hours,
mice were euthanized with CO₂, the peritoneal cavity was flushed with 6 ml of RPMI for 3 min. Leukocytes were quantified in a CASY counter. Similarly, wild-type (B6) mice received JWH-133 one hour before injection of 4% thioglycollate. The resulting leukocyte migration was measured after 4 and 72 hours.

Mass spectrometry - pharmacokinetics of JWH-133

Mice were subjected to i.p. injection of JWH-133 (5 mg/kg body weight) on day 1, 3, 6, 8 and 10. On day 10 retro-orbital blood was taken at 2, 12, 24, and 48 hours after the last injection of JWH-133. Mouse sera (100 μl, N = 6) were added to 3 μl internal standard (d₃-THC; 5 ng/ml in ethanol), followed by 2.9 ml acetic acid 0.1 M, followed by automated solid phase extraction using Aspec GX-274 (Gilson, Middleton, USA), reversed phase C₁₈ SPE-cartouche (type chromabond), and a 500 μg column bed (Macherey-Nagel, Düren, Germany). Samples were eluted with 1 ml acetonitrile, evaporated in a stream of nitrogen at 40°C, and incorporated in 25 μl ethyl acetate.

Frozen aortic tissue was incubated in 500 μl ethanol in an ultrasound bath twice for 15 min. 25 ng internal standard was added (d₃-THC), samples were evaporated in a stream of nitrogen at 40°C, and reincubated with 25 μl ethyl acetate.

Extraction using Aspec GX-274 (Gilson, Middleton, USA), reversed phase C₁₈ SPE-cartouche (type chromabond), and a 500 μg column bed (Macherey-Nagel, Düren, Germany). Samples were eluted with 1 ml acetonitrile, evaporated in a stream of nitrogen at 40°C, and incorporated in 25 μl ethyl acetate.

Perfusion system (temperature gradient 100°C for 1 min, increase to 290°C for 2.5 min, increase to 310°C for 4 min, fragmentation energy 70 eV). The following fragments were chosen. For JWH-133: m/z 302 (quantifier), m/z 317 and m/z 269 (quantifier), m/z 312 and m/z 229 (qualifier), retention time 70 eV. For d₃-THC: m/z 302 (quantifier), m/z 317 and m/z 254 (qualifier), retention time 7.4 min. The detection limit was 25 ng/ml.

Quantitative real-time PCR. Aortas from CB₂⁻/⁻ and CB₂⁺/⁻ mice consuming HCD for 16 weeks were harvested and stored in RNalater (Qiagen, Venlo, Netherlands) at −80°C. Total RNA was extracted using TRIzol Reagent (Invitrogen, San Diego, CA) and glycosyn as a co-precipitator (Roche, Basel, Switzerland). Homogenization was performed using a rotor-stator dispergator (IKA®, Staufen, Germany). 1 μg of total RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Subsequent quantitative real-time PCR was performed with a LightCycler 480 System using the LightCycler 480 SYBR Green I Master (Roche) detection format. mGAP-DH was performed with a LightCycler 480 System using the LightCycler 480 SYBR Green I Master (Roche) detection format. mGAP-DH served as endogenous control. Primer sequences: mCNR1: 5'-TCC TTT TAT GAG AGA GCC AGG C-3' (forward), 5'-GCC AGG CTC AAC GTG ACT GAG A-3' (reverse); mGAP-DH: 5'-TGC ACC ACC AAC TGC TTA G-3' (forward), 5'-GAT GCA GGG ATG ATG TTC-3' (reverse).

Statistical analysis

Data are expressed as means ± SEM of absolute or normalized values. Groups were compared employing the Student’s t-test. A value of P<0.05 was considered significant. Data sets were analysed using GraphPad Prism® (GraphPad Software Inc, La Jolla, CA).

Results

Treatment with the selective CB₂ receptor agonist JWH-133 does not attenuate atherogenesis in mice

To explore the contribution of direct CB₂ receptor stimulation LDLR⁻/⁻ mice consuming a high-cholesterol diet (HCD) for 16 weeks were treated with intraperitoneal injections of the selective CB₂ receptor agonist JWH-133 or vehicle three times a week. JWH-133 was detectable in mouse serum after 2, 12, and 24 hours by mass spectrometry, proving bioavailability in vivo (Fig. 1). More importantly, JWH-133 could also be detected directly in aortic tissue of treated animals 48 hours after administration at a level of 2.2±0.67 ng/mg while it was undetectable in vehicle-treated animals. Weights, cholesterol levels, and total leukocyte numbers did not differ between the study groups at baseline and end of feeding. Both groups also showed no difference in visceral fat mass, blood pressure, heart rate, and leukocyte subtypes as quantified at the end of the study (Table 1). Surprisingly, intimal lesion size in aortic roots was similar between JWH-133-treated mice and those receiving vehicle control (0.316±0.030 mm², N = 10 vs. 0.312±0.044 mm², N = 8, P = 0.94; Fig. 2A). Similar results were obtained in aortic arches (0.091±0.024 mm², N = 11 vs. 0.066±0.013 mm², N = 8, P = 0.41; Fig. 2B). Also, lipid deposition in en face analysis of abdominal aortas did not differ between both groups (Fig. 2C), demonstrating that CB₂ receptor stimulation does not attenuate atherogenesis in mice. Similarly, JWH-133 treatment did not modulate the content of lipids, macrophages, collagen, T cells, smooth muscle cells, and the cellular apoptosis rates in atherosclerotic plaques (Fig. 2D).

CB₂ receptor deficiency does not affect the development of atherosclerotic lesions in mice

Consistent with our results for selective CB₂ receptor stimulation, CB₂⁻/⁻/LDLR⁻/⁻ mice consuming HCD for 16 weeks developed lesions of similar size as respective CB₂⁺/⁻/LDLR⁻/⁻ control animals in aortic roots (0.261±0.038 mm², N = 12 vs. 0.223±0.023 mm², N = 13, P = 0.40, Figure 3A), aortic arches (0.095±0.022 mm², N = 12 vs. 0.075±0.022 mm², N = 13, P = 0.54, Figure 3B), and abdominal aortas (Fig. 3C). Again, there was no change in the degree of apoptosis, the content of collagen, T cells, and smooth muscle cells within the atherosclerotic plaque. However, we could detect increased lipid and macrophage content (Fig. 3D). CB₁ expression quantified by RT-PCR did not differ between both groups rendering a CB₁-driven bias unlikely (0.0013±0.0002 vs. 0.0018±0.0004, P = 0.34, N = 5).
Figure 2. Treatment with the CB2 agonist JWH-133 does not modulate atherosclerosis in mice. A and B, LDLR\(^{-/-}\) mice consuming high cholesterol diet for 16 weeks (HCD) received intraperitoneal injections of 5 mg/kg JWH-133 (N = 10) or vehicle control (Tocris, N = 8) three times a week. Intimal lesion area in the aortic root (A) and arch (B) are displayed as pooled data \(\pm\) SEM; representative images stained for lipid deposition (Oil-red-O) are shown below the corresponding graph. C, The abdominal aortas of mice treated as described above underwent en face analysis of lipid deposition. Oil-red-O-positive staining in relation to total wall area was quantified and is displayed as pooled data \(\pm\) SEM (N = 8 and 10); representative images are shown below. D, Sections of aortic roots of mice treated as described above were analyzed for lipid-, macrophage-, collagen-, T cell-, smooth muscle cell- and apoptotic cell content. Oil-red-O-, Mac-3-, picosirius red-, CD4-, \(\alpha\)-actin- and TUNEL-positive staining in relation to total wall area is given as mean \(\pm\) SEM (N = 8 and 10).

doi:10.1371/journal.pone.0019405.g002

Table 1. Characteristics of study animals before and after feeding.

|                | Tocris (N = 12) | JWH-133 (N = 17) | p-value | CB2\(^{+/-}\)/LDLR\(^{-/-}\) (N = 17) | CB2\(^{+/-}\)/LDLR\(^{-/-}\) (N = 18) | p-value |
|----------------|-----------------|-------------------|---------|-----------------|-----------------|---------|
| Weight (g)     | BF 20.95 ± 0.91 | 19.17 ± 0.74      | 0.14    | 21.85 ± 0.45    | 22.36 ± 0.42    | 0.42    |
|                | AF 31.28 ± 1.27 | 30.89 ± 0.93      | 0.80    | 33.03 ± 1.07    | 35.57 ± 1.12    | 0.11    |
| Cholesterol (mg/dl) | BF 191.5 ± 11.82 | 201.0 ± 11.82 | 0.59 | 194.7 ± 11.47 | 183.2 ± 7.66 | 0.41 |
|                | AF 907.6 ± 120.9 | 1064.0 ± 195.7   | 0.54    | 777.0 ± 48.55   | 852.0 ± 108.3   | 0.54    |
| Triglycerides (mg/dl) | BF 98.84 ± 11.67 | 146.0 ± 15.78 | 0.04 | 163.9 ± 21.29 | 137.1 ± 9.23 | 0.26 |
|                | AF 255.8 ± 50.95 | 219.2 ± 23.13    | 0.48    | 194.5 ± 21.81   | 202.7 ± 21.36   | 0.79    |
| Visceral fat pads (g) | BF 1.2 ± 0.21 | 1.01 ± 0.16 | 0.68 | 1.31 ± 0.17 | 1.53 ± 0.20 | 0.41 |
| Systolic Blood Pressure (mmHg) | AF 102.8 ± 3.77 | 107.1 ± 6.04 | 0.57 | 98.96 ± 2.49 | 104.2 ± 3.86 | 0.27 |
| Heart rate (bpm) | AF 679.8 ± 17.78 | 634.6 ± 17.01 | 0.08 | 615.2 ± 16.16 | 659.1 ± 18.79 | 0.09 |
| Leukocytes \((\times 1000/\mu l)\) | BF 10.73 ± 0.79 | 10.71 ± 0.85 | 0.98 | 9.77 ± 0.57 | 10.93 ± 0.55 | 0.15 |
|                | AF 4.5 ± 1.02 | 6.49 ± 0.72 | 0.11 | 6.92 ± 0.89 | 6.36 ± 0.55 | 0.59 |
| CD3\(^+\) (% leukocytes) | AF 26.45 ± 3.46 | 21.73 ± 2.71 | 0.29 | 19.47 ± 2.40 | 20.13 ± 1.85 | 0.83 |
| CD4\(^+\) (% leukocytes) | AF 12.55 ± 1.71 | 9.4 ± 0.66 | 0.07 | 8.47 ± 0.87 | 9.75 ± 0.78 | 0.28 |
| CD8\(^+\) (% leukocytes) | AF 9.36 ± 1.05 | 7.53 ± 0.58 | 0.12 | 7.13 ± 0.62 | 7.68 ± 0.59 | 0.52 |
| CD20\(^+\) (% leukocytes) | AF 12.27 ± 2.27 | 19.13 ± 3.52 | 0.15 | 12.0 ± 1.85 | 19.19 ± 4.71 | 0.18 |

doi:10.1371/journal.pone.0019405.t001
Study characteristics were similar between both groups at baseline and end of feeding (Table 1).

**CB2 receptor signaling differentially affects inflammatory cell recruitment**

Since previous reports implicated the CB2 receptor in the recruitment of inflammatory cells, we investigated a potential role of CB2 receptor signaling in Murine peritonitis [12,23,24]. 72 hours after intraperitoneal injection of thioglycollate peritoneal macrophage numbers were significantly reduced in JWH-133-treated mice compared with vehicle controls (Fig. 4A N = 5 per group). In contrast, JWH-133 treatment did not affect short term (4 h) thioglycollate-induced peritonitis predominated by neutrophils (Fig. 4A, N = 9 per group). Similar amounts of leukocytes accumulated in the peritoneal cavity of CB2^+/+^/LDLR^−/−^ mice and CB2^−/−^/LDLR^−/−^ control animals after 72 and 4 hours (N = 5 and N = 13 per group, respectively, Fig. 4B). In accord, CB2 receptor signaling did not affect adhesion of inflammatory cells in the flow chamber (Fig. 4C), expression of ICAM-1 as assessed by Western blotting (Fig. 5A) and FACS (Fig. 5B), as well as chemokine expression in cultured endothelial cells (Fig. 5 C). JWH-133 did not modulate apoptosis (Fig. 5D) and cytotoxicity of the cells tested (Fig. 5E).

**Discussion**

The present study made the surprising finding that selective CB2 receptor stimulation did not affect the development of atherosclerotic plaques in LDLR-deficient mice. Accordingly, deletion of the CB2 receptor in LDLR-deficient mice did neither increase nor decrease atherosclerotic burden. There was a significant elevation of lipid and macrophage content in plaques of CB2^−/−^/LDLR^−/−^ mice compared to CB2^+/+^/LDLR^−/−^ mice. We also did not observe an effect of CB2 receptor signaling on atherosclerosis, a chronic inflammatory disease, we sought to explore its role in an acute model of inflammation. Interestingly, neither genetic deficiency nor selective stimulation of CB2 by JWH-133 modulated the expression of IL-6, MCP-1, IL-10, IFN-γ, or IL-12p70 in mice challenged intraperitoneally with TNF-α (Fig. 6A). However, JWH-133-treated mice recruited lower numbers of monocytes with an inflammatory, GR1^high^ subtype to the blood pool upon stimulation with TNF-α. Of note, no difference in numbers of this cellular subtype could be measured between CB2^−/−^/LDLR^−/−^ and CB2^+/+^/LDLR^−/−^ mice.
mice, though. A recent study also showed no significant difference in atherosclerotic lesion area between CB2\(^{+-}\)/LDLR\(^{-/-}\) and CB2\(^{-/-}\)/LDLR\(^{-/-}\) mice after 8 or 12 weeks on an atherogenic diet. In accordance with our findings, plaques of these CB2-deficient animals contained more macrophages [25]. One possible explanation is that CB2 receptor deficiency reduces the susceptibility of macrophages to oxidized LDL-induced apoptosis in vitro [26]. Therefore, the elevated macrophage levels in plaques of CB2\(^{-/-}\)/LDLR\(^{-/-}\) mice might be the result of reduced apoptosis. Indeed, Netherland et al. observed decreased cellular apoptosis rates in atherosclerotic plaques from CB2\(^{-/-}\)/LDLR\(^{-/-}\) mice [25]. In contrast, we could not detect CB2-dependent changes in apoptosis.

Figure 4. Inflammatory cell recruitment is differentially affected by CB2 receptor stimulation. A, Wild-type mice received intraperitoneal injections of 4% thioglycollate after pre-treatment with JWH-133 or vehicle control. Leukocyte recruitment into the peritoneal cavity was quantified after 72 and 4 h. Data represent mean ± SEM. Asterisks indicate significant change, defined as p<0.05. B, In parallel, thioglycollate-elicited accumulation of leukocytes in the peritoneal cavity was quantified in CB2\(^{-/-}\)/LDLR\(^{-/-}\) mice and CB2\(^{+-}\)/LDLR\(^{-/-}\) control animals. Data for both 72 and 4 h stimulation are expressed as mean ± SEM. C, PMA-activated thioglycollate-elicited peritoneal leukocytes obtained from wild-type (B6) mice were allowed to adhere on TNF\(\alpha\)-activated endothelial cells (EC) isolated by magnetic bead separation from wild-type mice in the presence or absence of 40\(\mu\)M JWH-133. Adhering leukocytes were quantified under microscope after the indicated time points in the flow chamber (N = 3 each).
in our study animals. Increased macrophage content is a feature associated with more unstable plaques in humans. However, plaque stability also depends on collagen and smooth muscle content, which were both not modulated in our study. Furthermore, if CB2 deficiency results in more plaque inflammation and less stability, one would expect that CB2 agonism promotes less inflammation, more stable lesions. We could not observe such an effect in animals treated with JWH-133. In accord, i.p. application of the direct CB2 antagonist SR144528 in HCD-consuming ApoE−/− mice did not modulate atherosogenesis in another report [27]. Thus, while we cannot rule out that CB2 signaling may affect macrophage biology, in the context of atherosclerosis this does not appear to be relevant.

Since CB1 receptor signaling is thought to be proatherogenic [8,28,29], we also quantified CB1 mRNA expression via RT-PCR, showing no significant difference between the CB2−/−/LDLR−/− and CB2−/−/LDLR+/− mice. This makes a CB2-driven bias unlikely, however we cannot rule out a change of receptor activation due to receptor internalization.

Our data challenge two previous reports suggesting CB2-dependent anti-atherosclerotic properties of endocannabinoids [3,17]. Both studies observed only indirect evidence for a CB2-dependent effect and lacked the use of highly selective CB2 agonists or genetic CB2 knock-out animals. They demonstrated attenuation of atherosclerotic lesion formation by the CB1/CB2 agonists tetrahydrocannabinol (THC) and WIN55212-2, effects partially reversed by treatment with the CB2 antagonists SR144528 and AM630. Some reports claim selectivity of CB2 receptor agonist, with a Ki of 3.4 nM and a 200-fold higher affinity for CB2 over CB1 receptors [31]. Numerous studies used JWH-133 in vivo at concentrations ranging from 0.015–15 mg/kg [14,32,33,34,35]. In the present study, we administered JWH-133 three times a week by intraperitoneal injection for the complete duration of high cholesterol diet, e.g. 16 weeks. This regimen resulted in detectable serum and aortic concentrations of JWH-133 as assessed by mass spectrometry, demonstrating bioavailability.

Imbalance in the ratio of the T cell subgroups and inflammatory monocytes as well as in their effector cytokines can modulate atherogenesis and plaque composition in mice [36,37]. Several studies have shown that THC regulates Th1/Th2 cytokine balance in activated human T cells [7,38,39]. The expression of IFNγ was dose-dependently reduced in splenocytes after THC stimulation in a report whereas only a modest, non-significant down-regulation of IL-10 and TGFβ was detected, leading the authors to the conclusion that THC induces a dose-dependent shift in the Th1/Th2 balance [17]. Cytokine levels were too low to be quantified in our atherosclerosis model. To investigate whether deficiency or stimulation of the CB2 receptor has an influence on cells and cytokines also known to be involved in atherosclerosis, we chose a cytokine challenge model of acute Murine inflammation. The present study found no difference in IL-6, MCP-1, IL-10, IFNγ, and IL-12p70 expression after intraperitoneal TNFα challenge in both JWH-133-pretreated and CB2−/−/LDLR−/− mice compared with respective controls. Therefore, in contrast to the non-selective THC, selective CB2 stimulation or deletion of the CB2 receptor has no influence on the expression of these cytokines in vivo. However, mice treated with JWH-133 for 10 days recruited lower numbers of inflammatory Gr1biH monocytes to the blood pool after intraperitoneal TNFα challenge, suggesting that CB2 stimulation may indeed have short term anti-inflammatory effects.

The recruitment of inflammatory cells (e.g., monocytes and T lymphocytes) to the intima is an essential step in the development and progression of atherosclerosis [40]. Rolling, adhesion, and trans-endothelial migration of leukocytes are triggered by local production of chemokines, chemokine receptors, and adhesion molecules [41]. Several previous in vitro studies have investigated the role of CB2 receptor activation on baseline or stimulated inflammatory cell migration, with both increases and decreases of cell migration reported, depending on the endocannabinoid, synthetic agonist/antagonist, and cell type used [for review, see 42]. Intraperitoneal injection of HU-210 and WIN-55,212-2 reduced the influx of neutrophils into peritoneal cavity in mice in one report [43]. However, both substances are considered to be both CB1 and CB2 agonists [44,45,46]. Using the highly selective CB2 agonist JWH-133, we detected a significant decrease of macrophage accumulation in the peritoneum of JWH-133-treated mice 72 hours after thioglycollate injection, suggesting anti-inflammatory properties of this drug in vivo at the dosage employed. In contrast, JWH-133 did not affect peritoneal neutrophil accumulation 4 hours after thioglycollate exposure. Accordingly, exposure of isolated LDLR−/− cells to increasing concentrations of JWH-133 followed by TNFα stimulation did not mitigate MCP-1 and ICAM-1 expression. We could also not detect any significant differences in the expression of MCP-1 in TNFα-stimulated endothelial cells, isolated from CB2−/−/LDLR−/− mice compared to respective controls. In contrast, Rajesh et al. found a significant decrease of MCP-1 and ICAM-1 in TNFα-stimulated human coronary artery endothelial cells after incubation with JWH-133 [12]. This might be due to cell type specific differences and methodical differences.

There are several limitations of this study that need to be considered: 1. Despite detection of JWH-133 in mouse serum and aortas by mass spectrometry, we cannot rule out that the dose of JWH-133 applied was insufficient to adequately stimulate the CB2 receptor in vivo. This is, however, unlikely since several other studies applied doses in a similar range to mice in vivo and observed biological effects [32,33,34,35,47]. Also, even if the dosage was insufficient one would still expect the genetic deficient animals to show an opposite effect which they did not in our study. 2. It is...
possible that CB2 receptor signaling affects initial but not later stages of atherosclerosis as tested in this study. However, one may question the biological and therapeutic relevance of such effects if they do not hold up through the course of atherogenesis. Also, plaques in the aortic arch are generally regarded to be at an earlier stage of development whereas those in the aortic root are considered to be more advanced [18]. Since we did not observe any modulation of atherosclerosis at both sites a stage-dependent effect is unlikely.

In summary, the present study made the novel observation that neither CB2 receptor stimulation nor its genetic deficiency modulates atherogenesis. Therefore, therapies targeting the CB2 receptor may not be beneficial in reducing atherosclerotic burden.
We thank Sandra Ernst and Christian Münkell for excellent technical support.

Acknowledgments

20. Missiou A, Kostlin N, Varo N, Rudolf P, Aichele P, et al. (2010) Tumor necrosis factor-α (TNF-α) production and cytokine expression in aortic smooth muscle cells is increased in hypercholesterolemic mice. Atherosclerosis 209: 60–68.

18. Steffens S, Veillard NR, Arnaud C, Pelli G, Burger F, et al. (2005) Low dose oral tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol 165: 373–380.

17. Zhang M, Martin BR, Adler MW, Razdan RK, Jallo JI, et al. (2007) Cannabinoid CB2 receptor activation decreases cerebral infarction in a mouse model of ischemia/reperfusion injury. Stroke 38: 2120–2130.

16. Montecucco F, Matias I, Lensl S, Petrosino S, Burger F, et al. (2009) Cannabinoid 2 receptors protect against cerebral ischemia by inhibiting neutrophil recruitment. Free Radic Biol Med 47: 788–798.

15. Xu H, Cheng CL, Chen M, Manivannan A, Cabay L, et al. (2007) Anti-inflammatory property of the cannabinoid receptor-2-selective agonist JWH-133 in a rodent model of autoimmune uveoiritis. J Lab Invest 102: 332–342.

14. Murikinati S, Juttler E, Keinert T, Ridder DA, Muhammad S, et al. (2010) Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. Free Radic Biol Med 47: 788–798.

13. Jonsson KO, Persson E, Foderj C (2006) The cannabinoid CB2 receptor selective agonist JWH133 reduces mast cell oedema in response to compound 48/80 in vivo but not the release of beta-hexosaminidase from skin slices in vitro. Life Sci 78: 598–606.

12. Shnida K (2009) Immune system and atherosclerotic disease: heterogeneity of leukocyte subsets participating in the pathogenesis of atherosclerosis. Circ J 73: 994–1001.

11. Galkina E, Ley K (2009) Immune and inflammatory mechanisms of atherosclerosis (*). Am Rev Respir Dis 176: 169–175.

10. Han KH, Lim S, Ryu J, Lee CW, Kim Y, et al. (2009) CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. Cardiovasc Res 84: 378–386.

9. Zaidi A, Kostlin N, Varo N, Rudolf P, Aichele P, et al. (2010) Tumor necrosis factor-α (TNF-α) production and cytokine expression in aortic smooth muscle cells is increased in hypercholesterolemic mice. Atherosclerosis 209: 60–68.

8. Steffens S, Veillard NR, Arnaud C, Pelli G, Burger F, et al. (2005) Low dose oral tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol 165: 373–380.

7. Zhang M, Martin BR, Adler MW, Razdan RK, Jallo JI, et al. (2007) Cannabinoid CB2 receptor activation decreases cerebral infarction in a mouse model of ischemia/reperfusion injury. Stroke 38: 2120–2130.

6. Montecucco F, Matias I, Lensl S, Petrosino S, Burger F, et al. (2009) Cannabinoid 2 receptors protect against cerebral ischemia by inhibiting neutrophil recruitment. Free Radic Biol Med 47: 788–798.

5. Netherland CD, Pickle TG, Bales A, Thewske DP (2010) Cannabinoid receptor type 2 (CB2) deficiency alters atherosclerosis lesion formation in hyperlipidemic LDL-/-mice. Atherosclerosis 207: 102–108.

4. Han KH, Lim S, Ryu J, Lee CW, Kim Y, et al. (2009) CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. Cardiovasc Res 84: 378–386.

3. Di Marco V (2008) Targeting the endocannabinoid system: to enhance or reduce? Nat Rev Drug Discov 7: 438–455.

2. Pacher P, Batki S, Kunos G (2006) The endocannabinoid system as an emerging target of pharmaceutical therapy. Pharmacol Rev 58: 389–462.

1. Libby P (2002) Inflammation in atherosclerosis. Nature 420: 868–874.