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Inhibition of monocyte chemotaxis by VB-201, a small molecule lecinoxoid, hinders atherosclerosis development in *ApoE*$^{-/-}$ mice

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ABSTRACT

Objective: Monocytes are motile cells which sense inflammatory stimuli and subsequently migrate to sites of inflammation. Key players in host defense, monocytes have nevertheless been implicated as requisite mediators of several chronic inflammatory diseases. Inhibition of monocyte chemotaxis is therefore an attractive anti-inflammatory strategy. Oxidized phospholipids (OxPL) are native regulators of inflammation, yet their direct effect on monocyte chemotaxis is poorly defined. In this study, we investigated the direct effect of natural and synthetic phospholipids on monocyte chemotaxis.
Methods: Exploring various phospholipids using *in vitro* chemotaxis assays, we found that the natural phospholipid 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC) can decrease monocyte chemotaxis by 50%, while other tested OxPL had no effect. We generated a library of synthetic OxPL designated lecinoxoids, which was screened for anti-inflammatory properties.
Results and conclusions: VB-201, a small-molecule lecinoxoid, exhibited up to 90% inhibition of monocyte chemotaxis *in vitro*. Molecular analysis revealed that the effect of VB-201 was not restricted to a specific chemotactic ligand or receptor, and resulted from inhibition of signaling pathways required for monocyte chemotaxis. Interestingly, VB-201 did not inhibit monocyte adhesion or phagocytosis and had no effect on chemotaxis of CD4^{+} T-cells or neutrophils. *In vivo*, oral treatment with VB-201 reduced monocyte migration in a peritonitis model and inhibited atheroma development in *ApoE*$^{-/-}$ mice, without affecting cholesterol or triglyceride levels. Our findings highlight a novel role played by native and synthetic phospholipids in regulation of monocyte chemotaxis. The data strengthen the involvement of phospholipids as key signaling molecules in inflammatory settings and demonstrate their potential therapeutic applicability.

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1. Introduction

Monocytes are key players in the immune system, with critical roles in innate and adaptive immunity, immune surveillance and particle scavenging [1,2]. Circulating monocytes can sense inflammatory stimuli and quickly migrate through the vascular or lymphatic endothelium to the periphery [2]. While playing a necessary role in host defense, monocytes were nonetheless identified as critical mediators of several inflammatory disorders, including atherosclerosis, rheumatoid arthritis and multiple sclerosis [3–5]. Suppressing the accumulation of unwanted monocytes/macrophages in a chronically inflamed tissue has a therapeutic potential, and migration inhibitors have accordingly demonstrated beneficial therapeutic results in animal models and clinical trials.

Extensive research has shown that chemokine receptors and adhesion molecules play a key role in regulation of leukocyte trafficking (reviewed in Refs. [1,6]). A complex array of chemokine receptors, G-protein coupled receptors (GPCRs) that are differentially expressed on leukocyte lineages and subsets, regulates which cell types would migrate and to which tissue, under different conditions. Chemokines are secreted proteins that regulate

Abbreviations: DC, dendritic cell; FACS, fluorescence-activated cell sorting; GPCR, G-protein coupled receptor; LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein-1; OxPL, oxidized phospholipid; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; OxPAPC, oxidized PAPC; PGPC, 1-palmitoyl-2-glutaryl phosphatidylcholine; ROS, reactive oxygen species.

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migration and activation of leukocytes and stromal cells [1,3,7]. In the case of inflammatory monocytes, diapedesis and intravasation are processes dependent on C–C motif receptor 2 (CCR2) signaling, in response to activation by the CCL2 (also known as MCP-1) and CCL7 (MCP-3).

Phospholipids are much more than structural components of cellular membranes. Subjected to enzymatic and chemical modifications, modified phospholipid species serve as key signaling molecules and secondary messengers affecting multiple biological systems, including innate and adaptive immune responses [8]. Oxidized phospholipids (OxPLs) are abundantly found at sites of inflammation and are involved in modulation of the immune response. Most studies have attributed a pro-inflammatory role to OxPLs, as in the case of low density lipoprotein (LDL) oxidation products which accumulate in vessel walls and promote atherosclerosis [9,10]. Minimally-modified and oxidized LDL components bind to scavenger, nuclear, Toll-like, and G-protein coupled receptors on endothelial, smooth muscle cells and macrophages, subsequently inducing secretion of multiple chemokines and facilitating chemotaxis of pro-inflammatory leukocytes [9–12]. However, multiple different OxPL species can be found *in vivo* and while some promote inflammation, other OxPLs exhibit anti-inflammatory effects [13]. Several groups have demonstrated OxPL-mediated inhibition of Toll-like receptor signaling, production of anti-inflammatory compounds and protection of endothelial cell barrier [14–16], properties which may indirectly reduce leukocyte recruitment.

Additional phospholipid species were shown to affect cell migration. The lipid mediators, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) which regulate a complex array of GPCRs, were reported to either enhance or reduce chemotaxis of different cell types [17]. Taken together, these studies point to an important role played by physiological phospholipids in regulation of immune cell chemotaxis. Yet, to the best of our knowledge, a direct and clear *in vivo* inhibition of monocyte migration by a phospholipid molecule has not been reported thus far.

Atherosclerosis is a complex disorder involving lipid retention, inflammation, oxidative stress and endothelial dysfunction. Monocytes play a key role in initiation and progression of atherosclerosis. Evidence to their critical role can be found in studies of the M-CSF mutation in mice, which abolished aortic atherogenesis in the *ApoE* knockout (*KO*) model in spite of high lipid levels [18,19]. Conditions of atherosclerosis induce a rapid influx of inflammatory monocytes to the vessel wall which is followed by differentiation of the monocytes to inflammatory macrophages and DCs. Migrating as well as resident macrophages and DCs, along with infiltrating T-cells, undergo phenotypic changes and modulate the inflammatory milieu within the arterial wall [20].

VB-201 is a novel, orally available, non-oxidative synthetic phospholipid analog of the lecinoxoid (lecin for lecithin, i.e. a phospholipid and oxoid for oxidized) family. Structurally related to the native compound 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC), VB-201 was engineered towards enhanced *in vivo* stability, through chemical modifications at sn-1 and sn-2 positions of the glycerol backbone. Recently, we demonstrated that VB-201 has a profound effect on the onset and severity of experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis, reducing the number of immune cells infiltrating the spinal cord and restraining the Th1 response of treated animals [21]. Furthermore, VB-201 was found to attenuate inflammation in additional experimental immune-mediated models (unpublished data) and after successful completion of phase I, is currently tested in phase II clinical trials. Yet, the mechanism of action by which VB-201 (and the lecinoxoids in general) reduces inflammation was unknown. Here we report findings that unravel a cellular and molecular anti-inflammatory role for lecinoxoids in general and VB-201 in particular.

2. Materials and methods

2.1. Materials

VB-201 and VB-207 (an inactive derivative of VB-201, used as a negative control) are proprietary molecules synthesized by VBL Therapeutics. Chemoattractants were purchased from Peprotech. GW5074 was purchased from Sigma. Rapamycin and Wortmannin were from EMD-Millipore. 1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC) were purchased from Avanti Polar Lipids. PAPC was oxidized for 24 or 72 h prior to use, as indicated. THP-1 cells were purchased from ATCC. RPMI-1640 medium and fetal bovine sera were from Biological Industries Ltd., Israel. Ficoll–Paque plus was from GE Healthcare. HUVEC were freshly isolated from blood cords of donors and grown in EGM-2 medium (Lonza). Thioglycollate solution was purchased from Hy-Labs, Israel.

2.2. Animals

Apolipoprotein E knockout (*ApoE KO*) mice were bred in-house at the Bert W. Strassburger Lipid Center Sheba Medical Center, Tel-Hashomer, Israel. C57B6/J WT mice were purchased from Harlan (Rehovot, Israel). All mice were fed a chow diet. For *in vivo* examination of monocyte migration, C57B6/J WT mice (age 9–12 weeks) were orally administered with VB-201 at dose of 0.04, 0.4 or 4 mg/kg once daily for 8 days. On day 5, thioglycollate was injected to the peritoneal cavity of the mice to induce monocyte migration. Three days later mice were sacrificed and cells were collected from the peritoneum and counted by hemocytometer.

For atherosclerosis study, 9–11-week old male *ApoE KO* mice were administered with VB-201 (1.5 mg/kg) or PBS by oral gavage. Dosing was performed once a day, for 8 weeks ($n = 11$–12/group). Atherosclerotic lesions were quantified by calculating the lesion size in the aortic sinus as previously described [1]. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Sheba Medical Center, Ramat Gan, Israel.

2.3. Human specimens

Blood samples and umbilical cords were obtained from healthy donors. The study design was approved by the ethics committee of Sheba Medical Center, and all subjects provided written informed consent.

2.4. Cell viability assays

Following incubation with the indicated doses of VB-201 for 16 h, cell viability was analyzed using CellTiter MTS assay (Promega) according to the manufacturer's protocol.

2.5. Western blot analysis

For Western blot analyses, cells were washed twice with PBS, harvested in denaturing lysis buffer containing protease & phosphatase inhibitor cocktails (Pierce) and boiled for 5 min. Lysates were resolved on Any-KD Criterion gels (Biorad) and transferred to nitrocellulose membranes. For immune-blotting, phospho-AKT (Ser473)(D9E), AKT, p-MEK1/2 (Ser217/221) (41G9) and p-p38(Thr180/Tyr 182)(D3F9) were purchased from Cell Signaling Technology; ERK1/2 and p-ERK1/2(diphosphorylated) antibodies were from Sigma; p38 (A-12) and MEK1 (H-8) antibodies were

from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were from Jackson Immunoresearch. Signal was developed using ECL kit (Pierce).

2.6. Immunohistochemistry

Immunostaining was performed on frozen sections which were fixed with cold acetone and blocked with 4% normal rabbit serum/PBS (Vector Lab). Following incubation with rat anti-mouse CD68 (Serotec NCA1957; 1:250) and anti-rat biotinylated antibody (Vector Lab AB-4001; 1:200), signal was developed using Vectastain ABC-alkaline phosphatase kit and Vector Red Substrate (Vector Lab). Mayer's hematoxylin was used for counterstaining.

2.7. In vitro cell migration assay

Human monocytes ($CD14^+$ cells), $CD4^+$ T-cells and neutrophils were isolated from whole blood of healthy donors. Following PBMCs separation from whole blood using Ficoll–Paque and leucosep tubes (Greiner Bio-One), $CD14^+/CD4^+/CD66^+$ cells were separated using specific anti-CD14/anti-CD4/anti-CD66abce micro-beads, respectively (Miltenyi Biotec). Purity of the isolated populations was validated using specific antibodies by FACS analysis. Isolated cells were pre-incubated for 30 min with solvent (0.005% ethanol/PBS) or VB-201 as indicated. For chemotaxis of monocytes, RANTES (100 ng/ml), MIP-1α (50 ng/ml) or MCP-1 (50 ng/ml) were dissolved in 0.5% FBS/RPMI-1640 and placed at the lower chamber of QCM™ 24-well migration assay plate (Corning-Costar; 5 μm pores). For chemo-attraction of $CD4^+$ cells, 0.5% FBS/RPMI-1640 solution containing RANTES (100 ng/ml) and SDF-1α (50 ng/ml) was used, using the same plate setting. Chemo-attraction of neutrophils was done using 2% FBS/RPMI-1640 solution containing LPS (100 ng/ml), or 10% FBS/RPMI-1640 supplemented with MCP-3 (100 ng/ml). Migration assay was conducted by seeding 300,000 treated cells in the upper chamber, followed by incubation of 3–4 h. Subsequently, the number of cells which migrated to the medium in the lower compartment was determined by FACS. For analysis of chemotaxis in the THP-1 line, cells were suspended in 0.5% FBS/RPMI-1640 and pre-incubated for 1 h with the PI3K inhibitor Wortmannin (1 μmol/l), the mTOR inhibitor Rapamycin (1.1 μmol/l) or the RAF inhibitor GW5074 (1 μmol/l), followed by addition of solvent (0.005% ethanol/PBS) or VB-201 (5 μg/ml) for 30 min. Assays were performed for 3 h, using 2% FBS/RPMI-1640 supplemented with RANTES (100 ng/ml) and MCP-1 (100 ng/ml) for cell attraction.

2.8. Quantification of chemokine receptor expression level

Human $CD14^+$ monocytes were incubated for 3.5 h in 2% FCS/RPMI medium with solvent or VB-201. Cells were then washed, resuspended in PBS containing 2% FCS and 0.02% sodium azide (FACS buffer) and stained for 30 min at 4 °C with anti-CCR1-Alexa Fluor 647 (BD Biosciences), anti-CCR5-PE, anti-CXCR1-PE, anti-CXCR2-Alexa Fluor 647, anti-CCR7-PE and anti-CCR2-PerCP-Cy5.5 (all from BioLegend). Cells were washed, resuspended in FACS buffer and analyzed on a FACS-Calibur device.

2.9. GPCR activation assay

FLIPR assays were conducted by Millipore GPCR Profiler Service to screen VB-201 and VB-207 (inactive derivative of VB-201, as a negative control) for dose-dependent agonist and antagonist activities on the GPCRs listed. Sample compounds were plated in serial dilutions in assay buffer (1× HBSS with 20 mmol/l HEPES and 2.5 mmol/l Probenecid), with a top concentration of 10 μmol/l. Agonist percentage activation determinations were obtained by assaying the test compounds and referencing the E_{max} control for the GPCRs profiled. Antagonist percentage inhibition determinations were obtained by assaying the test compounds and referencing the control EC_{80} wells for the GPCRs profiled.

2.10. Isolation and staining of mouse peritoneal cells

Three days after intraperitoneal injection of C57B6/J mice (8–10 weeks) with 1.5 ml of thioglycollate solution (Hy-labs, Israel), mice were euthanized by CO_2 and cells were collected from the peritoneal cavity by 3–4 washes with ice-cold PBS. Following removal of erythrocytes with RBC lysis buffer (Biological Industries, Israel), cells were resuspended in RPMI medium and counted by light microscopy. For characterization of the cell population, cells were stained with anti-GR-1 (neutrophils marker) conjugated to PE and anti-F4/80 (macrophages marker) conjugated to PerCP-Cy5.5 (both from eBioscience). Fluorescence intensity was quantified by FACS.

2.11. Statistical analysis

Mean ± standard deviation/standard error was calculated using Excel or Sigma-Stat software, and the statistical significance was calculated by two-tailed Student *t*-test. To compare between experimental groups one way ANOVA or *t*-test was used. p value of <0.05 was considered as statistically significant.

3. Experimental results

3.1. PGPC and VB-201 inhibit human monocyte chemotaxis in vitro

Investigating potential anti-inflammatory roles of OxPLs, we were interested in testing possible effects of native phospholipids on monocyte chemotaxis directly. OxPLs were previously reported to increase monocyte adhesion to endothelial cells [10,22], however, these effects resulted from activation of the endothelial cells which was followed by cytokine secretion and enhanced expression of adhesion molecules, while the monocytes themselves were not treated or studied. We therefore chose to avoid the presence of other cell types and performed *in vitro* monocyte chemotaxis assays using isolated human monocytes and recombinant chemoattractants.

For this purpose, monocytes from healthy donors were pre-incubated for 30 min with either solvent (0.005% ethanol/PBS), phosphatidylcholine (PC; 5 μg/ml), partially oxidized PAPC (24 h oxidation; 40 μg/ml) or PGPC (5 μg/ml), and allowed to migrate towards recombinant human MCP-1 (CCL2; ligand for CCR2) and RANTES (CCL5; ligand for CCR1, CCR3, CCR5, US28) as attractants (50 ng/ml each). Interestingly, whereas PC and partially oxidized PAPC did not reduce the number of migrating cells, treatment with pure PGPC, an oxidized product of PAPC, significantly attenuated monocyte chemotaxis by ~50% (Fig. 1A). This unexpected result led us to screen through a library of Lecinoxoids for additional compounds that may inhibit monocyte chemotaxis. The screen yielded VB-201, a synthetic saturated phospholipid which is structurally related to PGPC (Supplementary Fig. 1 A,B), that demonstrated profound inhibition of monocyte chemotaxis (Fig. 1A). Increased potency of VB-201 over PGPC was further validated using dose-escalating chemotaxis assays (Supplementary Fig. 1C).

We have repeated the assays, employing MCP-1, RANTES or MIP-1α (CCL3; ligand for CCR1,CCR5) as chemoattractants and found that compared to the solvent control, VB-201 significantly and profoundly reduced monocyte migration by approximately 80% for MCP-1, 72% for RANTES and 68% for MIP-1α (Fig. 1B). VB-201 also inhibited in a dose-dependent fashion monocyte migration towards growth medium taken from a culture of human umbilical

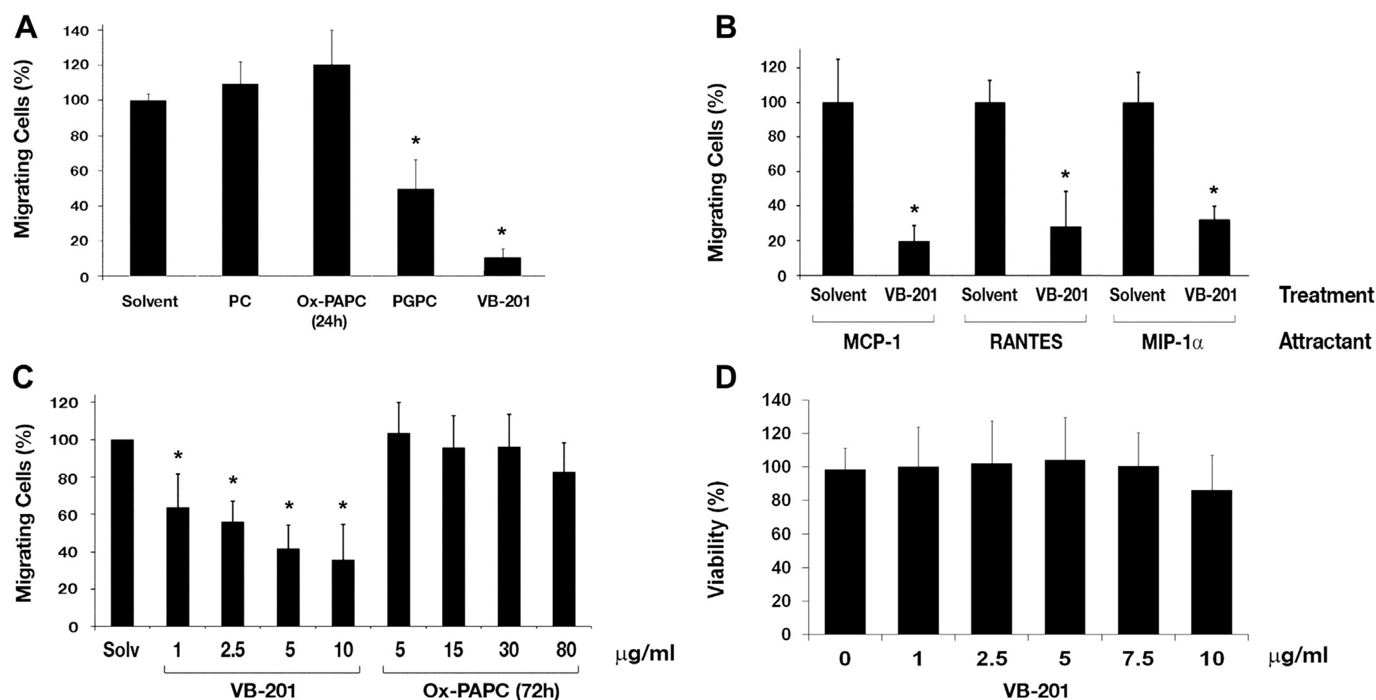

Fig. 1. VB-201 inhibits chemotaxis of human monocyte *in vitro*. (A) Human monocytes ($CD14^+$) isolated from blood of healthy donors were pre-treated for 30 min with either solvent, phosphatidylcholine (PC; 5 μg/ml), oxidized PAPC (40 μg/ml, 24 h oxidation), PGPC (5 μg/ml) or VB-201 (5 μg/ml), and then subjected to transwell chemotaxis assay, using MCP-1 and RANTES mix (50 ng/ml each) for attraction. The number of cells migrating to the lower compartment was determined by FACS, and normalized to the solvent control. Data are mean ± SD from a representative experiment performed in triplicates. (B) $CD14^+$ cells were treated with solvent or VB-201 (5 μg/ml) prior to chemotaxis assay using MCP-1 (50 ng/ml), MIP-1α (50 ng/ml) or RANTES (100 ng/ml) as attractants. Data are mean ± SD ($n = 3$, in triplicates). (C) Effect of escalating doses of VB-201 or oxidized-PAPC (Ox-PAPC) on $CD14^+$ migration towards HUVEC supernatant. Data represent mean ± SD ($n = 3$, in triplicates). (D) MTS assay in human monocytes subjected to escalating doses of VB-201 for 16 h, demonstrates that VB-201-inhibited chemotaxis is not due to reduced cell viability (mean ± SD; $n = 7$ in quadruplicates). Statistically significant ($p < 0.05$) differences relative to solvent are marked with asterisk.

cord endothelial cells (HUVEC). Inhibition of migration was not seen with fully oxidized PAPC for any of the doses tested (Fig. 1C).

To exclude the possibility that inhibition of monocyte migration by VB-201 results from cell death, monocytes were treated with escalating doses of VB-201 for 16 h, after which cell viability was determined by MTS assay. As shown in Fig. 1D, VB-201 had no effect on monocyte viability at doses of 1–10 μg/ml. Along that line, VB-201 also inhibited chemotaxis of monocytic THP-1 cells without affecting their viability (Supplementary Fig. 2).

To ensure that VB-201 is not an oxidative molecule which may induce ROS and damage cells, human monocytes were incubated overnight with PBS, VB-201 (10 μg/ml), PGPC (10 μg/ml) or the positive control OxPAPC (100 μg/ml; oxidized for 24 h) and ROS formation was tested as described in the Methods section. As expected, OxPAPC significantly elevated ROS in human monocytes relative to PBS-treated cells. However, VB-201 and PGPC had no effect on ROS formation in primary monocytes (Supplementary Fig. 3). Similarly, VB-201 did not affect monocyte secretion of IL-6, IL-8 or MCP-1 (Supplementary Fig. 4, and data not shown), in contrast to the reported stimulation of inflammatory cytokine release by certain OxPL [23].

Collectively, these findings indicate that some phospholipid molecules, such as PGPC and VB-201, can inhibit monocyte chemotaxis *in vitro*. Notably, this effect is compound-specific and does not result from a general phospholipid-related phenomenon (a class effect).

3.2. Monocyte phagocytosis and adhesive properties are not affected by VB-201

Given the inhibition of monocyte chemotaxis by VB-201, we were interested to test whether VB-201 affects additional monocytic functions. To this end, phagocytosis assay was performed on whole blood from healthy donors, in presence or absence of VB-201. Interestingly, VB-201 doses of 5 or 10 μg/ml that were effective in inhibition of monocyte migration did not have any significant effect on phagocytosis capacity of monocytes or granulocytes (Supplementary Fig. 5 A,B). In addition, treatment of monocytes with 1–10 μg/ml VB-201 had no effect on monocyte adhesion to HUVEC compared to the solvent control (Supplementary Fig. 5C). These experiments suggest that the effect of VB-201 is process-specific, regulating monocyte chemotaxis but not their phagocytosis or adhesive properties.

3.3. VB-201 does not affect the expression level of chemokine receptors in monocytes

Looking into possible mechanism/s by which VB-201 reduces monocyte chemotaxis, we first analyzed the expression level of key monocyte chemokine receptors. To this end, monocytes were treated for 3.5 h with 1–5 μg/ml VB-201, conditions which are sufficient to inhibit monocyte migration. Subsequently, cells were stained with fluorescently-labeled antibodies against CXCR1, CXCR2, CCR1, CCR2, CCR5 or CCR7 and were analyzed for receptor expression of by flow cytometry. We were unable to detect any change in the expression level of any of the tested chemokine receptors in VB-201 treated samples relative to the solvent control (Fig. 2A–C), suggesting that reduction of chemokine receptors is not the mechanism of action used by VB-201 to inhibit monocyte migration.

3.4. VB-201 is not a chemokine receptor agonist or antagonist

Next, we asked whether VB-201 regulates activation of chemokine receptors, either as an agonist or as an antagonist. Using

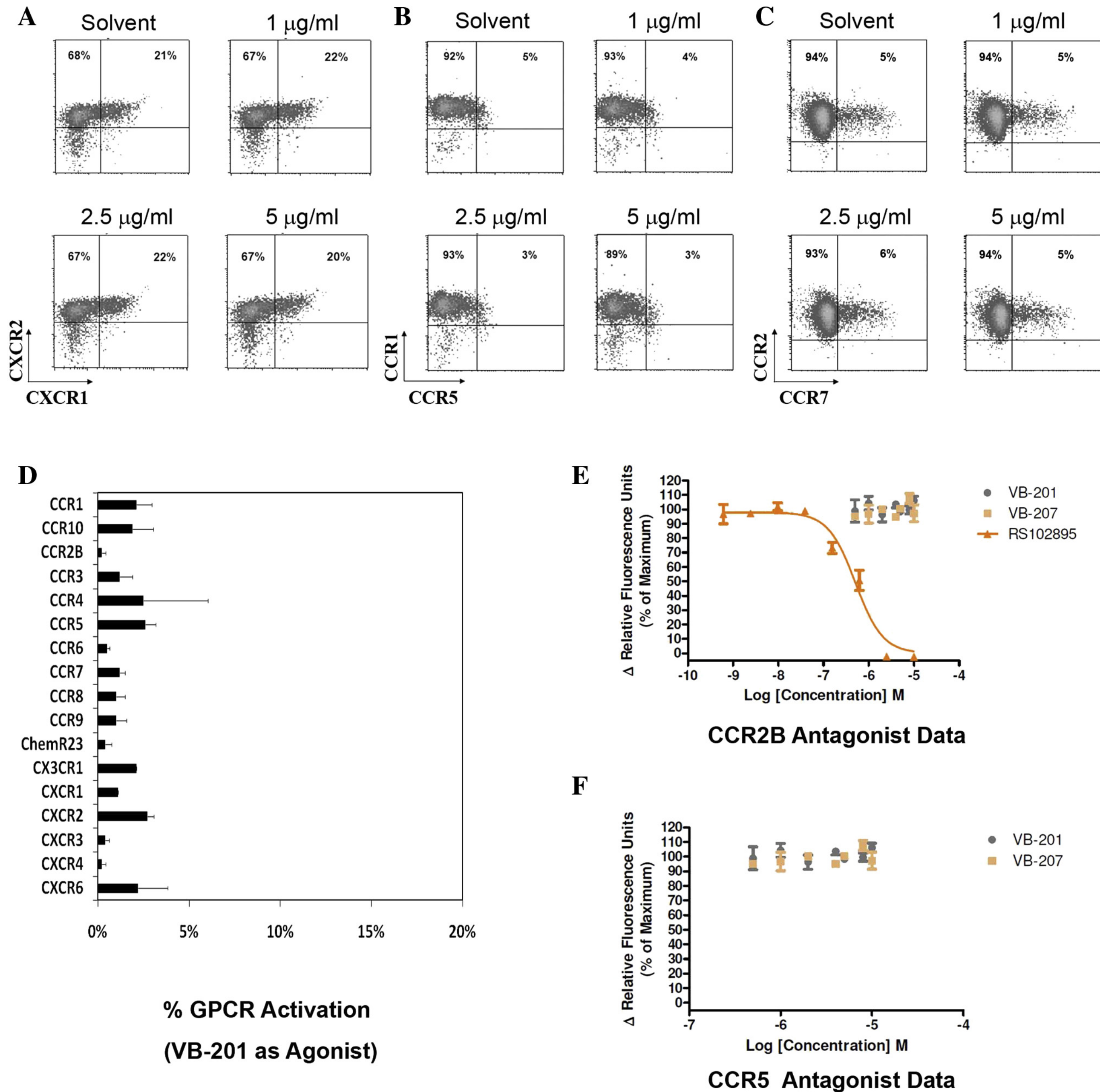

Fig. 2. VB-201 does not modulate chemokine receptors expression or activity. Human monocytes were treated with solvent or escalating doses of VB-201 for 3.5 h, after which the expression levels of (A) CXCR1 and CXCR2, (B) CCR1 and CCR5 and (C) CCR2 and CCR7 was quantified by FACS. (D) GPCR activation assay measuring the effect of 7.35 μg/ml (~12.5 μmol/l) VB-201 on Ca++ flux in a panel of cell lines, each expressing a different chemokine receptor. (E) CCR2B antagonist assay performed in the presence of escalating doses of VB-201, VB-207 (inactive molecule, negative control) or RS102895 (CCR2B antagonist, positive control). (F) CCR5 antagonist assay performed in the presence of escalating doses of VB-201 or VB-207.

Millipore's GPCR profiling services, ChemiScreen stable cell lines expressing different chemokine receptors were treated with VB-201 to assess receptor activation or inhibition by measurement of Ca^{++} flux. Treatment with VB-201 yielded less than 5% activation relative to positive controls (Fig. 2D), suggesting that it is not an agonist of any of the tested receptors. Similarly, VB-201 did not induce antagonistic effect on CCR2B and CCR5 receptors expressed in ChemiScreen cell lines (Fig. 2E,F), in spite of its ability to inhibit chemotaxis induced by ligands of these receptors (MCP-1 and RANTES, respectively, Fig. 1B). These experiments suggest that VB-201 is not a direct regulator of chemokine receptors activ ity.

3.5. *Chemokine receptor signaling in monocytes is inhibited by VB-201*

Since VB-201 had no effect on chemokine receptors expression and direct activation, we decided to explore signaling pathways activated downstream of chemokine receptors. For this purpose, we pre-treated human monocytes with solvent or 5 μg/ml VB-201 and then stimulated the cells with various chemokines, including MCP-1, MCP-3 (CCL7; ligand for CCR1, CCR2, CCR3), MIP-1α, Fractalkine (CX3CL1; CX3CR1 ligand) and RANTES. Following chemokine stimulation, whole cell lysates were collected at different time points and the activation state of AKT, the MEK–ERK axis and p38

was compared as readout for receptor signaling activity. Interestingly, VB-201 pretreatment caused profound reduction in the phosphorylation level of AKT, MEK1/2 and ERK1/2 in MCP-1 or MCP-3 stimulated cells. The p38 pathway was not activated under these settings and was unaffected by VB-201 (Fig. 3A). VB-201-mediated inhibition of chemokine receptor downstream signaling was seen also for monocytes stimulated with MIP-1α, Fractalkine or RANTES (Fig. 3B). Overall, VB-201-treatement either blocked

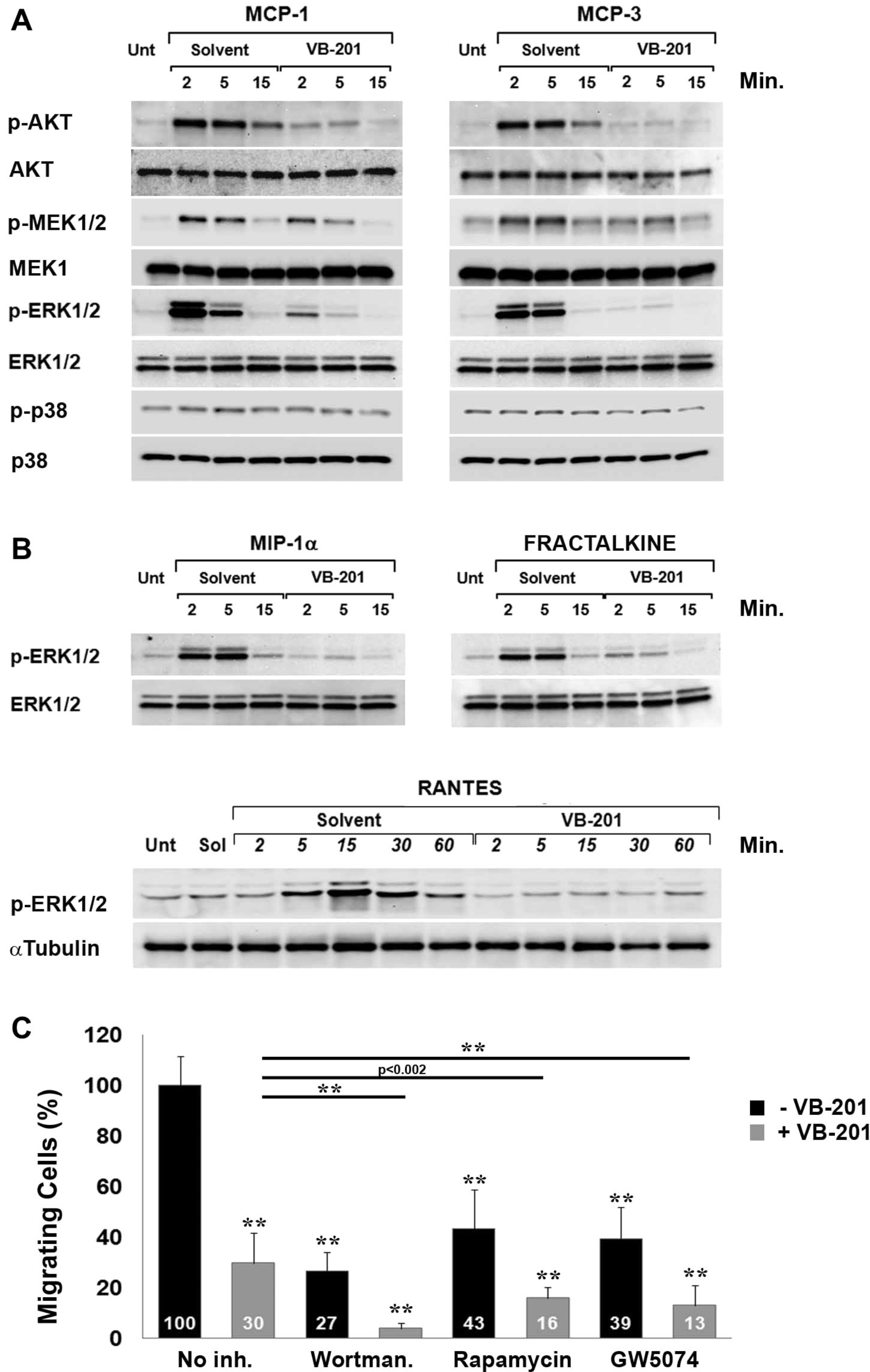

Fig. 3. VB-201 inhibits chemokine-induced signaling in human monocytes. (A) Monocytes were incubated for 20 min with solvent or VB-201 prior to stimulation with MCP-1 (20 ng/ml) or MCP-3 (50 ng/ml). Untreated cells serve as a reference for chemokine stimulation. (B) Monocytes were incubated for 20 min with solvent or VB-201 prior to stimulation with MIP-1α (50 ng/ml), Fractalkine (50 ng/ml) or RANTES (50 ng/ml). Phosphorylation of ERK1/2 was tested at different time points post stimulation. (C) Wortmannin (1 μmol/l), Rapamycin (1.1 μmol/l) and the RAF inhibitor GW5074 (1 μmol/l) decrease chemotaxis of THP-1 cells *in vitro*, but their effect (at sub-maximal doses; black bars) is further enhanced by VB-201 (gray bars). Data are mean ± SD from 4 experiments in triplicates, normalized to untreated cells. $^{**}p < 0.001$. The concentration of VB-201 in all settings was 5 μg/ml, except for the RANTES stimulation which was performed with 2 μg/ml.

downstream signaling, or reduced its intensity, yielding weaker phosphorylation in over shorter period of time.

How are these signaling findings relevant for functional monocyte chemotaxis? To address this question, we examined the effect of the PI3K–AKT and the RAF–MEK–ERK pathways on chemotaxis of THP-1 cells using commercially available inhibitors. Cells were pre-incubated with solvent, the PI3K inhibitor Wortmannin, the mTOR inhibitor Rapamycin, or the RAF inhibitor GW5074, alone (all at sub-maximal doses) or in combination with VB-201, and subjected to chemotaxis assay using 2% FBS/RPMI-1640 supplemented with MCP-1 and RANTES (100 ng/ml each) for attraction. Treatment of THP-1 cells with VB-201 (5 μg/ml) reduced chemotaxis by ~70% relative to a solvent control (as also shown in Supplementary Fig. 2A). Similarly, Wortmannin (1 μmol/l), Rapamycin (1.1 μmol/l) and GW5074 (1 μmol/l) significantly reduced THP-1 chemotaxis, demonstrating the importance of PI3K, mTOR and RAF for THP-1 chemotaxis. Notably, a combination of VB-201 with each of these inhibitors yielded significantly stronger inhibition relative to inhibitor alone or VB-201 alone (Fig. 3C). Collectively, these data suggest that VB-201 inhibits activation of the MEK–ERK and PI3K–AKT pathways in chemokine-stimulated monocytes, resulting in reduced chemotactic capacity.

3.6. Cell specificity of VB-201

Since chemokine receptors are expressed on different immune cells, we asked whether the effect of VB-201 is of broad 'phospholipid' nature, or perhaps is cell-specific. At dose of 5 μg/ml of VB-201, which dramatically inhibits monocytes chemotaxis, we were unable to find any inhibition of $CD4^+$ cell chemotaxis in multiple tested donors (Supplementary Fig. 6A). Similarly, VB-201 had no effect on chemotaxis of human neutrophils towards LPS or RPMI-1640 medium supplemented with 10% FBS/MCP-3 (Supplementary Fig. 6B). The effect of VB-201 on cell motility seems to be cell-specific.

3.7. VB-201 inhibits in vivo monocyte migration in the mouse peritonitis model

To test whether VB-201 can inhibit monocyte migration *in vivo*, we took advantage of the well-characterized mouse model of thioglycollate-induced peritonitis. In this model, injection of thioglycollate to the peritoneal cavity induces a "sterile" inflammatory response recruiting peripheral monocytes to the peritoneum, where they differentiate into macrophages [24]. To this end, mice were fed with different doses of VB-201 for 5 days prior to thioglycollate injection. Upon sacrifice, the number and identity of the cells accumulating in the peritoneum was determined by FACS, and plasma samples were taken for determination of VB-201 levels. As expected for this experimental model, three days after thioglycollate injection 95% of the cells in the peritoneum were macrophages (F4/80 positive, GR-1 negative cells) (Fig. 4B).

Comparing the number of accumulating macrophages in the control animals versus VB-201-treated groups, we noticed that the lowest dose of 0.04 mg/kg/day of VB-201 had no inhibitory effect on macrophage accumulation, as was expected with such a low dose. However, a dose of 0.4 mg/kg, which yielded 0.8 ± 0.3 μmol/l VB-201 in mice plasma, resulted in ~25% inhibition. Notably, when mice were fed with 4 mg/kg VB-201, dosing which yielded plasma level of 8.5 ± 1.5 μmol/l VB-201 that was active in human monocytes, we observed a profound (>60%) significant reduction in the number of peritoneal macrophages relative to the PBS-treated animals (Fig. 4A). These data demonstrate a dose-dependent *in vivo* effect of VB-201 on monocyte migration in a mouse peritonitis model. Furthermore, the effective dose of VB-201 in mice plasma correlated with the dose of VB-201 that inhibited chemotaxis of human monocytes *in vitro*.

3.8. VB-201 treatment inhibited atherosclerosis development in ApoE KO mice

Atherosclerosis is an inflammatory disorder in which monocyte dynamics play a key role. To test our hypothesis that VB-201 may act to modulate inflammation via inhibition of monocyte trafficking, VB-201 was employed in the mouse *ApoE KO* model of atherosclerosis. To evaluate the possible effect of VB-201 on atherosclerosis development, VB-201 or PBS was administered by daily oral gavage to young *ApoE KO* mice (9–11 weeks old) for 8 weeks after which animals were scarified and lesions were examined.

Since *ApoE KO* mice do not exhibit atheromatous lesions along the aorta at this age, atherogenesis was evaluated at the aortic root. Interestingly, quantification of aortic sinus lesion area revealed that

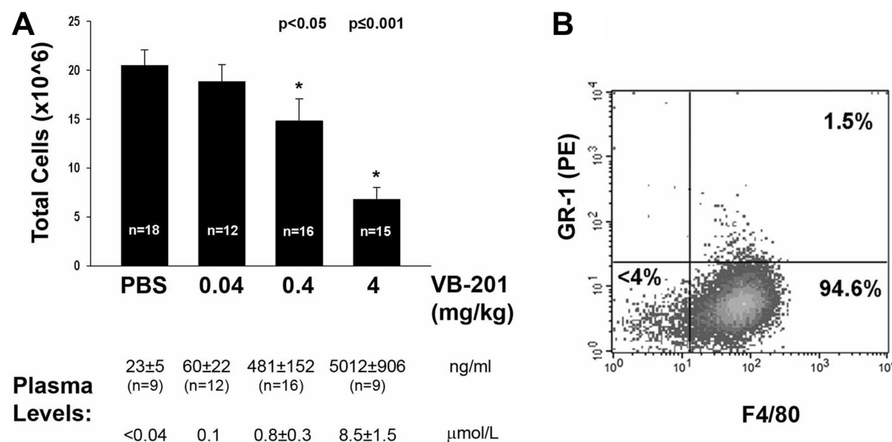

Fig. 4. VB-201 inhibits monocyte chemotaxis *in vivo*. (A) C57B6/J WT mice were orally administered with escalating doses of VB-201 as indicated. After 5 days of administration, thioglycollate was injected to the peritoneal cavity of the mice to induce monocyte migration. Four days later, mice were sacrificed and migrating cells were collected from the peritoneum and counted by hemocytometer. Data are mean ± SE, collected from 3 to 4 independent experiments. Plasma concentrations of VB-201 are shown for each group. (B) To characterize their identity, migrating cells were stained with the macrophage marker F4/80 and the neutrophils marker GR-1. FACS analysis from a representative animal demonstrates that ~95% of the cells isolated from the peritoneum are macrophages.

treatment with 1.5 mg/kg of VB-201 significantly reduced plaque formation relative to the PBS control (Fig. 5A). This effect was clear in spite of the fact that VB-201 administration did not modify mouse weight or metabolic parameters associated with elevated risk for the development of atherosclerosis such as total plasma cholesterol or triglycerides (Fig. 5B–D) and their lipoprotein FPLC profile (Supplementary Fig. 7).

Increased bone marrow and blood monocytosis is a known feature of *ApoE KO* mice [25]. Looking into the possibility that VB-201 reduces monocyte mobilization from bone-marrow to the blood, we performed monocyte counts in the bone-marrow, as well as blood cell counts, in VB-201-treated mice versus controls. However, there was no significant effect of VB-201 on the number of monocytes in the bone marrow or in the circulation, either in *ApoE*$^{-/-}$ mice or in thioglycollate-treated wild-type C57B6/J mice. Similarly, VB-201 had no significant effect on blood counts of neutrophils, lymphocytes, eosinophils, basophils and platelets (Supplementary Figs. 8 and 9).

To test whether the VB-201-mediated inhibition of atherosclerosis development correlated with reduced monocyte/macrophage accumulation, aortic sinus lesion specimens were sectioned and stained for macrophage presence using anti-CD68 antibody. As presented in Fig. 5E, macrophage accumulation in the aortic sinus lesion was reduced in VB-201-treated animals relative to controls. These data indicate that treatment with VB-201 inhibited macrophage accumulation in atherosclerosis plaques, which correlated with smaller lesions.

4. Discussion

Inhibition of leukocyte chemotaxis towards inflammatory sites is an attractive anti-inflammatory approach which could be implemented in various chronic diseases. Accordingly, pharmaceutical companies have put in a significant effort in this direction, developing inhibitors, blocking antibodies and antagonists of chemokine receptors, including CCR1-5, CCR9 and CXCR1-4. Data

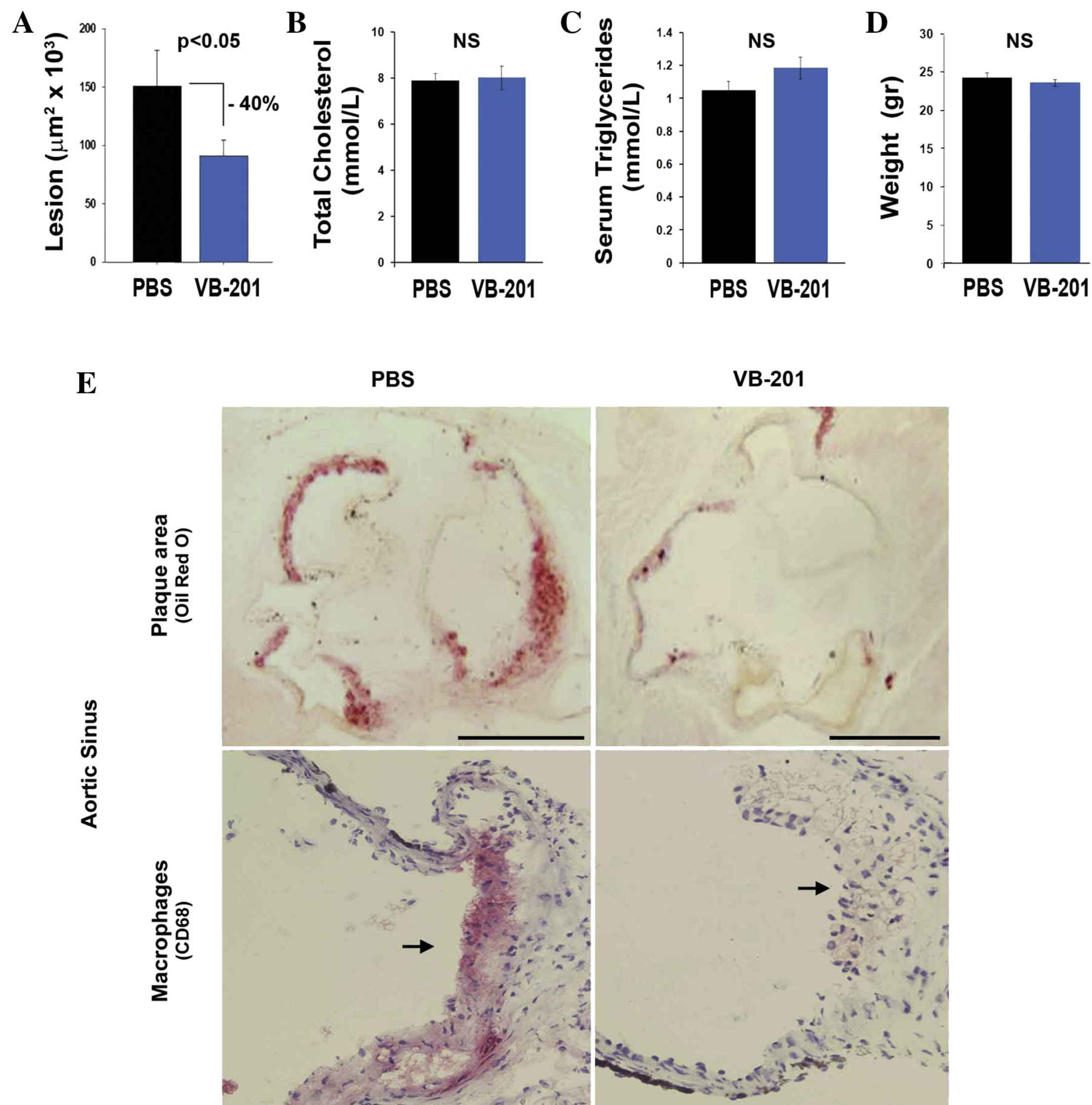

Fig. 5. VB-201 inhibits atherosclerosis development in *ApoE* knockout mice without affecting their lipid profile. (A) *ApoE KO* mice were treated with a daily oral dose of VB-201 (1.5 mg/kg) or PBS as a control from age of 9–11 weeks for 8 weeks. Aortic sinus lesion area was assessed by oil red O staining. Data are mean ± SE (n = 11–12/group). (B) Total cholesterol, (C) serum triglyceride and (D) body weight values of treated groups. Data are mean ± SE (n = 11–12/group). NS = not significant. (E) Representative pictures of oil red O staining (4× lens) showing an atherosclerotic plaque in the aortic sinus of PBS-treated animal, which is correlated by high macrophage content (10× lens). The scale bar represents 0.5 mm. The plaque and macrophages stains are dramatically weaker in VB-201-treated animals. Arrows point to a lesion site in a PBS-treated animal and to a corresponding site in a section from VB-201-treated mouse.

emerging from clinical trials have provided proof-of-concept to this strategy. Nevertheless, over the recent years there have been several phase II clinical trial failures with chemokine receptor antagonists, possibly due to redundancy of the target receptor and the complexity of heterogeneous diseases such as multiple sclerosis and rheumatoid arthritis [26]. It has been suggested that development of dual or promiscuous antagonists, which may simultaneously inhibit two or more different receptors may overcome the redundancy hurdle [26,27].

VB-201 is rationally designed, oxidized but non-oxidative phospholipid analog which was developed by VBL Therapeutics along with other phospholipid pipeline molecules of the lecinoxoid family. When tested in experimental animal models *in vivo*, VB-201 ameliorated the severity of experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) peptide MOG35–55[21] and showed promising efficacy in rheumatoid arthritis and colitis models (data not shown). Yet, it was unclear how VB-201 induces such anti-inflammatory effects. Our previous work has shown that VB-201 limited inflammatory cell infiltration into the CNS and restricted Th1 cell polarization [21]. In this study, we report anti-atherogenic activity of VB-201 and provide mechanistic insight to VB-201-mediated inhibition of monocyte chemotaxis *in vitro* and *in vivo*.

The MEK–ERK pathway is important for monocyte chemotaxis and its inhibition significantly hampers monocyte migratory properties [28,29]. The PI3K–AKT pathway was also implicated in cell chemotaxis, and PI3K inhibitors reduced monocyte migration *in vitro* [29,30]. Furthermore, knockout of PI3Kγ or Akt1 reduced monocyte/macrophage chemotaxis [31,32]. *In vitro* kinase assays indicate that VB-201 does not inhibit human PI3Ks, AKT1, c-RAF, MEK1 or ERK2 enzymatic activity (data not shown), however it leads to blunted AKT and ERK1/2 activation upon chemokine stimulation, in a manner which can be enhanced by combination with inhibitors of PI3K, mTOR or RAF (Fig. 3).

Our data demonstrate that VB-201 does not block the initial event of chemokine receptor activation but rather inhibits signaling events downstream of the receptor in a mechanism yet to be defined. In this sense, VB-201 induces a polypharmacological effect independent of the ligand/receptor employed. Nevertheless, the fact that VB-201 did not alter other monocytic activities such as phagocytosis or adhesion to activated endothelial cell and did not inhibit granulocyte phagocytic activity (Supplementary Fig. 5) or the chemotactic capacity of T-cells and neutrophils (Supplementary Fig. 6), suggest that the inhibitory effect of VB-201 is process- and cell-specific.

Two different models were employed in this study to test the hypothesis that VB-201 may inhibit monocyte chemotaxis *in vivo*. In the thioglycollate-induced mouse peritonitis model, VB-201-treatment resulted in dose-dependent inhibition of macrophage accumulation in the peritoneum (Fig. 4). In the second model, VB-201 inhibited atherosclerosis development in *ApoE KO* mice with clear evidence of reduced lesional macrophage staining (Fig. 5E). The observation that VB-201 treatment reduced lesion size irrespective of the lipid profile, further supports the contention that the immunomodulatory properties of VB-201 are responsible for its anti-atherogenic effects. Intriguingly, such properties may be shared by native phospholipid species, as *in vitro* inhibition of monocyte chemotaxis was also seen with the native oxidation-product PGPC (Fig. 1). It is conceivable that the net pro- versus anti-inflammatory effect of phospholipids *in vivo* is dependent on the identity, abundance and half-life of the phospholipid species found in the circulation and lesion site. By shifting the balance from pro- to anti-inflammatory molecules, for example through delivery of a stable anti-inflammatory lecinoxoid such as VB-201, the inflammatory process may be restrained.

Over the past few decades, prevention and treatment of atherosclerosis have been mostly focused on regulation of cholesterol and blood-pressure. Statins, anti-coagulation and blood-pressure-lowering drugs have helped millions worldwide, and multiple drugs in development continue to pursue the lipid angle of atherosclerosis as a target. Nevertheless, numerous recent studies have emphasized that inflammation plays a significant role in atherosclerosis, and this key vector has not been adequately met thus far. Our findings demonstrate for the first time *in vivo* inhibition of monocytes migration by an oral phospholipid-based small molecule, which reduces macrophage accumulation and decreases experimental atherosclerotic lesion development even without affecting the cholesterol or triglyceride levels. Such data may pave the way towards modulation of chronic inflammatory disorders via regulation of monocyte trafficking, using oral anti-inflammatory synthetic phospholipid analogs.

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Disclosures

The authors are employees and stock options holders of VBL therapeutics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.06.005.

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