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Decreased M1 macrophage polarization in dabigatran-treated *Ldlr*-deficient mice: Implications for atherosclerosis and adipose tissue inflammation

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HIGHLIGHTS

- The direct oral thrombin inhibitor dabigatran induces adipocyte hypertrophy via a PAR-4 dependent mechanism.
- Dabigatran decreases proinflammatory M1 macrophage polarization in visceral AT by alterations in adipocytes secretory profile.
- Dabigatran decreases M1 macrophages also in the aortic wall.
- Dabigatran effects on AT secretome may inhibit macrophage-driven inflammation during atheroprogression.

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ABSTRACT

Background and aims: The non-vitamin K oral anticoagulant dabigatran etexilate (dabigatran) is increasingly prescribed to patients with non-valvular atrial fibrillation and venous thromboembolism. Adipose tissue (AT) inflammation during obesity plays a crucial role in the development of insulin resistance, type II diabetes and atherogenesis. The aim of the present study was to investigate the effects of thrombin inhibition by dabigatran in a combined model of diet-induced obesity and atherosclerosis.
Methods: Female L*ow density lipoprotein receptor* knockout (*Lldr*$^{-/-}$) mice were fed a high-fat diet containing 5 mg/g dabigatran or matching control for 20 weeks.
Results: Dabigatran-treated animals showed increased adipocyte hypertrophy, but reduced numbers of pro-inflammatory M1-polarized macrophages in the adipose tissue. Abundance of pro-inflammatory M1 macrophages was also decreased in the aortic wall of dabigatran-fed mice. Multiple circulating cytokines were reduced, indicating an effect in systemically relevant secretory compartments such as the AT.
Conclusions: Dabigatran treatment reduces pro-inflammatory M1 macrophages in atherosclerotic lesions, thereby contributing to plaque stabilizing and atheroprotective effects of the thrombin inhibitor. This finding is not restricted to the vascular wall but is also present in AT where dabigatran treatment reduced the release of pro-inflammatory cytokines and accumulation of M1 macrophages.

1. Introduction

The direct thrombin inhibitor dabigatran has been shown to exert coagulation-independent benefits in experimental atherosclerosis [1–3], in part by limiting thrombin-driven inflammatory signalling through protease-activated receptors (PAR).

Clinical and experimental studies have associated visceral adiposity and type II diabetes with a procoagulant state, characterized by an

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increased capacity for thrombin generation and augmented thrombin activation markers [4–8]. Visceral obesity is moreover implicated in atherosclerosis development [9]. An underlying factor in obesity-associated insulin resistance, diabetes [10,11] and atherosclerosis [12] is chronic low-grade inflammation of visceral adipose tissue (VAT), with accumulation of pro-inflammatory M1-polarized macrophages driving secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [13,14]. How VAT inflammation contributes to atherosclerosis development, and how a thrombin inhibitor will impact on this, has not been fully elucidated.

We sought to investigate the effects of pharmacological thrombin inhibition with dabigatran on inflammatory processes in a murine model of combined obesity-induced AT inflammation and accelerated atherosclerosis [15], and to elucidate a possible interrelationship between inflammation in these two compartments.

2. Materials and methods

Detailed methods are provided in the Supplementary Materials and the Major Resource Table.

2.1. Animals

Female 10 week-old *Low-density lipoprotein receptor* knockout (*Ldlr*$^{-/-}$) mice (Jackson Laboratory, Sacramento, CA, USA) and C57BL/6J mice (Janvier, Le Genest St Isle, France) received control chow (3438, Provimi Kliba AG, Kaiseraugst, Switzerland) or a high-fat diet (HFD; D12451; Research Diets, Inc., New Brunswick, NJ, USA) supplemented ± dabigatran etexilate (5 mg per g HFD) for 20 weeks (Supplementary Fig. I A). Experiments conformed with guidelines for the use of experimental animals of the Deutsches Tierschutzgesetz and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, Bethesda, MD, USA).

2.2. Dabigatran plasma concentrations and anticoagulant activity

Dabigatran plasma levels were measured by mass spectrometry (Nuvisan, Neu-Ulm, Germany). Mean (± standard deviation, SD) dabigatran plasma levels were 226 ± 149 ng/ml. Anticoagulation was assessed by Endogenous Thrombin Potential (ETP) and the Hemoclot Thrombin Inhibitors Assay (CoaChrom Diagnostica GmbH, Maria Enzersdorf, Austria).

2.3. Sample preparation

Subcutaneous and visceral adipose tissue (VAT), hearts and total aortas were analysed by immunohistochemistry, flow cytometry and molecular biology. For conditioned medium, VAT was incubated for 24 h in Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Life Technologies™, Paisley, UK) under cell culture conditions, then stored at −80 °C.

2.4. VAT histology and immunohistochemical analysis

Three consecutive paraffin-sections (5 µm) separated by 300 µm were analyzed. Adipocyte size was assessed in H&E stained sections using Image J 1.37v software (NIH), Fiji and the BioVoxxel Image Processing and Analysis Toolbox (BioVoxxel, Mutterstadt, Germany). Macrophages were stained with galectin-3 (Mac 2). Aortic roots assessed by Sirius red birefringence analysis [16].

2.5. Aortic plaque score

Aortic plaque score was determined in Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) stained aortas using ImageJ.

2.6. Quantitative real-time PCR (qPCR)

qPCR was performed with the Applied Biosystems 7300 Real-Time PCR System (aortic and 3T3-L1 samples) or the StepOnePlus Real-Time PCR System (VAT) using SYBR Green PCR master mix (Life Technologies GmbH, Carlsbad, CA, USA) as described [17]. Primer sequences are shown in Supplemental Table 1.

2.7. Flow cytometric analysis

Stromal vascular cells from VAT [18] and aortic immune cells [19] were assessed as described, with protocol modifications detailed in the methods supplement. Circulating immune cells were assessed in blood collected by cardiac puncture according to a modified protocol [20]. Measurements were performed with a Gallios™ Flow Cytometer, and analyzed using Kaluza® Flow Analysis Software (both Beckman Coulter Inc., Krefeld, Germany).

2.8. Multiplex analysis

Plasma cytokine levels were analyzed by multiplex bead-based immunoassay Bio-Plex suspension array system (Biorad, Hercules, CA, USA) as instructed. Protein concentrations were calculated from optimized standard curves with Bio-Plex Manager Software version 6.0 (Biorad).

2.9. In vitro adipogenesis

3T3-L1 preadipocytes (Sigma-Aldrich) were differentiated in absence and presence of human α-thrombin (3 U/ml; Sekisui Diagnostics, LLC, Stamford, CT, USA), human lung β tryptase (0.1 ng/mL, Promega, Mannheim, Germany), human leukocyte cathepsin G (0.01 U/mL, BioCentrum, Krakau, Poland) or synthetic activating peptides (AP, 100 µmol/L) for murine PAR-2 (SLIGRL-NH2) or PAR-4 (AYPGKF-NH2, both synthesized by the BMFZ, University Hospital Düsseldorf, Germany). Lipid accumulation was assessed by Oil Red O staining, proliferation with Rotitest® Vital (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

2.10. In vitro differentiation and polarization of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated and differentiated after a modified protocol [21] for 7 days with 10 ng/ml macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA). M1 polarization was induced with 10 U/ml interferon (IFN)–γ (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and 1 µg/ml lipopolysaccharide (LPS from *Salmonella enterica, serotype minnesota*; Sigma-Aldrich) [22]. M2 polarization was induced with 20 ng/ml IL-4 (Sigma-Aldrich) [23]. Polarization markers were assessed by flow cytometry in cells stimulated ± thrombin (24 h). Antibodies are listed in the Major Resource Table.

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SD. Statistical significance was set at the level of $p \leq 0.05$.

3. Results

3.1. Anticoagulation in dabigatran-fed mice

ETP and Hemaclot assays confirmed reduced thrombin activity with significantly prolonged lag time, time to peak and clotting time in dabigatran-fed mice (Supplementary Fig. IB-F).

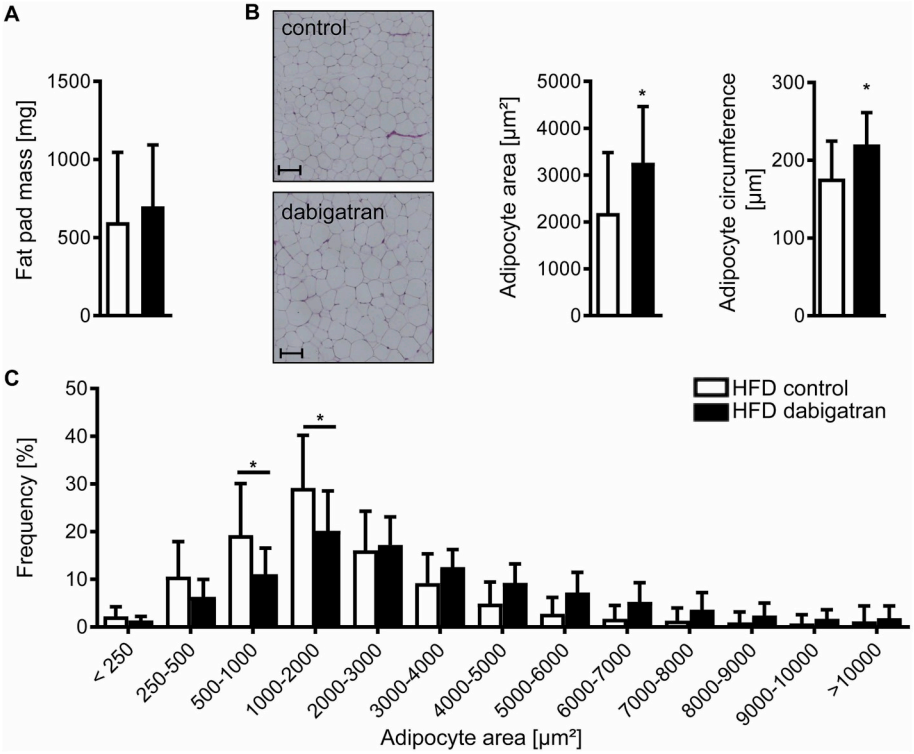

Fig. 1. Dabigatran increases adipocyte hypertrophy in *Ldlr*$^{-/-}$ mice.
(A) Visceral adipose tissue (VAT) mass (n ≥ 10) and (B) VAT morphology (representative photomicrographs of n ≥ 22, 100-fold magnification, scale bar = 100 µm) and calculated adipocyte area, circumference and (C) frequency distribution of adipocyte area. Mean ± SD, *$p \leq 0.05$ *versus* high-fat diet (HFD) control.

3.2. Metabolic response to HFD in female *Ldlr*$^{-/-}$ mice

HFD increased body weight gain and body mass index compared to control chow (Supplementary Fig. II A-C, all n ≥ 5). VAT histology showed a trend towards increased adipocyte area under HFD (Supplementary Fig. I D-F). Fasting blood glucose levels were unaltered, but oral glucose tolerance test (GTT p.o.) showed impairment (Supplementary Fig I G-I).

3.3. Adipocyte hypertrophy in dabigatran-treated mice

Dabigatran did not alter average food consumption, weight gain, body mass index or total body fat content compared to control HFD (Supplementary Fig. III). Visceral fat pad weight did not differ between the groups (Fig. 1 A), but dabigatran increased adipocyte size in VAT (Fig. 1B, C) and subcutaneous AT (Supplementary Fig. IV). Fasting blood glucose, fasting insulin or systemic glucose tolerance and insulin sensitivity were unaffected (Supplementary Fig. V). PAR-1 (*F2r*), PAR-3 (*F2rl2*) and PAR-4 (*F2rl3*) mRNA was comparable in VAT of both groups (Supplementary Fig. VI A-C). *Ki67* mRNA expression or PARP cleavage (Supplementary Fig. VI D, E) were also not different. Dabigatran modestly raised lipoprotein lipase (*Lpl1*), fatty acid translocase (FAT, *Cd36*), fatty acid transport protein 1 (FATP1, *Slc27a1*) and acyl-CoA synthetase (*Acsl1*), and slightly decreased perilipin (*Plin1*) mRNA (Supplementary Fig VII). *Cd36* and *Slc27a1* mRNA showed the strongest trend but no regulation was observed at the protein level. Plasma membrane fatty acid-binding protein (FABPpm, *Got2*), adipose triglyceride lipase (ATGL, *Pnpla2*) and hormone-sensitive lipase (*Lipe1*) were not regulated by dabigatran (Supplementary Fig. VII I, J).

3.4. Anti-adipogenic action of thrombin in 3T3-L1 adipocytes

Adipocyte hypertrophy was examined in differentiating 3T3-L1 cells *in vitro*. Thrombin stimulation during differentiation strongly inhibited accumulation of Oil Red O-stained lipids (Fig. 2A). Thrombin did not drive preadipocyte proliferation (Fig. 2B). PAR-1 (*F2r*) and PAR-4 (*F2rl3*) are expressed in 3T3-L1 cells but only *F2rl3* is transcriptionally upregulated during maturation (Fig. 2C). Western blot analysis confirmed upregulation at the protein level (Fig. 2 D). Anti-adipogenic effects of thrombin were mimicked by PAR-4AP and cathepsin G (Fig. 2 E, Supplementary Fig. VIII A-B). *F2rl1* mRNA and PAR-2 was upregulated during 3T3-L1 differentiation (Supplementary Fig. VIII C, D). PAR-2AP or tryptase further increased lipid accumulation and leptin gene expression in differentiating cells (Supplementary Fig. VIII E, F).

3.5. Dabigatran decreases pro-inflammatory macrophages in VAT of *Ldlr*$^{-/-}$ mice

Dabigatran suppressed pro-inflammatory CD11b^{+}F4/80^{+}CD11c^{+} M1-like macrophages without affecting total macrophage numbers, as determined by flow cytometry and quantification of Mac2-positive crown-like structures (Fig. 3A–C). Other immune cell populations were unaltered (Supplementary Fig. IX; gating schemes in Supplementary Figure X). Multiplex bead-based immunoassay of VAT supernatants showed reduced secretion IL-6, IL-5 and granulocyte (G)-CSF with dabigatran (Fig. 3D).

3.6. Thrombin promotes M1 polarization by modulating at cytokine secretion

To evaluate if thrombin directly influences macrophage phenotype, BMDM differentiated with M-CSF were polarized using IFN-γ/LPS (M1) or IL-4 (M2), alone or with thrombin. Flow cytometry gating schemes are shown in Supplemental Figure XI. Thrombin did not directly influence macrophage polarization (Fig. 4A), but conditioned medium from thrombin-stimulated 3T3-L1 adipocytes shifted BMDM towards a pro-inflammatory CD86^{+} phenotype (Fig. 4B). Conditioned medium from VAT of dabigatran-treated mice shifted the M1/M2 ratio towards less M1-polarized BMDM (Fig. 4C).

3.7. Dabigatran reduces systemic and aortic inflammation and atherosclerosis

Dabigatran treatment did not modify circulating immune cell

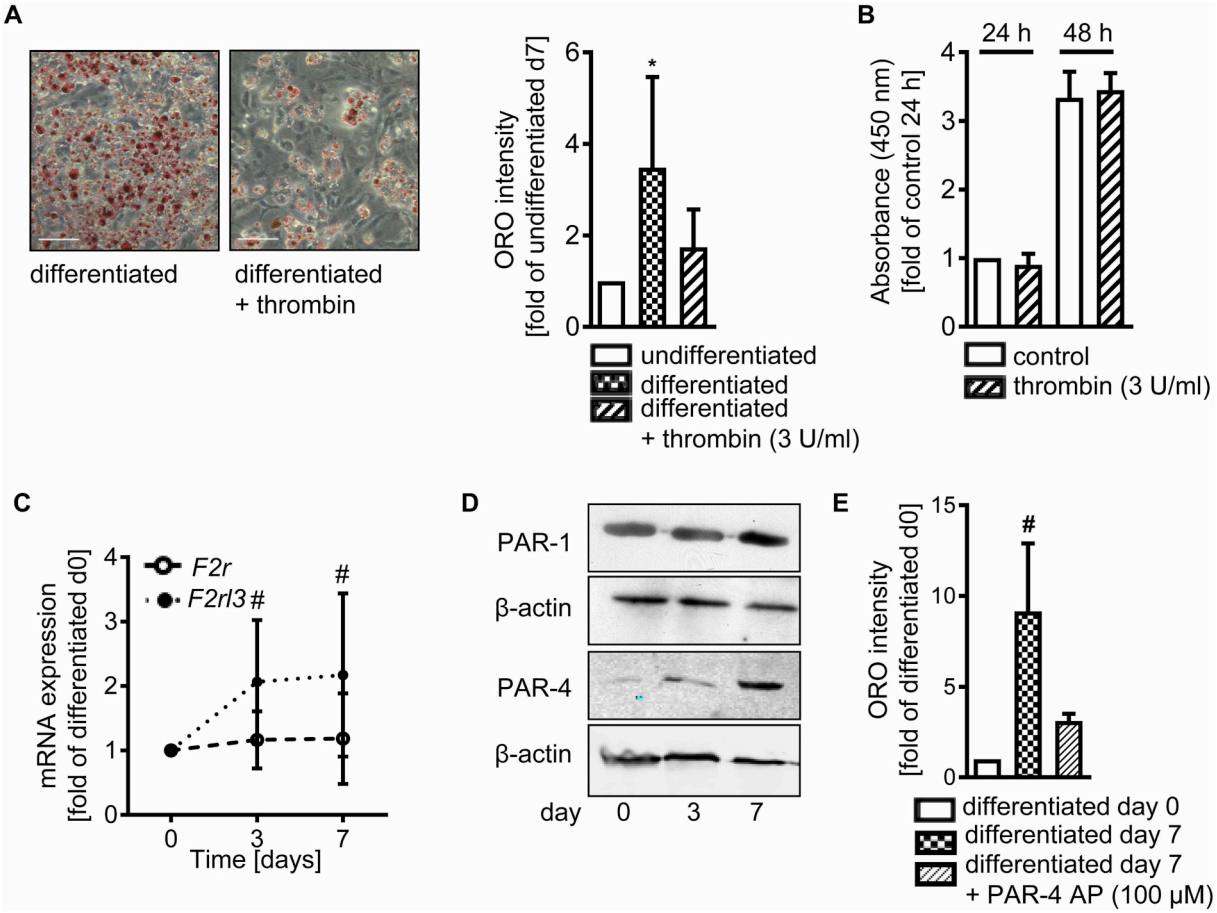

Fig. 2. Thrombin inhibits adipocyte lipid accumulation.
(A) Representative photomicrographs (scale bar = 100 μm) and quantification of Oil Red O (ORO)-stained lipids in 3T3-L1 cells differentiated 7 days in the absence and presence of thrombin (3 U/ml, n = 6). (B) Proliferation of 3T3-L1 preadipocytes stimulated 24 or 48 h with thrombin (n = 3).
(C) PAR-1 (*F2r*) and PAR-4 (*F2rl3*) mRNA (n = 8) and (D) protein expression (representative Western blots of n = 3 independent experiments). (E) ORO-stained lipids in 3T3-L1 cells differentiated in the absence and presence of PAR-4 AP (100 μM, n = 4). Mean ± SD; $^{*}p \leq 0.05$ *versus* undifferentiated d7, $^{\#}p \leq 0.05$ *versus* differentiated d0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

subsets (Supplementary Fig. XII; gating scheme Supplementary Fig. XIII) or plasma lipids (Supplementary Fig. XV) in mice fed HFD, but reduced multiple circulating cytokines (Fig. 5A, Supplementary Fig. XIV). Atherosclerotic lesion formation (Fig. 5B) and pro-inflammatory M1-polarized macrophage ($CD11b^+F4/80^+CD86^+$) numbers were reduced under treat dabigatran; total macrophage ($CD11b^+F4/80^+$) and anti-inflammatory M2 macrophages ($CD11b^+F4/80^+CD206^+$) numbers were unaltered (Fig. 5C, D; gating schemes in Supplemental Figure XVI). Dabigatran did not affect aortic lipid deposition (Supplementary Fig. XVII A) or hyaluronan content (data not shown). Total collagen content was increased only slightly but tightly-arranged collagen fibril abundance was significantly higher with dabigatran (Supplementary Fig. XVII B). Expression of *collagen type I, alpha 1 (collagen1a1)* and *collagen type III, alpha 1 (collagen 3a1)* mRNAs were unaffected (Supplementary Fig. XVII C), suggesting post-transcriptional matrix stabilisation. The small leucine-rich proteoglycan decorin was not regulated by dabigatran (data not shown) but biglycan was upregulated (Supplementary Fig. XVII D) while matrix metalloproteinase MMP-13 was downregulated (Suppl Fig. XVII F). *Biglycan* mRNA upregulated only modestly (Supplementary Fig. XVII E).

4. Discussion

The thrombin inhibitor dabigatran improves local and systemic inflammatory under HFD, in part through modification of macrophage phenotype in adipose tissue and aorta. This likely contributes to the atheroprotective effects of thrombin inhibition.

Female $Ldlr^{-/-}$ mice fed HFD for 20 weeks developed typical metabolic sequelae (moderate obesity, body fat accumulation, impaired glucose and insulin homeostasis) and systemically elevated cytokine burden. Dabigatran supplementation led to a significant anticoagulant effect but did not affect food intake, weight gain, body mass index, proportion of body fat or visceral fat pad weight. However, adipocyte area and circumference was strikingly increased in visceral (VAT) and subcutaneous adipose tissues. Since VAT mass was not altered, total adipocyte numbers may be reduced under dabigatran, through enhanced apoptosis, decreased preadipocyte proliferation and/or altered lipid handling. Dabigatran did not regulate a spectrum of lipid transporters, lipases or other genes controlling lipid metabolism, and did not affect PAR peptides (PARP) cleavage. The proliferation marker *Ki67* was modestly suppressed. Presumably, a constellation of small long-term changes including altered proliferation and transcriptional and post-transcriptional regulation of factors involved in lipid transport and storage, underlie the enhanced adipocyte hypertrophy under dabigatran-treatment.

Adipocyte size is essentially determined by the need to store energy as lipids and triglycerides. Thrombin markedly suppressed lipid accumulation in differentiating 3T3-L1 adipocytes *in vitro*, an effect directly counteracted by dabigatran (500 nmol/L, data not shown). 3T3-L1 express the major thrombin receptors *F2r* (PAR-1), *F2rl2* (PAR-3), *F2rl3* (PAR-4) and *Thbd* (thrombomodulin, not shown), but only PAR-4 is upregulated during differentiation. A synthetic PAR-4AP and the PAR-

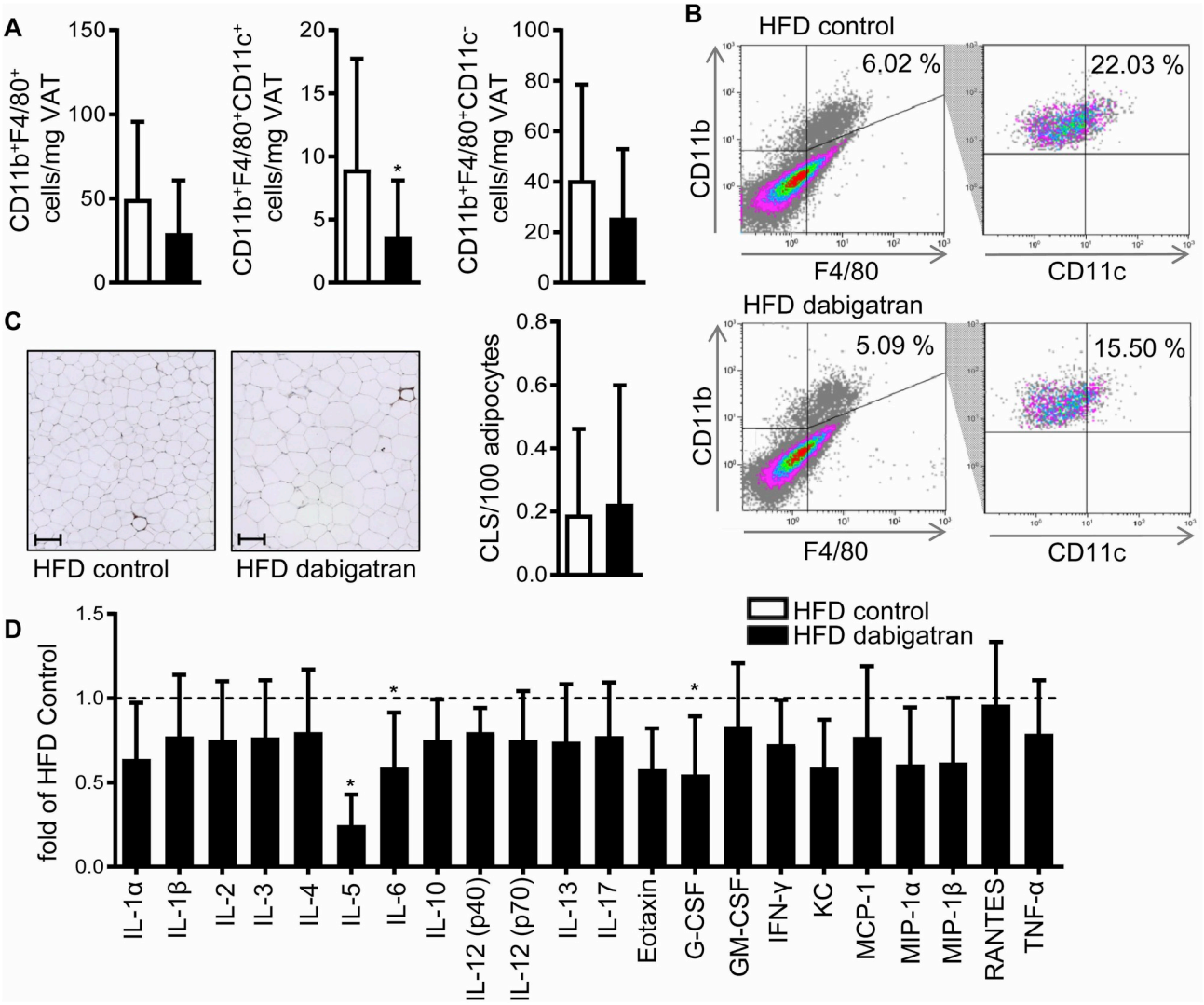

Fig. 3. Dabigatran reduces pro-inflammatory macrophages in visceral adipose tissue of *Ldlr*$^{-/-}$ mice.
(A) Total (CD11b^{+}F4/80^{+}), CD11c^{+} and CD11c^{-} macrophages in stromal vascular cells of visceral adipose tissue (VAT, n ≥ 11). (B) Representative plots of CD11b^{+}F4/80^{+} (gated on living cells) and CD11c^{+} macrophages. (C) Representative photomicrographs of galectin-3 (Mac-2)-stained macrophages (100-fold magnification, scale bar = 100 μm) and crown-like structures (CLS) per 100 adipocytes (n ≥ 12). (D) Cytokine multiplex analysis of VAT-conditioned medium (n ≥ 7). Mean ± SD,*$p \leq 0.05$ *versus* high-fat diet (HFD) control. IL, interleukin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; KC, platelet-derived growth factor-inducible protein keratinocyte chemoattractant; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; TNF, tumor necrosis factor.

4-activating protease cathepsin G mimic the anti-adipogenic effect of thrombin. Together with reports that PAR-1 deficiency does not influence obesity development *in vivo,* [24,25] these observations strongly implicate PAR-4 as a key mediator of adipocyte responses to thrombin. *F2rl1* (PAR-2), which does not respond to thrombin, is also upregulated in differentiating 3T3-L1 cells, but unlike PAR-4 further drives adipogenic maturation in response to PAR-2AP or tryptase, in keeping with the reported role of PAR-2 in metabolic disease *in vivo* [24]. The relative levels of PAR and activating proteases will likely determine net outcome *in vivo*. The thrombin inhibitor argatroban was recently reported to downregulate PAR-1 and PAR-4 in diabetic mouse hearts [26], in the mouse model used here, dabigatran only modestly lowered VAT *F2r* and *F2rl3* gene expression. Dabigatran effects *in vivo* are therefore attributable more likely to inhibition of thrombin-stimulated activation of available PAR, rather than receptor downregulation. Additionally, inhibition of thrombin-regulated fibrinogen/fibrin deposition may contribute. Thrombin was recently shown to promote diet-induced obesity through fibrin/fibrinogen-driven inflammation, with modulation of macrophage polarization discussed as a candidate mechanism [27].

We here show that thrombin inhibition with dabigatran reduces inflammatory macrophage abundance in VAT and suppresses VAT-secreted cytokines such as IL-6. This fits with observations that thrombin stimulates cytokine secretion from human adipose tissue *ex vivo* [28], and suggests that the effects observed here in mice may be extrapolated to patients treated with dabigatran.

Clearly, adipocyte enlargement must not always associate with VAT inflammation and dysfunction, in line with the concept of “healthy obese adipose tissue”. The inhibitory influence of dabigatran was specific for the inflammatory macrophage subset, leaving total macrophages and other leukocytes unchanged. Mechanistically, this implies a selective influence of thrombin on macrophage polarization, as described in human monocytes and macrophages [29,30]. In this study, thrombin did not directly influence polarization of murine BMDM, so the phenotypic switch is controlled indirectly via modulation of the

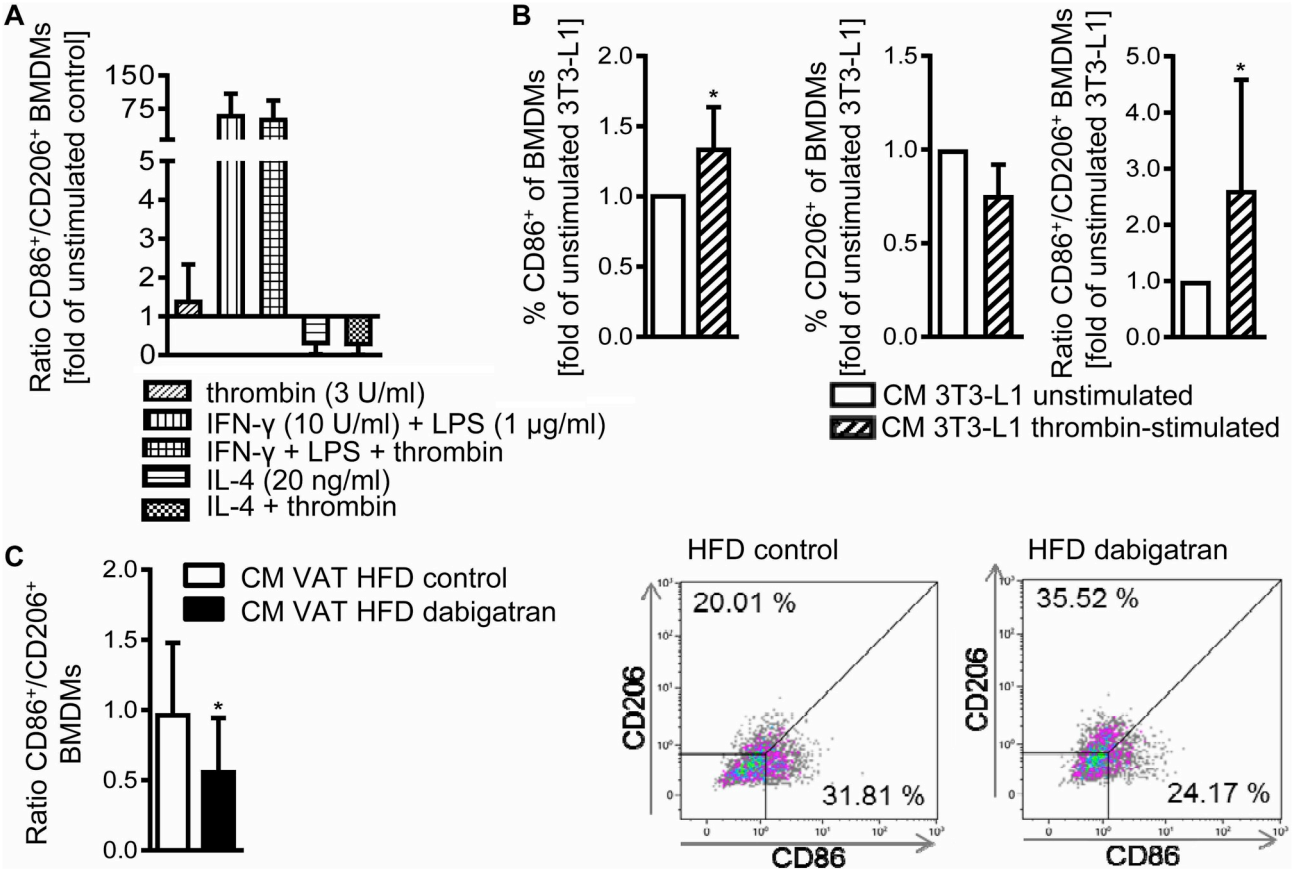

Fig. 4. Dabigatran modifies adipose tissue macrophage polarization in *Ldlr*$^{-/-}$ mice.
(A) Polarization assessed as ratio of CD86$^+$ to CD206$^+$ bone marrow-derived macrophages (BMDMs) stimulated 24 h at indicated concentrations interferon (IFN)-γ and lipopolysaccharide (LPS) or interleukin (IL)-4, with or without thrombin (n = 6), or (B) conditioned medium (CM) of 3T3-L1 cells differentiated with or without thrombin for 7 days, shown as percent CD86$^+$ and CD206$^+$ macrophages and as ratio of CD86$^+$ to CD206$^+$ BMDM (n = 7). (C) Ratio of CD86$^+$ to CD206$^+$ BMDM incubated for 24 h with CM from visceral adipose tissue (VAT) of *Ldlr*$^{-/-}$ mice fed high fat diet (HFD) with and without dabigatran for 20 weeks (n = 13). Representative plots gated on CD11b$^+$F4/80$^+$ cells. Mean ± SD,*$p \leq 0.05$ *versus* CM 3T3-L1 unstimulated (B) or CM VAT HFD control (C).

secretory profile of adipocytes, and possibly other cells in the local microenvironment. In line with this, conditioned medium of thrombin-treated 3T3-L1 adipocytes increased M1 polarization of BMDM, while conditioned medium of VAT from dabigatran-treated animals reduced the ratio of M1 to M2-polarized macrophages *in vitro*. Multiplex analysis of secreted VAT cytokines identified IL-6, G-CSF and IL-5 as candidate mediators reduced by dabigatran.

The observed effects of dabigatran on adipocyte hypertrophy without changes in body weight, body fat content and glucose metabolism might be specific for our mild model of diet-induced obesity. Two recent studies showed significantly lower body weight in male C57BL/6J mice on HFD plus dabigatran [27,31]. No change in body weight was seen with argatroban in male *Ldlr*$^{-/-}$ mice with established fatty liver disease, at least over the 4-week study period [32], or *Lepr*^{db} mice with pronounced obesity and type II diabetes [33]. In the latter study, adipocyte hypertrophy was reduced, and insulin sensitivity improved. In all these studies, HFD-driven weight gain was much higher than in the female HFD-fed *Ldlr*$^{-/-}$ mice used here. Therefore, differences in the severity of the metabolic phenotype as well as sex-dependent differences might account for the variable findings. The use of female mice with a mild metabolic phenotype is specific to the present study.

The modulatory impact of dabigatran on M1 macrophage phenotype was also seen in the atherosclerosis-prone aorta, which likely contributes to the atheroprotective effect. Analysis of atherosclerotic plaque composition revealed increased biglycan expression in dabigatran-treated mice, presumably resulting from reduced degradation by MMP-13. In line with the role of biglycan in stabilizing the vascular collagen matrix [34], tightly-arranged collagen fibrils were increased. Notably, biglycan can directly inhibit thrombin and suppress macrophage-driven inflammation *in vivo* [35].

Besides this direct impact of dabigatran in the vascular wall, an indirect action might result from the altered VAT cytokine/adipokine secretion profile. Notably IL-6 was markedly reduced both in plasma and in VAT-conditioned media under dabigatran treatment. Human subcutaneous AT is an important source for circulating IL-6,[36] the same may hold for VAT. Cytokine secretion may therefore represent one means by which adipocytes can influence inflammatory signalling and immune function both locally and in distant target organs. Accordingly, pro-inflammatory cytokine secretion from VAT is increased in patients with coronary artery disease [37]. Finally, pleiotropic coagulation- and/or thrombin-independent effects of dabigatran cannot be excluded.

In summary, we show that thrombin inhibition with dabigatran in female *Ldlr*$^{-/-}$ induces adipocyte hypertrophy without concomitant inflammatory dysfunction. Mechanistically, dabigatran appears to limit thrombin-triggered PAR-4 activation and cytokine secretion, leading to reduced M1 macrophage polarization. This phenotypic modulation is also seen in aortic macrophages, likely contributing to the atheroprotective effect of dabigatran.

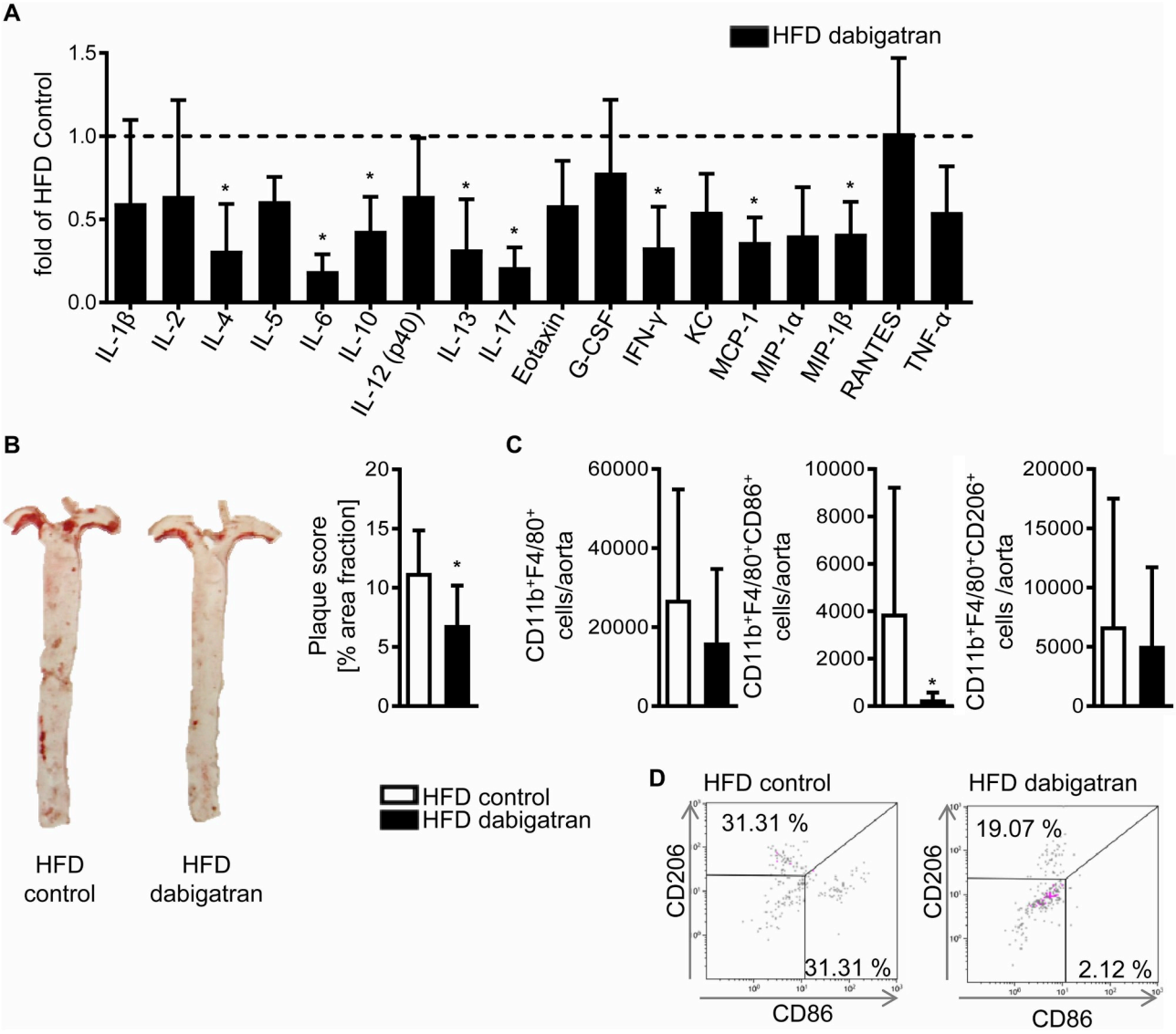

Fig. 5. Dabigatran reduces inflammatory burden in *Ldlr*$^{-/-}$ mice.
(A) Plasma cytokines after 20 weeks of dabigatran. IL, interleukin; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; KC, platelet-derived growth factor-inducible protein keratinocyte chemoattractant; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; TNF, tumor necrosis factor. (B) Representative images of Oil Red O-stained aortas and quantification of aortic plaque score. (C) Total (CD11b$^+$F4/80$^+$), proinflammatory CD86$^+$ and anti-inflammatory CD206$^+$ macrophages per aorta are shown. (D) Representative plots gated on CD11b$^+$F4/80$^+$ cells. Mean ± SD, *$p \leq 0.05$ *vs.* high-fat diet (HFD) control, all n ≥ 8. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

K.F. performed experiments and reviewed the manuscript. M.G. and J.W.F. wrote the manuscript, contributed to the discussion and reviewed/edited the manuscript. C.K., B.V., S.G. and N.N. performed experiments. A.C.F. performed experiments, wrote and reviewed the manuscript. S.H. and S.L. performed analysis by multiplex array. J.W.F. takes full responsibility for the work as a whole.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.06.897.

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