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## **Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization**

Bot, I.

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## Low Dose FK506 Blocks Collar-Induced Atherosclerotic Plaque Development and Stabilizes Plaques in ApoE<sup>-/-</sup> Mice

\*Ilze Bot, \*Marjo M.P.C. Donners<sup>§</sup>, Leon J. De Windt<sup>#</sup>, Theo J.C. van Berkel, Mat J.A.P. Daemen<sup>§</sup>, Erik A.L. Biessen, Sylvia Heeneman<sup>§</sup>.

\*both authors contributed equally to this study

Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands, <sup>§</sup>Dept. of Pathology, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, Maastricht, the Netherlands, <sup>#</sup>Hubrecht Laboratory and the Interuniversity Cardiology Institute of the Netherlands, Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands.

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### Abstract

Since atherosclerosis is a chronic inflammatory disease, we tested the hypothesis that the immunosuppressive drug FK506 would attenuate the development of atherosclerosis using a mouse model of collar-induced atherosclerosis. ApoE<sup>-/-</sup> mice were treated for 4 weeks with the immunosuppressive drug FK506 (0.05 mg/kg/day), yielding sustained blood levels (~0.2 ng/mL) without systemic side-effects. Atherosclerotic plaque development of FK506-treated mice was significantly reduced (63%) while plaque cell density was increased (52%) compared to controls. Importantly, FK506 also blocked progression of pre-existing atherosclerotic plaques. Plaque area of pre-existing plaques was 35% reduced by FK506. Cell density (35%) and collagen content (51%) were significantly increased, whereas necrotic core content was decreased (42%), indicating a more stable plaque morphology. Similar results were found during spontaneous atherosclerotic plaque development in ApoE<sup>-/-</sup> mice (treatment 17-25 weeks of age). Flow-cytometric analysis showed no peripheral effects on blood cell count or T-cell activation after FK506-treatment. *In vitro*, FK506 decreased vascular smooth muscle cell (VSMC) apoptosis and inhibited nuclear factor of activated T-cells (NFAT)-luciferase reporter activity at concentrations in the range of the *in vivo* concentration. Low dose FK506 inhibits collar-induced atherosclerotic plaque development and progression and induces more stable plaque phenotypes in ApoE<sup>-/-</sup> mice without any peripheral side-effects.

## Introduction

Atherosclerosis is a chronic inflammatory disease, involving several inflammatory cells such as macrophages and T-lymphocytes, and often complicates transplant biology. Immunosuppressives such as Cyclosporin A (CsA) and FK506 (tacrolimus) suppress inflammation by inhibiting the activation of calcineurin, a calcium/calmodulin-dependent serine/threonine protein phosphatase. Upon activation, calcineurin dephosphorylates the transcription factor NFAT (nuclear factor of activated T cells), which then translocates to the nucleus and regulates the expression of several cytokines such as IL-2, IFN $\gamma$ , TNF $\alpha$  and CD40Ligand<sup>1</sup>.

CsA and FK506 are often used to suppress graft-versus-host disease after transplantation. However, it is not clear whether the use of these immunosuppressives either inhibits or ameliorates the development of (transplant) atherosclerosis. Previous studies on the effect of FK506 and CsA, on transplant arteriosclerosis<sup>2,3</sup> and *de novo* atherosclerosis in hyperlipidemic animals<sup>4-6</sup> were contradictory. Drew *et al.*<sup>4</sup> showed a reduction in atherosclerosis in cholesterol-fed rabbits after CsA treatment, whereas others showed an increase in atherosclerosis in cholesterol-fed rabbits treated with either CsA or FK506<sup>5,6</sup>.

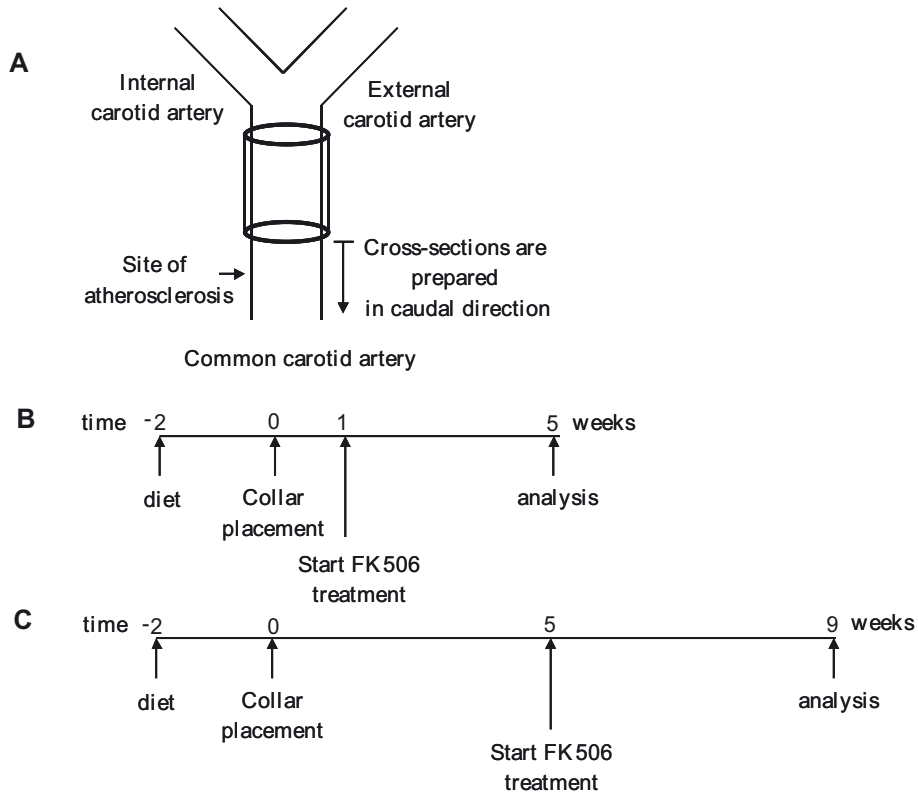
In this study, we tested the effect of FK506 on *de novo* atherosclerosis and progression of established atherosclerotic plaques in ApoE<sup>-/-</sup> mice. In view of reported side-effects we used a low dose of FK506 (0.05 mg/kg/day). Rapid atherogenesis was induced by perivascular collar-placement which generates complex heterogeneous, lipid-rich lesions<sup>7</sup>. Treatment with FK506 blocked initial atherosclerotic plaque development as well as progression of pre-existing lesions. Besides these effects on collar-induced atherosclerosis, we also examined the effects of low dose FK506 on spontaneous development of atherosclerosis in the conventional ApoE<sup>-/-</sup> mouse model. In the absence of an atherogenic diet, low dose FK506 also reduced atherosclerotic plaque development. Our results suggest an important role for calcineurin in the initiation and progression of murine atherosclerosis and show that calcineurin-inhibition by a low dose of the immunosuppressive FK506 has a favorable effect on the development of this wide-spread disease.

## Materials and Methods

### *Mouse model of collar-induced atherosclerosis*

Male ApoE<sup>-/-</sup> mice (10-12 weeks of age) were obtained from TNO-PG (Leiden, the Netherlands) and fed a Western-type diet throughout the experiment. Carotid atherosclerotic lesions were induced by bilateral perivascular collar placement as described by von der Thüsen *et al.*<sup>7</sup> (Figure 1A).

To assess the effect of FK506 on lesion initiation, one week after collar-placement osmotic minipumps (Alzet type 2004, Durect Corporation, Cupertino, USA) containing either 0.17  $\mu\text{g}/\mu\text{L}$  (20 mM) FK506 (Fujisawa GmbH, Munchen, Germany) in PBS (0.05 mg/kg/day, n=9) or PBS alone (n=8), were placed subcutaneously on the back of the mice (Figure 1B). To determine the effect of FK506 treatment on lesion progression, identical osmotic minipumps containing FK506 (0.05 mg/kg/day, n=15) or PBS (n=9) were inserted 5 weeks after collar placement (Figure 1C). In both treatment protocols, mice were treated with FK506 for 4 weeks.



**Figure 1.** (A) Schematic representation of the collar-model. Atherosclerotic lesions develop caudal to the collar, therefore cross-sections were made of the common carotid artery in a caudal direction from the collar and collected in a parallel series of slides. (B) Time schedule of the experiment to study the effect of calcineurin-inhibition on atherosclerotic plaque development. (C) Time schedule of the experiment to study the effect of calcineurin-inhibition on pre-existing atherosclerotic lesions.

#### *ApoE<sup>-/-</sup> mouse model of spontaneous atherosclerosis*

Male ApoE<sup>-/-</sup> mice (17 weeks of age, n=10 per group) obtained from Charles River Laboratories (Maastricht, the Netherlands) were treated with FK506 (0.05 mg/kg/day) or PBS (controls) for 8 weeks by placing osmotic

minipumps subcutaneously on the back of the mice. The osmotic minipumps were replaced after 4 weeks. Mice were fed a normal chow throughout the experiment.

#### *Cholesterol and triglyceride assay*

Blood samples (~100  $\mu$ L) were taken weekly from the mice on the atherogenic diet by tail-cut and plasma was obtained by centrifugation at 8,000 rpm for 10 min. Levels of total plasma cholesterol and triglycerides were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, the Netherlands).

#### *Tissue harvesting and analysis*

Five or nine weeks after collar placement, mice were subjected to *in situ* perfusion-fixation through the left cardiac ventricle, cryosections were prepared and stained with hematoxylin and eosin (HE). Cross-sections with maximal stenosis were used for morphometric analysis using a Leica system, as described previously<sup>8</sup>.

For analysis of spontaneous atherosclerosis, the aortic arch including its main branch points (brachiocephalic trunk/right common carotid artery, left common carotid artery and left subclavian artery) was removed and embedded longitudinally in paraffin. The entire aortic arch was cut into approximately 40 sections (4  $\mu$ m thick), of which 20 consecutive sections, representing the central area of the arch as described previously<sup>9</sup>. The brachiocephalic trunk was used for analysis of atherosclerotic plaque morphometry and morphology.

(Immuno)histochemical stainings were performed to detect macrophages (MOMA-2), VSMC ( $\alpha$ -smooth muscle actin (ASMA)), lipid (Oil red O), T-cells (CD3) and collagen (picosirius red) as described previously<sup>8</sup>.

For macrophages, cell numbers (by counting MOMA-positive cells in one cross-section, expressed as a percentage of total cell numbers) as well as MOMA-positive cell area (expressed as a percentage of plaque area) were assessed. For VSMC and collagen, the area of ASMA-positive cells and picosirius red staining, respectively, was measured and expressed as a percentage of total plaque area. Analysis was performed by one blinded investigator (MMPCD) with an intra-observer variability of <10%.

#### *Assessment of FK506 blood concentration*

Whole blood samples, taken after 4 weeks of FK506-treatment were hemolyzed, deproteinized and analyzed using an on-line solid-phase extraction combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described by Vogeser *et al.*<sup>10</sup>.

#### *Fluorescence-activated cell sorting (FACS)-analysis*

ApoE<sup>-/-</sup> mice (n=10 per group) were treated with FK506 or PBS as described above. After 4 weeks, mice were sacrificed and peripheral blood was obtained from the inferior vena cava. Spleen and lymph nodes (superficial

and deep cervical nodes, brachial nodes and mesenteric nodes) were isolated and used to make single-cell suspensions. Erythrocytes in peripheral blood and spleen were removed by hypotonic lysis with  $\text{NH}_4\text{Cl}$ . Blood cells ( $2 \times 10^5$  cells/sample), spleen and lymph node cells ( $1 \times 10^6$  per sample) were stained with anti-CD3-FITC (17A2, 1:100 diluted), anti-CD8 $\alpha$ -PE (53-6.7, 1:20 diluted) and anti-CD4-Cyc (RM4-5, 1:10 diluted), with anti-CD25-PE (PC61, 1:40 diluted) and anti-CD4-Cyc or with anti-CD69-PE (H1.2F3, 1:20 diluted) and anti-CD3-FITC (all antibodies from BD-Biosciences Pharmingen, San Diego, California). Blood cells ( $2 \times 10^5$  cells/sample) were also stained with anti-CD3-FITC and anti-B220-PE (RA3-6B2, 1:300 diluted) or with anti-Gr1-FITC (RB6-8C5, 1:375 diluted) and anti-Mac1-PE (M1/70, 1:300 diluted). FACS-analysis was performed on a FACS-Calibur and analyzed with the CellQuest software (BD-Science, San Jose, California). Data of  $1 \times 10^4$  CD3 $^+$  cells were collected when stained with anti-CD3-FITC/anti-CD8 $\alpha$ -PE/anti-CD4-Cyc, with anti-CD3-FITC/anti-CD69-PE or with anti-CD3-FITC/anti-B220-PE, of  $1 \times 10^4$  CD4 $^+$  cells when stained with anti-CD25-PE/anti-CD4-Cyc or of  $1 \times 10^4$  Gr1 $^+$  cells when stained with anti-Gr1-FITC/anti-Mac1-PE.

#### *In vitro analysis*

The murine macrophage cell-line RAW 264.7 and VSMC, isolated from thoracic aortas of male C57Bl/6 mice, were used for proliferation and apoptosis assays as described previously<sup>8</sup>. In brief, proliferation of both cell types was determined by [ $^3\text{H}$ ]-thymidine incorporation, while apoptosis was assessed by measuring DNA-fragmentation using FACS-analysis.

#### *Luciferase reporter assay*

VSMC and RAW 264.7 were seeded at a density of  $10^5$  cells/cm $^2$  and allowed to attach for 24 hours. Cells were transfected with 1  $\mu\text{g}$  pNF $\kappa$ B-luciferase reporter plasmid containing the p65/p65 and p50/p65 responsive HIV kB enhancer and TATA box (kindly provided by Dr O.C. Meijer, LACDR, Leiden, Netherlands) or pNFAT-luciferase reporter plasmid (kindly provided by Dr L.J. de Windt, Hubrecht Laboratory, Utrecht, Netherlands) and 0.1  $\mu\text{g}$  phRL (Renilla Luciformis)-luciferase reference plasmid (Promega) using Exgen 500 in vitro transfection reagent (Fermentas). After 24 hours, cells were pretreated with 0-2000 ng/mL FK506 in medium for 2 hours followed by stimulation with 20 ng/mL PMA and 1  $\mu\text{M}$  ionomycin for 5 hours. Cells were lysed with Passive Lysis Buffer and luciferase-activity was measured with a luminometer according to the manufacturer's protocol (Dual Luciferase Reporter Assay System, Promega).

#### *Gene expression analysis*

To assess the effects of FK506 dose on gene expression, male ApoE $^{-/-}$  mice (14-15 weeks of age) were fed a Western-type diet for 2 weeks. Mice were then treated for 2 weeks by 0 (controls), 0.05 mg/kg/day (low dose) or 0.5 mg/kg/day (high dose) FK506 using osmotic minipumps. Mice were injected

intravenously with 50 µg/kg LPS, 3 hours before sacrifice, after which the aorta (including the aortic arch and thoracic aorta) was harvested and immediately frozen in liquid nitrogen. Total RNA of the aorta (arch and thoracic aorta) was extracted using the RNeasy kit (Qiagen). For cDNA synthesis, 500 ng RNA was used. 1kB gene expression was analyzed by quantitative real-time PCR, using the following primers and probe: forward 5'-TGGAAGTCATTGGTCAGGTGAA-3'; Reverse 5'-CAGAAGTGCCTCAGCAATTCCT-3'; Probe 5'-FAM-AGACCTGGCCTTCCTCAACTTCCGAACA-TAMRA-3'

To standardize for the amount of cDNA, expression levels of the housekeeping gene  $\beta$ -actin were analyzed, using the following primers and probe: forward 5'-GACAGGATGCAGAAGGAGATTACTG-3'; reverse 5'-CCACCGATCCACACAGAGTACTT-3'; probe 5'-TET-ATCAAGATCATTGCTCCTCCTGAGCGC-TAMRA-3'. A standard curve for each amplicon was obtained using serial dilutions of cDNA from bone-marrow derived macrophages of C57Bl6/J mice. Samples (30 ng) were analyzed in duplo in the 7700 Sequence Detector (Applied Biosystems) using the Sequence Detection Software version 1.9 according to the Relative Standard Curve Method (Applied Biosystems)

#### Statistical Analysis

Values are expressed as mean  $\pm$  SEM and Mann-Whitney-test or one-way ANOVA were used to compare individual groups of animals or in vitro experiments, respectively. Probability values of  $<0.05$  were considered significant.

## Results

Steady-state FK506 concentrations in the blood of mice after subcutaneous infusion for 4 weeks with 0.05 mg/kg/day were  $0.2 \pm 0.04$  ng/mL ( $\sim 25$  nM). ApoE<sup>-/-</sup> mice on normal chow, treated for 8 weeks with the same dose of FK506 had whole blood concentrations of  $0.11 \pm 0.01$  ng/mL. FK506-treatment did not affect body weight, hematocrit, total cholesterol and triglyceride levels of the mice (Table 1). No nephrotoxicity, the most frequently documented side-effect of FK506, was found in treated mice (data not shown).

**Table 1.** Effects of FK506-treatment (0.05 mg/kg/day) on (gain of) body weight, hematocrit, cholesterol and triglyceride levels of mice.

	ApoE <sup>-/-</sup> Atherogenic diet		ApoE <sup>-/-</sup> Normal chow	
	Control	FK506	Control	FK506
FK506 concentration (ng/mL)	-	$0.2 \pm 0.04$	-	$0.11 \pm 0.01$
Body weight gain (g)	$5 \pm 1$	$4 \pm 1$	$3.8 \pm 0.2$	$3.5 \pm 0.3$
Cholesterol levels (mg/dL)	$813 \pm 56$	$728 \pm 46$	$375 \pm 27$	$387 \pm 19$
Triglyceride levels (mg/dL)	$119 \pm 9$	$96 \pm 13$	$57 \pm 8.9$	$69 \pm 6.7$
Hematocrit (%)	$32 \pm 1$	$35 \pm 1$		

*Low dose FK506 inhibits collar-induced atherosclerosis*Plaque morphometry

Plaque size of FK506-treated mice 5 weeks after collar-placement was significantly lower (63%,  $P=0.01$ ) compared to controls (Figures 2A, B and C). Intima/lumen ratio of FK506-treated mice was also significantly reduced (56%,  $P=0.01$ ), as was the medial area. Lumen size was larger in the FK506-treated mice compared to controls. No significant differences were found in intima/media ratio (Table 2).

**Table 2.** Effect of calcineurin-inhibition by FK506 (0.05 mg/kg/day) on plaque morphometry. Results are shown for both the effects of Calcineurin-inhibition on de novo atherosclerosis and pre-existing lesions.

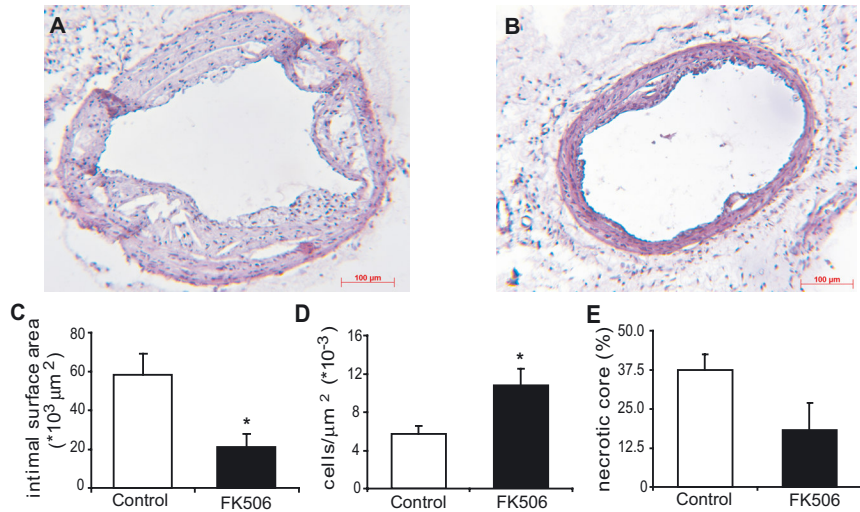
	<i>De novo</i> atherosclerosis		pre-existing lesions	
	Control (n=8)	FK506 (n=9)	Control (n=9)	FK506 (n=15)
Plaque size ( $\times 10^3 \mu\text{m}^2$ )	58.5 $\pm$ 10.6	21.4 $\pm$ 6.6	76.7 $\pm$ 9.8	49.7 $\pm$ 7.6
Medial area ( $\times 10^3 \mu\text{m}^2$ )	33.8 $\pm$ 2.6	23.3 $\pm$ 1.8*	50.2 $\pm$ 12.1	42.0 $\pm$ 6.2
Intima/Media ratio	1.8 $\pm$ 0.4	0.94 $\pm$ 0.3	1.9 $\pm$ 0.3	1.2 $\pm$ 0.2
Intima/Lumen ratio	0.7 $\pm$ 0.1	0.3 $\pm$ 0.1*	0.9 $\pm$ 0.1	0.6 $\pm$ 0.1*
Lumen size ( $\times 10^3 \mu\text{m}^2$ )	24.6 $\pm$ 5.8	44.1 $\pm$ 5.3	7.5 $\pm$ 2.2	30.5 $\pm$ 4.1**

To study the effect of calcineurin-inhibition on pre-existing lesions, ApoE<sup>-/-</sup> mice were treated with FK506 for 4 weeks, beginning 5 weeks after collar-placement (Figure 1C). Plaque size of FK506-treated mice was significantly lower (35%,  $P=0.02$ ) compared to controls (Figures 4A, B and C) as was the intima/lumen ratio. Lumen size was markedly increased (75%,  $P<0.001$ ) in FK506-treated mice compared to controls. Medial area, intima/media ratio and total vessel area (outward remodelling) did not differ between groups (Table 2). Remarkably, plaque size of pre-existing lesions of FK506-treated mice (at 9 weeks) was comparable to that of control mice at 5 weeks after collar-placement (49,665  $\pm$  7,597  $\mu\text{m}^2$  versus 58,499  $\pm$  10,627  $\mu\text{m}^2$  respectively), suggesting that FK506 completely blocked lesion progression.

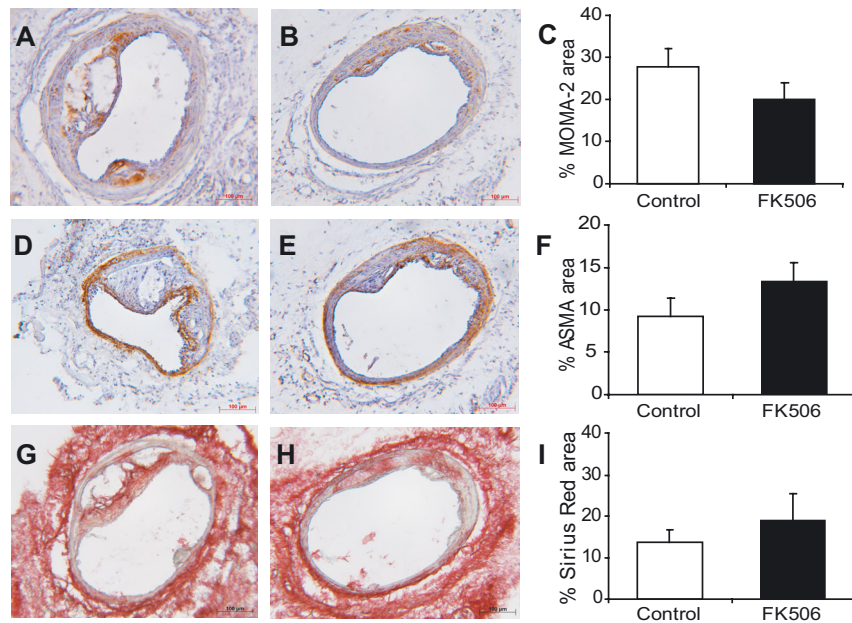
Plaque morphology

Figures 2-5 show representative examples of cross-sections stained with hematoxylin and eosin (HE), MOMA (macrophages), ASMA ( $\alpha$ -smooth muscle actin-containing VSMC), and picrosirius red (collagen). Since plaque area of FK506-treated mice was significantly reduced compared to controls, the amount of cells, necrotic core area and collagen content of the plaques are expressed relative to the total amount of cells in the plaque or to total plaque area.

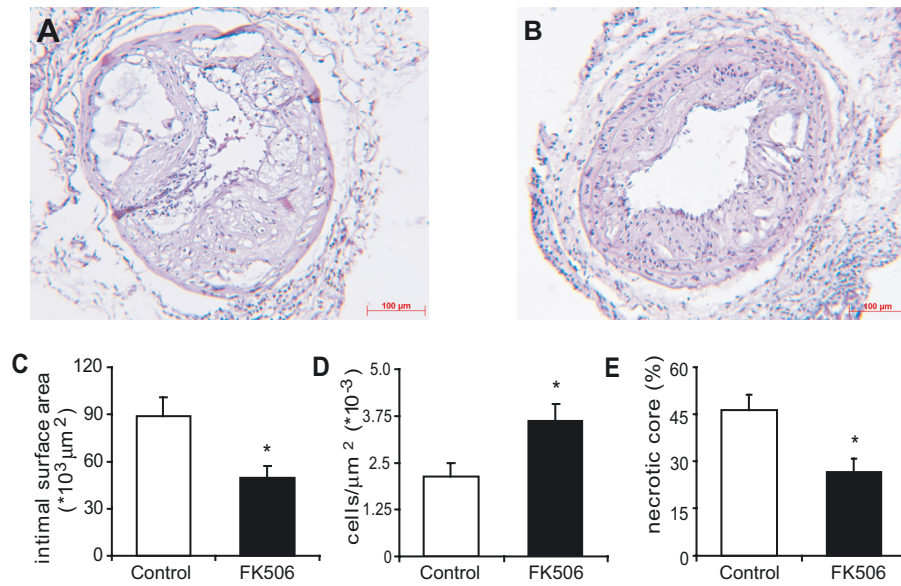




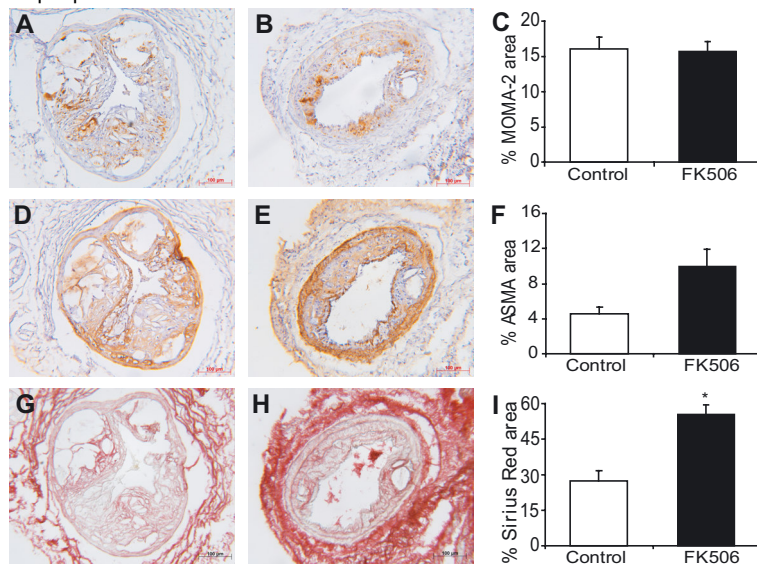
**Figure 2.** Morphological and morphometric analysis of the effect of FK506-treatment on collar-induced atherosclerotic plaque development in common carotid arteries of ApoE<sup>-/-</sup> mice. Panels A and B show representative HE stained cross-sections of control versus FK506-treated mice, respectively. Panel C: plaque size, panel D: cell density and panel E: necrotic core area relative to plaque area.



**Figure 3.** Analysis of the effect of FK506-treatment on plaque morphology. Panel A-C: macrophage-specific MOMA-staining, panel D-F: VSMC-specific ASMA-staining and panel G-I: sirius red staining for collagen content of plaques of FK506-treated mice (B, E and H) compared to controls (A, D and G).



**Figure 4.** Morphological and morphometric analysis of the effect of FK506-treatment on the development of pre-existing atherosclerotic plaques in common carotid arteries of ApoE<sup>-/-</sup> mice. Panels A and B show representative HE stained cross-sections of control versus FK506-treated mice, respectively. Panel C: plaque size, panel D: cell density and panel E: necrotic core area relative to plaque area.



**Figure 5.** Analysis of the effect of FK506-treatment on morphology of pre-existing atherosclerotic plaques in FK506-treated mice (B,E and H) compared to controls (A, D and G). Panel A-C: macrophage specific MOMA-staining, panel D-F: VSMC-specific ASMA-staining and panel G-I: sirius red staining for collagen content.

Cell density of the plaques at 5 weeks after collar-placement was significantly increased in FK506-treated mice compared to controls (Figure 2D). However, no significant differences were found in either relative amount (Figure 3C) or relative area of macrophages ( $35.7 \pm 4.8\%$  for controls versus  $27.5 \pm 6.8\%$  for FK506-treated mice,  $P=0.396$ ). Furthermore, no differences were found in the relative area of ASMA-stained VSMC (Figures 3F) and in collagen content of the plaques (Figure 3I). Necrotic core area in FK506-treated mice tended to be decreased ( $P=0.08$ , figure 2E). No differences in the (relative) amount of T-cells were found between FK506-treated mice and controls, albeit that the T-cell content of carotid artery plaques was too low to draw firm conclusions (data not shown).

The effect of calcineurin-inhibition on the morphology of pre-existing lesions was even more pronounced. FK506-treatment of pre-existing plaques resulted in more stable plaque phenotypes compared to plaques of control mice. Cell density of the plaques was significantly increased in FK506-treated mice (Figure 4D) as was the relative amount of collagen in the plaques (Figure 5I). The relative area of VSMC was increased, although not significant ( $P=0.1$ , Figure 5F). The relative necrotic core area of FK506-treated mice was significantly decreased compared to controls (Figure 4E). No differences were observed in the relative macrophage number (Figure 5C) or area ( $10.7 \pm 1.7\%$  versus  $11.4 \pm 1.8\%$  for FK506 and control group, respectively,  $P=0.751$ ) while the (relative) amount of T-cells in FK506-treated mice and controls was similar, albeit that the T-cell content of carotid artery plaques was again too low to draw firm conclusions (data not shown). Furthermore, lipid content of the plaques (Oil red O), was not affected by FK506-treatment ( $0.61 \pm 0.06$  for FK506-treated mice versus  $0.62 \pm 0.07$  for the controls,  $P=0.86$ ).

#### *FACS-analysis*

To investigate whether FK506 affects lesion formation indirectly via modulating peripheral immunity, we have analyzed the relative abundance of various leukocytes and the amount of activated T-cells by flow-cytometric analysis using single-cell solutions of blood, spleen and lymph nodes. No differences in amount of B-cells (B220+), granulocytes (Gr1+) and macrophages (Mac1+) were found between FK506-treated mice and controls (data not shown). Furthermore, CD3+ T-cell numbers, CD4/CD8 ratio (ratio of T-helper to cytotoxic T-cells) or activated T-cell numbers (CD25+ or CD69+) were not affected by FK506-treatment (Figure 6).

#### *In vitro experiments*

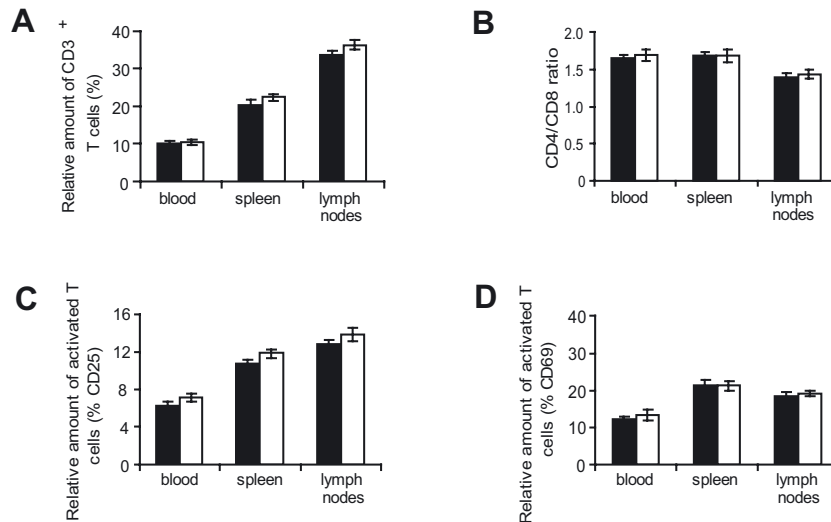
To address the effects of calcineurin-inhibition by FK506 on plaque cellularity *in vivo*, we investigated the effect of FK506 on cell proliferation and apoptosis *in vitro*. Our data clearly show that FK506 did not affect VSMC and macrophage proliferation at concentrations up to 42 ng/mL (Figure 7A and B), nor did it influence the rate of macrophage apoptosis

(Figure 7D). Conversely, incubation with FK506 significantly decreased the extent of VSMC apoptosis ( $P=0.002$ , Figure 7C).

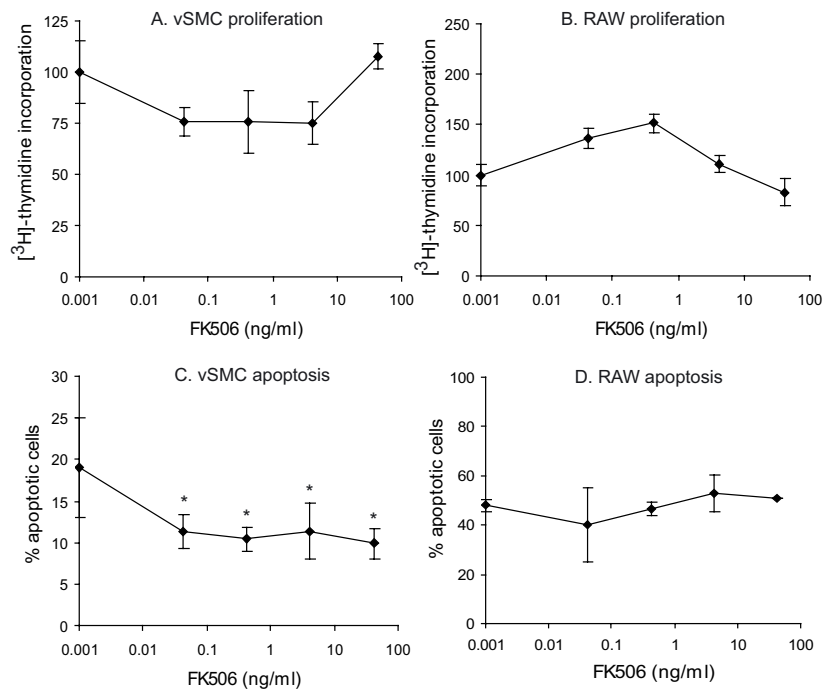
Effects of FK506 on transcriptional activation of downstream calcineurin-responsive transcription factors were assessed via luciferase-reporter assays. FK506 significantly and dose-dependently inhibited NFAT-activation in VSMC ( $P=0.02$  at 2 ng/mL). For RAW, NFAT-activation was significantly inhibited even at concentrations as low as 0.2 ng/mL, which corresponds with FK506-concentrations *in vivo* ( $P=0.03$ , Figure 8B). FK506 did not affect NF $\kappa$ B-activation in RAWs at concentrations of up to 2000 ng/mL (Figure 8C).

#### *Dose-dependent effect of FK506 on NF $\kappa$ B-activity*

To address the effects of FK506 doses on NF $\kappa$ B-activity, gene expression of the NF $\kappa$ B-responsive gene I $\kappa$ B was induced by stimulation with LPS and analyzed using quantitative real-time PCR. Mice treated with low dose FK506 (0.05 mg/kg/day) showed no differences in aortic I $\kappa$ B gene expression compared to control mice ( $n=10$ ), whereas I $\kappa$ B gene expression in the aorta of mice treated with a ten times higher dose of FK506 (0.5 mg/kg/day,  $n=8$ ) showed 1.5 fold increased I $\kappa$ B gene expression compared to controls ( $P=0.07$ ), which was also significantly increased compared to mice treated with low dose FK506 ( $P=0.03$ , Figure 8D). This indicates an increased NF $\kappa$ B-activity with a higher dose of FK506.



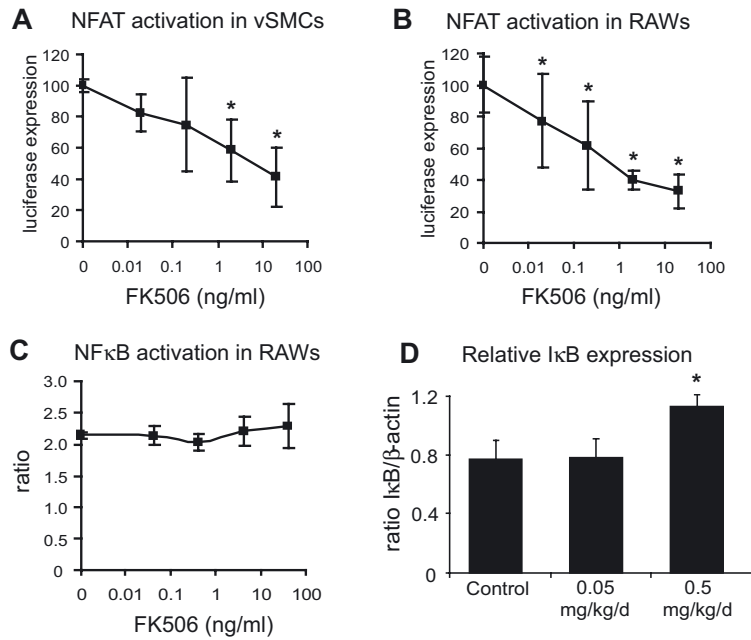
**Figure 6.** Flow-cytometric analysis of the effect of *in vivo* FK506-treatment on the relative amount of CD3<sup>+</sup> T-cells (A), the ratio CD4<sup>+</sup>/CD8<sup>+</sup> T-cells (B) and the amount of activated T-cells (C: CD25<sup>+</sup>; D: CD69<sup>+</sup>) in blood, spleen and lymph nodes of control (white bars) versus FK506-treated mice (black bars).



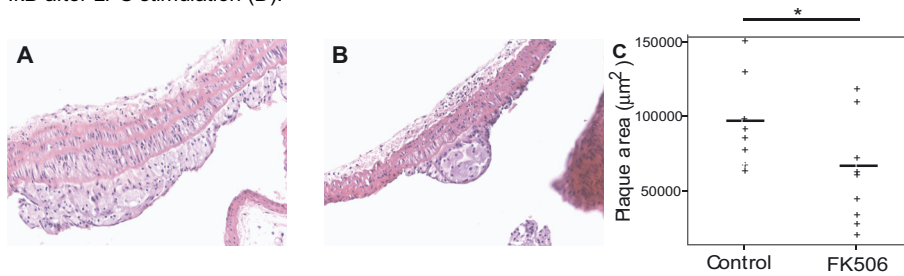
**Figure 7.** Effect of FK506 on VSMC proliferation (A), RAW proliferation (B) and VSMC apoptosis (C) and RAW apoptosis (D) *in vitro*. Results are expressed as percentage of the amount of apoptosis or [3H]-thymidine incorporation of controls (without FK506).

*Low dose FK506 also inhibits spontaneous development of atherosclerosis*

In addition to the effects of low dose FK506-treatment on collar-induced atherosclerosis, we also examined the effects on spontaneous development of atherosclerotic plaques in the brachiocephalic trunk of ApoE<sup>-/-</sup> mouse in the absence of an atherogenic diet. Plaque size was significantly (34%) reduced in FK506-treated mice compared to the controls ( $59,772 \pm 11,475 \mu\text{m}^2$  versus  $90,878 \pm 9,914 \mu\text{m}^2$ ,  $P=0.038$ , Figure 9). Effects on lesion morphology were less evident than the effects on collar-induced lesions. No significant differences were found on collagen content, necrotic core content, relative amount of ASMA-positive VSMCs or macrophages (data not shown). Nevertheless, we did find that the amount of T cells (relative to the total amount of plaque cells) was 68% decreased in FK506-treated mice ( $0.44 \pm 0.22$  % versus  $1.38 \pm 0.07$  % for the controls), albeit borderline significant ( $P=0.05$ ). This indicates that low dose FK506 also increased plaque stability in spontaneously developed atherosclerosis.



**Figure 8.** (A-C) Effect of FK506 on pNFAT-luciferase or pNFκB-luciferase reporter activity in VSMCs and RAW cells. Luciferase-activity was expressed relative to Renilla Luciformis luciferase-activity (used as an internal control). Effects of different FK506 doses on *in vivo* NFκB activity were measured by quantitative gene expression analysis of the NFκB-responsive gene IκB after LPS stimulation (D).



**Figure 9.** Morphometric analysis of the effect of low dose FK506 on spontaneous atherosclerotic plaque development in the brachiocephalic trunk of ApoE<sup>-/-</sup> mice on normal chow. Panels A and B show representative HE stained cross-sections of control versus FK506-treated mice, respectively. Panel C: plaque size (\*P=0.038).

## Discussion

Immunosuppressives such as CsA and FK506 (tacrolimus) are often used to suppress graft-versus-host disease after transplantation. However, it is not clear whether the use of these immunosuppressives either inhibits or ameliorates the development of (transplant) atherosclerosis. Both CsA and FK506 exert their immunosuppressive function by inhibition of the

calcineurin pathway, which is a major signaling pathway involved in the inflammatory response, especially in T cell activation and the regulation of cytokine gene expression in a variety of cell types<sup>1</sup>. Besides T cells, calcineurin and downstream transcription factors are also expressed in several other vascular cells such as endothelial cells, VSMC and macrophages<sup>11-13</sup>. Furthermore, calcineurin has been described in several other processes involved in atherosclerosis such as apoptosis<sup>14-15</sup> and angiogenesis<sup>12</sup>.

Since atherosclerosis is a chronic inflammatory disease, we tested the hypothesis that the immunosuppressive FK506, by inhibiting calcineurin signaling, would attenuate atherosclerotic plaque development in an ApoE<sup>-/-</sup> mouse model of collar-induced atherosclerosis. Similar to transplant arteriosclerosis, this is a model of rapid atherogenesis and therefore it may be very suitable to compare the effects of FK506 on the development of de novo atherosclerosis to the development of transplant arteriosclerosis.

This is the first study to report an atheroprotective effect of calcineurin-inhibition by FK506 in mice. Previously, the immunosuppressive CsA has been shown to accelerate fatty streak formation in cholesterol-diet fed C57Bl/6 mice<sup>16</sup>. However, the use of CsA has been shown to be associated with hyperlipidemia<sup>17</sup>, increased oxidizability of LDL<sup>18</sup> and hypertension<sup>19</sup>, which are all risk factors for development of atherosclerosis that are much less affected by FK506.

In view of the side-effects of these immunosuppressives, we decided to evaluate a low dose regimen of FK506 (0.05 mg/kg/day), which was subcutaneously administered by osmotic minipumps to yield blood concentrations of 0.2 ng/mL. This dose had no adverse effects on body weight, cholesterol and triglyceride levels, hematocrit or renal morphology nor did FK506 affect systemic T-cell numbers or T-cell activation as assessed by FACS-analysis. The administered dose was also well below the 0.5 mg/kg/day recently described by Ellis *et al.* as the upper limit for sub-immunosuppressive effects of FK506-administration in mice<sup>20</sup>. In addition, there is strong evidence that local FK506 responses are dictated by the relative expression profile of immunophilin isoforms (such as FKBP12) in a particular tissue<sup>21</sup>, implicating that identical doses of FK506 could differentially affect diverse cell-types. Interestingly, FKBP12 was found to be upregulated in human neointimal tissue retrieved by helix-cutter atherectomy, suggesting that the diseased human vascular wall may be more sensitive to FK506<sup>22</sup>. Combined, data from literature and our own data suggest that calcineurin-inhibition by FK506 exerts its regulatory actions on plaque phenotype at the level of the plaque itself and not primarily via a systemic immunosuppressive effect.

This study shows that calcineurin-inhibition significantly inhibited development of collar-induced atherosclerosis and blocked plaque-progression, even at a very low dose of FK506. Furthermore, FK506 induced a more stable plaque phenotype by reducing necrotic core content and increasing cell density and collagen content, which was most evident in



plaque progression. *In vitro* data showed FK506 to inhibit VSMC apoptosis which concurs with earlier studies<sup>14</sup>. FK506-mediated inhibition of VSMC apoptosis may at least in part explain the increased plaque cell density of FK506-treated mice. FK506 did not affect macrophage apoptosis consistent with the *in vivo* finding that the number of plaque macrophages was not affected by FK506-treatment.

Results of several studies using FK506 to examine its effects on atherosclerosis seem rather contradictory at first glance. Wu *et al.*<sup>3</sup> and Cramer *et al.*<sup>23</sup> showed an inhibitory effect of FK506 on post-transplant arteriosclerosis in rats, whereas Matsumoto *et al.*<sup>5</sup> showed a minor stimulation of atherosclerosis in cholesterol-fed rabbits. While the former study involves a different pathobiology, the latter has applied higher doses of FK506. Therefore, the contradictory results may have arisen from differences in experimental set-up or the dose of FK506 used.

In this study, we used a mouse model of atherosclerosis with a close resemblance to human atherosclerotic plaque morphology. We found an atheroprotective effect of FK506 in the ApoE<sup>-/-</sup> model of collar-induced model of atherosclerosis, but also in the ApoE<sup>-/-</sup> mouse model, in which atherosclerosis develops spontaneously. Furthermore, we used a very low dose of FK506.

In contrast to its activating effect in T-cells, calcineurin has been shown to induce an anti-inflammatory effect in macrophages by suppressing the expression of various inflammatory cytokines<sup>13</sup>. In macrophages, calcineurin-inhibition was found to activate the transcription factor NFκB, leading to enhanced expression of cytokine genes, whereas it suppresses cytokine gene expression in T-cells.

Interestingly, the FK506-concentration required for activation of NFκB and effector-gene expression in macrophages is much higher than required for inhibition of NFAT-activation and cytokine gene expression in T-cells. Indeed, our luciferase-reporter assays showed inhibition of NFAT-activation by FK506 even at a very low dose (0.2 ng/mL), whereas NFκB-activity was unaffected with up to 2 µg/ml FK506 (Figure 8A-C). Furthermore, in line with our hypothesis we found no effects on aortic expression of the NFκB-responsive gene IκB in mice treated with low dose FK506 compared to controls, whereas IκB gene expression was increased after treatment with the high dose FK506 (Figure 8D). This indicates an increase in NFκB-activity upon calcineurin-inhibition with a high dose of FK506 and stresses the importance of the low dose of FK506 used. This also might explain the differences on the effect of FK506 on the development of atherosclerosis in previous studies. In comparison, blood concentrations achieved in transplant patients are in the range 15-25 ng/mL<sup>24</sup>. The relatively high doses of FK506 used in transplantation might therefore stimulate development of atherosclerosis in these patients. Indeed, arteriosclerotic disease is still a major cause of morbidity and mortality in transplant patients.

Although FK506 and CsA mediate their effects primarily through calcineurin-inhibition, these drugs can also have calcineurin-independent effects.



However, prior research on effects of these drugs in the attenuation of cardiac hypertrophy has shown that interventions in the calcineurin-pathway (e.g. transgene and knock-out intervention studies) demonstrated the same phenotype as after the use of either FK506 or CsA<sup>25,26</sup>. These studies and our in vitro NFAT-reporter assay indicate that the results were mainly calcineurin-dependent.

In conclusion, calcineurin-inhibition by a low dose of the immunosuppressive agent FK506 reduced the development of collar-induced atherosclerosis in ApoE<sup>-/-</sup> mice and blocked progression of pre-existing lesions. Furthermore, it induced more stable plaque phenotypes by increasing cell density and collagen content while decreasing necrotic core content of plaques. These surprising findings may have important implications for the clinical use of (a low dose of) FK506.

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