Crosstalk between apoptosis and inflammation in atherosclerosis

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Chapter 6

Compromised Focal Adhesion Kinase Function Does Not Alter Atherogenesis in ApoE$^{-/-}$ Mice despite Profound Effects on Lipid Metabolism and Inflammatory Status

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Abstract

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase involved in cell survival, cell-matrix adhesion, migration and cell proliferation and apoptosis of all major cell types involved in atherosclerotic plaque development. While suggestive of a role in cellular homeostasis in atherosclerotic plaques, experimental evidence to support this hypothesis is lacking to date. The aim of this study was to address the role of impaired FAK function on inflammatory status and atherosclerotic lesion development in Western type diet fed $ApoE^{-/-}$ mice.

$FAK^{+/-}ApoE^{-/-}$ mice had significantly lower plasma total cholesterol levels, which was attributable to decreased hepatic VLDL production rather than an altered intestinal cholesterol absorption. Hepatic lipid content was unchanged in $FAK^{+/-}ApoE^{-/-}$mice. Furthermore, $FAK^{+/-}ApoE^{-/-}$ mice displayed splenomegaly (31% increase in weight) with a decreased number of white pulp nodules in the spleen and enlarged germinal centres, suggesting perturbed immune function. Macrophage content in spleen was found to be decreased whereas the number of lymphocytes was increased. In contrast, the peritoneal cavity and the circulation showed increased monocytes numbers accompanied by a reduced $CD4^+$ and $CD8^+$ T cell content. Surprisingly, despite these marked effects on lipid homeostasis and immune status, impaired FAK expression neither affected size and composition of collar induced carotid artery plaques nor that of spontaneous brachiocephalic artery and aortic root plaques.

We conclude that compromised FAK function beneficially affects cholesterol metabolism in Western type diet fed $ApoE^{-/-}$ mice and alters their inflammatory status. Despite these marked peripheral effects impaired focal adhesion does not translate in an altered atherogenic response.

Introduction

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor protein tyrosine kinase and is activated by (auto)phosphorylation upon integrin binding. Known targets of FAK include paxillin, Cas and the Src family of kinases[1]. In addition to activation by phosphorylation following integrin binding, FAK phosphorylation has been shown to occur in response to growth factor stimulation (VEGF and HGF)[2,3]. Several studies have established a role for FAK in cell-matrix adhesion, cell migration, proliferation and apoptosis[4]. FAK-deficient fibroblast-like cells were shown to have an increased number of focal adhesions, most likely to be caused by a decreased turnover, and to spread more slowly on extracellular matrix proteins[5]. Furthermore FAK deficient hematopoietic precursor cells display an impaired migratory response to chemotactic signals[6]. FAK is essential in embryonic development in particular of the vasculature, since FAK knockout embryo's die at 8.5 days of age due to perturbed vasculogenesis[5].

In the vasculature FAK was found to affect adherence, migration and proliferation of vascular smooth muscle cells (vSMC's)[7,8] and endothelial cells[9-11]. Moreover, study of endothelial cells from FAK knockout embryo's showed that endothelial FAK is required for tubulogenesis[9]. In addition, targeted FAK deletion in embryonic endothelial cells resulted in impaired vascular network stability and cell survival[10]. Consequently, FAK knockout embryo's exhibit severe defects in vascular development. The non-catalytic carboxyl-terminal protein binding domain of FAK called FRNK (FAK related non kinase) is independently and selectively expressed in vSMC's, acting as an endogenous inhibitor of FAK signaling. Following balloon-induced carotid artery injury FRNK is strongly upregulated in vSMC's indicating that following injury vSMC migration and adhesion complex turnover may be negatively regulated[7].

These findings combined with the recent observation that FAK is overexpressed in VSMC's in human intimal hyperplasia[12] might suggest a role for FAK in cellular homeostasis in atherosclerotic plaques. However to date the precise role of FAK in atherogenesis has not been addressed. In this study we therefore assessed the effect of compromised FAK function in ApoE$^{-/-}$ mice using perivascular collar placement around the common carotid arteries to induce atherosclerotic lesion development. Although our results surprisingly demonstrated no overt effect on atherosclerotic lesion size or composition, lipid metabolism and inflammatory status of these mice appeared to be seriously modified in the heterozygote status of FAK expression.

Materials and Methods

Animals

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. FAK$^{+/-}$ mice were obtained from the Department of Toxicology (LACDR, Leiden, The Netherlands) and were crossbred with ApoE$^{-/-}$ mice, obtained from the local animal breeding facility.

Carotid collar placement

At 16-18 weeks of age carotid atherosclerotic lesion development was induced by bilateral perivascular collar placement in female $FAK^{+/-}ApoE^{-/-}$ mice (n=10) and $FAK^{+/+}ApoE^{-/-}$ littermates (n=9) as described by Von der Thüsen *et al*.[13] Starting 4 weeks before collar placement and throughout the whole experiment mice were fed a Western type diet containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK). In a separate experiment bilateral perivascular collar were placed in female $FAK^{+/-}ApoE^{-/-}$ mice (n=11) and $ApoE^{-/-}$ littermates (n=11) at the age of 12-16 weeks. These mice were fed a regular chow diet throughout the experiment.

Cholesterol, triglyceride and phospholipid assay

Blood samples were taken by tail cut before start of (week 0), after four weeks of the Western type diet (week 4), and at the time of sacrifice (week 10). Hepatic lipids were extracted as described by Bligh and Dyer[14]. Total cholesterol, triglyceride and phospholipid content was measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands) in serum and hepatic extracts. Serum samples were fractionated using Superose 6 column (SMART System, Pharmacia Biotech, Uppsula, Sweden) to obtain total cholesterol distribution patterns.

Cholesterol absorption

To analyze cholesterol absorption from the intestine, $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates (n=5) were intravenously injected with 500 mg Triton WR1339/Tyloxapol per kg body weight (Sigma, Zwijndrecht, The Netherlands) to block lipoprotein lipase mediated lipolysis after overnight fasting. Subsequently the mice were gavaged with 1.2 µCi ^{14}C-cholesterol (GE Healthcare, Diegem, Belgium) and 200 µl unlabeled cholesterol (Sigma) in olive oil. Blood samples were taken by tail cut before and 1, 2, 3 and 4 hours after oral cholesterol administration. After 4 hours the animals were anesthetized and gal bladder, small intestine and liver were harvested. ^{14}C content was determined in serum, gall bladder, small intestine (20 cm from pylorus) and liver sections using a liquid scintillation analyzer (Tri-Carb 2900R, Packard).

VLDL production rate

For analysis of Very Low-Density Lipoprotein (VLDL) production rate, mice were intravenously injected with Triton WR1339/Tyloxapol (500 mg per kg body weight, Sigma). Blood samples were taken via tail vein before and 1 and 2 hours after injection. Serum triglyceride accumulation was measured as described above.

FAK gene and protein expression

$FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates (n=4) were anesthetized and blood was obtained by bleeding through the orbital sinus. Erythrocytes in whole blood were lysed to obtain white blood cells (WBC's). Various organs including liver, heart

and aorta were harvested in liquid nitrogen after perfusion with PBS. Total RNA was extracted using guanidine thiocyanate (GTC, Sigma). After RT-PCR, FAK gene expression in various organs and expression of genes involved in lipid homeostasis in liver were analyzed by quantitative real-time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) and SYBR-Green technology. The primer sequences are listed in table 1. HPRT, β-actin and/or acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.

Protein expression was analyzed by Western blotting using mouse anti-FAK, clone 4.47 (Upstate Biotech, Charlottesville, VA, USA) primary antibody and rabbit anti-mouse-HRP (DakoCytomation, Heverlee, Belgium) secondary antibody. Signal intensity was analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

Table 1. Primer sequences for realtime PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
18S	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
FAK	GAGAATCCAGCTTTGGCTGTT	GGCTTCTTGAAGGAACTTCT
CD36	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
FAS	GGCATCATTGGGCACTCCTT	GTCGCAAGCACAGCCTCTCT
SCD1	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA
SREBP-1	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC
SREBP-2	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC

Tissue harvesting and analysis

Six (for Western type diet fed mice) or ten weeks (for normal chow diet fed mice) after collar placement the animals were anesthetized and perfused with PBS followed by fixation with 4% formaldehyde (4.5 times diluted Zinc Formal-Fixx, Thermo Electron Corporation, Breda, The Netherlands). Carotid arteries, brachiocephalic artery, heart, spleen and liver were harvested and stored in 4% formaldehyde solution. Cryosections were prepared of carotid and brachiocephalic arteries, aortic valves, spleen and liver and stained with hematoxylin and eosin (HE) and/or Oil Red O. Lesion size was quantified using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma), VSMC (α-smooth muscle actin, Sigma), T cell (CD3, Serotec) and B cell (Cd45R/B220, Pharmingen) content. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics, Almere, The Netherlands).

Blood cell analysis and flow cytometry

Peripheral blood mononuclear cells, splenocytes and peritoneal leukocytes were isolated from FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed normal chow or Western type diet for 4 weeks and analyzed using a Sysmex blood cell analyzer (XT-2000i), or stained for cell surface markers and analyzed by flow cytometry. White blood cells

were isolated after erythrocyte lysis of whole blood samples obtained by bleeding through the orbital sinus, peritoneal leukocytes by peritoneal lavage with 10 ml PBS and splenocytes by passing (part of) the spleen through a 70 µm nylon cell strainer (BD Falcon). White blood cells were stained with fluorescently labeled antibodies against CD4, CD19, CD23, CD69, CD71, CD86, F4/80 and MHCII (eBioscience, San Diego, California) and CD8 and CD11b (BD Pharmingen, Breda, The Netherlands). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACS-Calibur and analyzed with CellQuest software (BD Biosciences).

Statistical analysis

Values are expressed as mean ± SEM. Analysis to compare two groups was performed by two-tailed Student's t-test. For analysis of relative mRNA expression data t-test was performed on ΔCt values. Statistical significance was set at $p<0.05$.

Results

FAK expression

To determine the level of FAK deletion in $FAK^{+/-}ApoE^{-/-}$ mice, gene and protein expression was analyzed in heart, aorta, liver and/or spleen of these mice and $ApoE^{-/-}$ littermates. FAK gene expression appeared to be reduced by 34-45 % in $FAK^{+/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ littermates as determined by quantitative real-time PCR (Fig. 1A). In keeping total FAK protein expression was reduced by a similar 35-50% in these organs as shown by Western blotting and Image J analysis software (Fig. 1B and C).

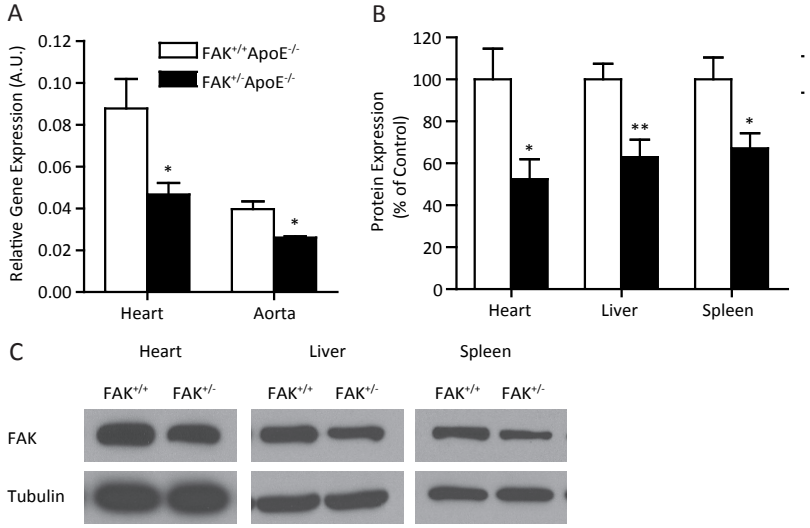

Figure 1. FAK expression in $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates. Total RNA was isolated from different organs with GTC and used for real-time PCR. Proteins were isolated with protein lysis buffer and used for Western Blotting. FAK gene expression in heart and aorta (A) and FAK protein expression in heart, liver and spleen (B) are reduced in $FAK^{+/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ littermates. (*$p<0.05$, **$p<0.01$)

Lipid metabolism

Throughout the study we have monitored lipid levels in FAK$^{+/-}$ApoE$^{-/-}$ and ApoE$^{-/-}$ mice by measuring serum total cholesterol (TC) and triglyceride (TG) levels before and after four or ten weeks of Western type feeding. Surprisingly, while total cholesterol levels were increased in both groups after Western type feeding, the diet induced increase in TC levels in FAK$^{+/-}$ApoE$^{-/-}$ mice was 18% lower than in ApoE$^{-/-}$ mice ($p<0.01$, Fig. 2A). Triglyceride levels were comparable in both groups as were total cholesterol levels when fed a regular chow diet. The reduced hypercholesterolemic response to Western type diet was not caused by apparent differences in health status or food intake, since total body weight remained unchanged, 30.3 ± 2.1 g for FAK$^{+/-}$ApoE$^{-/-}$ mice and 29.8 ± 2.1 g for ApoE$^{-/-}$ mice. As shown in fig. 2B, when serum was fractionated by Superose 6 column to analyze the TC distribution over the different lipoproteins, the total VLDL cholesterol levels were found to be responsible for the decrease in total cholesterol.

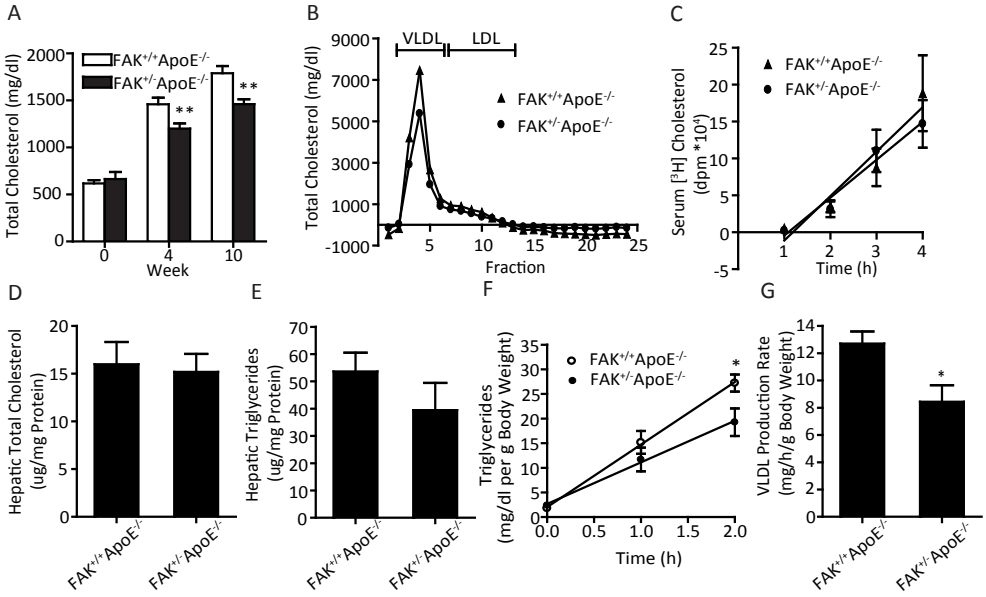

Figure 2. Lipid metabolism is altered in FAK$^{+/-}$ApoE$^{-/-}$ mice. Blood samples were taken by tail vein puncture before starting the Western type diet (WTD), after four and ten weeks of WTD feeding and at the time of sacrifice. Serum total cholesterol levels after high fat diet feeding are reduced (A), mainly due to reduced VLDL associated TC. Fractions 2-6 represent VLDL associated TC, fractions 7-13 represent LDL associated TC (B). This reduction was not caused by altered absorption of cholesterol from the intestine (C) or changes in hepatic lipid content (D,E) but is likely due to decreased VLDL production rate in FAK$^{+/-}$ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ littermates (F,G).

Liver morphology of FAK$^{+/-}$ApoE$^{-/-}$ mice did not reveal pathological changes or features of inflammation. Moreover the reduction in TC levels was not caused by changes in hepatic lipid content or cholesterol absorption rate from the intestine (fig. 2C-E). However hepatic VLDL production rate appeared to be decreased in FAK$^{+/-}$ApoE$^{-/-}$ mice from 12.71 ± 0.89 mg/dl/h/g body weight in ApoE$^{-/-}$ mice to 8.44

± 1.21 mg/dl/h/g body weight (p=0.02, fig. 2F-G), suggesting that decreased total cholesterol levels are caused by reduced VLDL production. Remarkably, assessment of hepatic gene expression of several genes involved in lipid metabolism by realtime PCR revealed an upregulation of key genes in lipogenesis in $FAK^{+/-}ApoE^{-/-}$ mice. Figures 3A-E show that expression of stearoyl-coenzyme A desaturase 1 (SCD1, 94% p=0.04) and sterol regulatory element-binding proteins (SREBP) 1 and 2 is increased (56%, p=0.01 and 32%, p=0.02 respectively) while fatty acid synthase (FAS) shows a tendency towards increased gene expression as well (59%, p=0.06). In addition CD36 expression was increased by 25% (p=0.003). Expression of several other genes involved in lipoprotein metabolism such as acetyl CoA carboxylase α (ACACA), ATP citrate lyase (ACLY), microsomal triglyceride transfer protein (MTP), lipoprotein lipase (LPL) and its cofactor apolipoprotein C2 (ApoC2) or transcription regulators like peroxisome proliferator-activated receptor α (PPARα) and PPARγ coactivator (PGC) 1a remained unchanged.

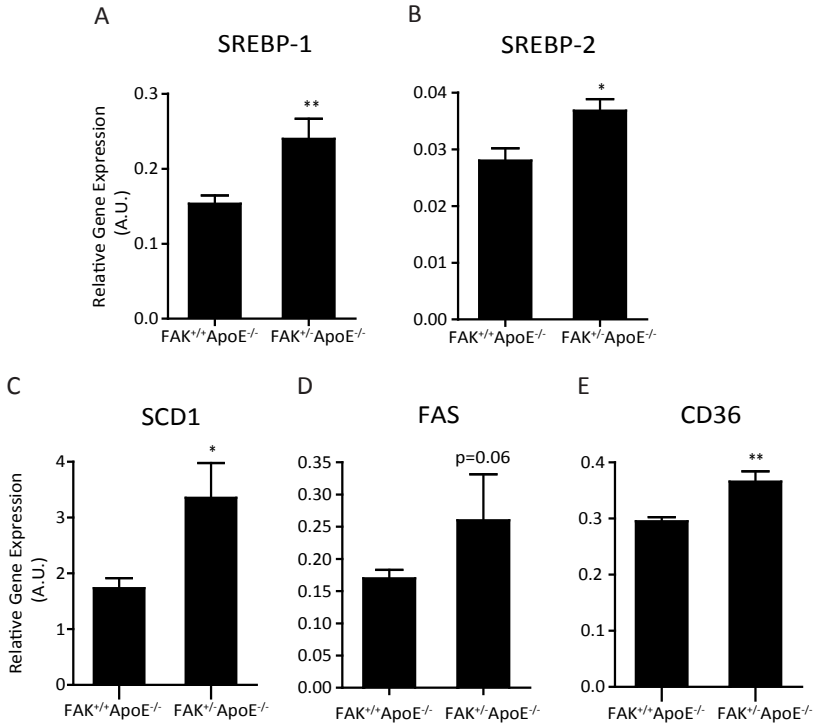

Figure 3. Hepatic expression of various key genes in lipogenesis is upregulated with reduced FAK levels. Relative gene expression of SREBP1 (A) and 2 (B), SCD1 (C), FAS (D) and CD36 (E). FAS, fatty acid synthase; SCD1, stearoyl-coenzyme A desaturase 1; SREBP, sterol regulatory element-binding protein. (*p<0.05, **p<0.01)

Spleen morphology and leukocyte composition

$FAK^{+/-}ApoE^{-/-}$ mice displayed moderate splenomegaly with a 31% increase in organ weight after Western type feeding for 10 weeks compared to $ApoE^{-/-}$ littermates (p=0.04, Fig. 4A). The spleen/body weight ratio was increased as well. The increase

in spleen weight appeared to be at least partly diet dependent, in that spleen weight of mice fed a regular chow diet was increased by only 12% (p=0.06, Fig. 4A). Further pathophysiological analysis revealed a decreased number of white pulp nodules in spleen while the germinal centers were enlarged, resulting in an increased germinal centre area. The germinal centre expansion was accompanied by an altered germinal center organization. As shown in Fig. 4B, germinal centers appear to be fused together exhibiting irregular arrangement.

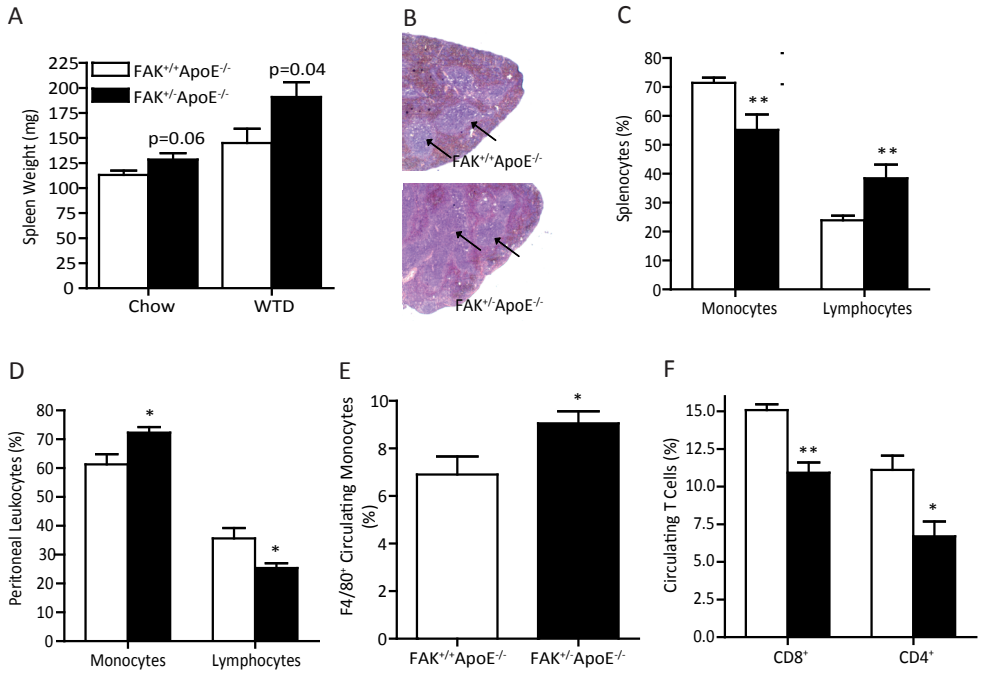

Figure 4. Reduced FAK levels lead to splenomegaly, altered spleen morphology and altered monocyte/macrophage and (T)-lymphocyte content in spleen, circulation and peritoneal cavity. Spleen size of approximately 5 month old FAK$^{+/-}$ApoE$^{-/-}$ mice (black bars) and ApoE$^{-/-}$ littermates (white bars) after normal chow or 10 weeks of Western type diet feeding (A). Cryosections of spleen were prepared and stained with hematoxylin and eosin. Spleens of FAK$^{+/-}$ApoE$^{-/-}$ mice show a decreased number of white pulp nodules in the spleen while the germinal centres (arrows) all seem to be enlarged (B). Peripheral blood mononuclear cells, splenocytes and peritoneal leukocytes were isolated from FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed normal chow or Western type diet for 4 weeks and analyzed by Sysmex blood cell analyzer (C,D) or flow cytometry (E,F). In spleen, reduced FAK expression resulted in decreased number of monocytes and elevated lymphocyte numbers (C) while the monocyte content in the peritoneal lavage was increased in FAK$^{+/-}$ApoE$^{-/-}$ mice accompanied by a decrease of peritoneal lymphocyte numbers (D). Also, reduced FAK expression led to an increase in circulating F4/80^{+} monocytes (E), and a decrease in circulating CD4^{+} as well as CD8^{+} T cells (F). (*p<0.05, **p<0.01)

To assess whether the increase in spleen weight was proportional between the different cell subsets, splenocytes were isolated and analyzed. In spleen, reduced FAK expression resulted in a 23% decreased relative number of monocytes (p=0.01, Fig. 4C) while the number of lymphocytes was elevated by 61% (p=0.01, Fig. 4C). In contrast, the leukocyte distribution profile in the peritoneal cavity showed the inverse pattern with increased monocyte numbers (+18%, p=0.02) and a concomitant

29% decrease in lymphocyte numbers (p=0.02) (fig. 4D) The changes in peritoneal leukocyte profile corresponded with those in circulation, where F4/80+ monocytes were up (+31%, p=0.04) and and CD4+ and CD8+ T cells were down by 40% (p=0.04) and 28% (p=0.005) respectively (fig. 4E-F). Reduced FAK expression did not affect macrophage phenotype and activation, T cell activation, circulating B cell and total white blood cell numbers or total peritoneal leukocyte numbers.

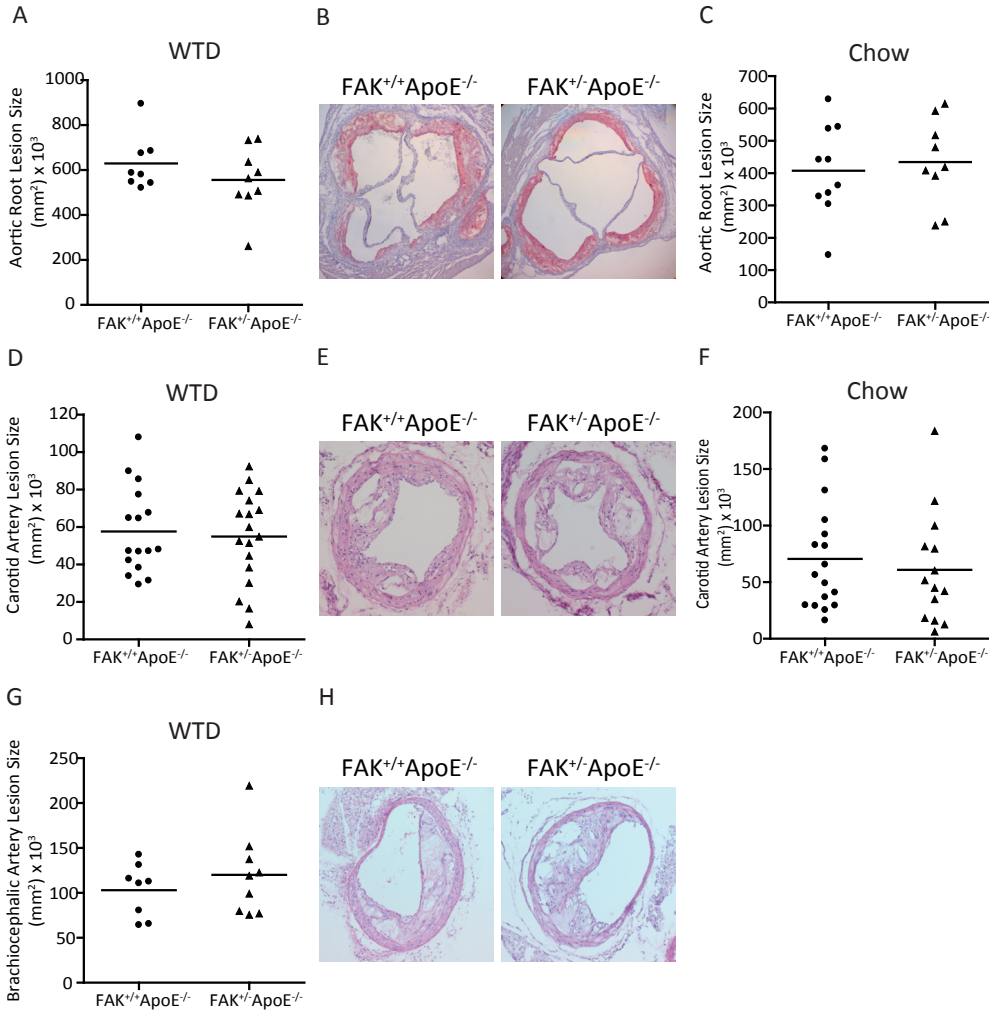

Figure 5. Reduced FAK expression does not affect atherogenesis. Cryosections were prepared of formaldehyde fixed aortic roots, carotid and brachiocephalic arteries and stained Oil Red O or HE. Aortic root (A,C), collar aided carotid artery (D,F) and brachiocephalic artery (G) atherosclerotic lesion size did not differ between FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed a Western type or chow diet. B, E and H show representative pictures of the lesions.

Atherosclerotic plaque size and composition

After ten weeks of high-cholesterol diet feeding and eight weeks after collar placement carotid and brachiocephalic arteries and aortic roots were isolated,

sectioned and stained with hematoxylin and eosin or Oil Red O. Remarkably, reduced FAK expression did not alter the atherosclerotic plaque size in collar aided carotid artery, brachiocephalic artery or aortic root (Fig. 5). Plaque composition as determined by immunohistochemical staining for monocytes/macrophages (MoMa-2) and vSMC's (alfa-SM-actin), for collagen (Sirius Red staining) and apoptotic cell content (TUNEL staining) at the three different assessed sites was essentially similar in FAK$^{+/-}$ApoE$^{-/-}$ versus ApoE$^{-/-}$ littermates. Moreover, lesion cellularity and necrotic core size were unaffected as well (fig. 6).

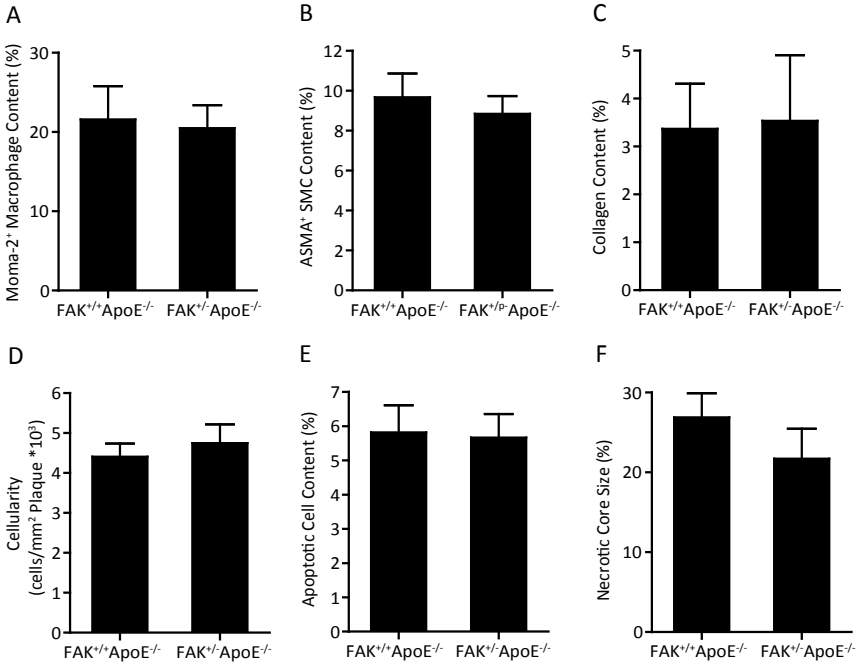

Figure 6. Atherosclerotic lesion composition is unchanged in FAK$^{+/-}$ApoE$^{-/-}$ mice. Moma-2+ macrophage (A), ASMA+ smooth muscle cell (B) and collagen (C) content, cellularity (D), apoptosis (E) and necrotic core size (F) did not differ between FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ controls. ASMA, α-smooth muscle-actin.

Because serum total cholesterol levels were reduced in FAK$^{+/-}$ApoE$^{-/-}$ mice after Western type feeding only, we have assessed whether this may have indirectly counteracted any pro-atherogenic responses of reduced FAK activity. However collar induced carotid artery formation as well as spontaneous brachiocephalic artery and aortic root plaque sizes of chow diet fed FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates were completely comparable (Fig. 5). Apparently differences in cholesterol load did not mask pro-atherogenic effects of FAK heterozygousity on leukocyte homeostasis.

Discussion

In this study we show that reduction of FAK expression in ApoE$^{-/-}$ mice, as achieved by cross breeding FAK$^{+/-}$ with ApoE$^{-/-}$ mice, leads to a reduction of total cholesterol (TC)

levels in response to Western type diet feeding. This hypocholesterolemic response was high fat diet feeding dependent, since it was not observed when mice were fed a regular chow diet and suggests an until now unknown involvement of FAK in cholesterol metabolism. Closer analysis of the cholesterol distribution over different lipoproteins, showed that mainly VLDL associated cholesterol was decreased. The absorption rate of cholesterol after oral administration of cholesterol did not differ between $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates. Hepatic total cholesterol, tryglyceride and phospholipid content, was comparable between the two groups as well. Thus the decreased TC levels are most likely caused by the observed lower hepatic VLDL production rate in $FAK^{+/-}ApoE^{-/-}$ mice. Of note, the hepatic expression of critical genes in lipogenesis, SREBP1 and 2, SCD1 and FAS, was increased in $FAK^{+/-}ApoE^{-/-}$ mice. SREBPs are strongly regulated by a feedback mechanism in cholesterol and fatty acid metabolism[15] and contribute to the regulation of various effector genes in lipogenesis, such as SCD1 and FAS[16,17]. Hepatic CD36 expression was slightly increased in response to FAK reduction as well. In a recent study oxidized LDL mediated CD36 signaling was shown to inhibit macrophage migration and stimulate cell spreading by continued FAK phosphorylation and activation[18], suggesting a role for FAK in trapping macrophages in the arterial wall and promoting atherogenesis. Thus the elevated hepatic expression of these genes may reflect a compensatory mechanism in response to lowered VLDL and TC levels in the case of lipogenesis genes and decreased FAK levels in the case of CD36.

In addition to cholesterol lowering, we found that reduced FAK expression in $ApoE^{-/-}$ mice leads to splenomegaly accompanied by altered spleen morphology. The increase in total spleen weight and in particular germinal centre area suggest an aberrant splenocyte composition, with a relative increase in germinal centre B and T lymphocytes content and a decrease in monocytes/macrophages numbers. This notion was confirmed by further analysis of splenocyte composition. Although initially FAK was reported to be absent in monocytes/macrophages[19,20], recent publications have conclusively proven this to be incorrect[21,22]. GM-CSF and M-CSF, key regulators of myeloid differentiation, were found to induce FAK phosphorylation in monocytes/macrophages while inhibition of FAK activity by FRNK led to impaired macrophage spreading and adhesion[21,22]. Deletion of FAK in monocytes/macrophages was seen to attenuate their migratory capacity, random motility as well as M-CSF, SDF-1α and MCP-1 directed migration[23]. The observed decrease in macrophage content in spleen in $FAK^{+/-}ApoE^{-/-}$ mice may likely be attributable to impaired ingress from circulation, even despite enhanced circulating monocyte numbers, whereas the increased peritoneal macrophage levels may be due to impaired migration from the peritoneal cavity. However, no reduction in plaque macrophages was observed.

In contrast to the change in splenocyte composition reduced FAK expression led to decreased circulating $CD4^+$ and $CD8^+$ T lymphocyte numbers, however activation of T cells or T cell subset numbers were unchanged. FAK is expressed in both human T and B lymphocytes[24]. In the present study we observed an increased spleen lymphocyte content in FAK heterozygous mice along with a seemingly paradoxical decrease in

relative circulating and peritoneal lymphocyte numbers. In vitro, in T-lymphocytes FAK has been shown to have an essential role in β_1 integrin dependent migration[25] and lymphocyte function-associated antigen-1 (LFA-1 or $\alpha_L\beta_2$ integrin) mediated T lymphocyte adhesion. FAK dephosphorylation and thus inhibited activity was shown to impair LFA-1 mediated adhesion[26,27]. Furthermore, FAK was indicated in chemokine mediated lymphocyte trafficking as FAK phosphorylation and activation in T cells is induced by RANTES[28]. Similarly SDF-1 induced FAK phosphorylation in T cells, hematopoietic progenitor cells and pro-B cells appeared to be essential for its chemotactic and adhesive responses[6,29,30]. In agreement with these findings, our study seems to point to enhanced retention of lymphocytes in spleen in the case of compromised FAK function.

Despite the previously reported involvement of FAK in migration and proliferation of vSMC's, macrophages and endothelial cells in the vasculature and the impaired hypercholesterolemic response to Western type diet and altered inflammatory cell distribution pattern in circulation, peritoneal cavity and spleen of FAK$^{+/-}$ApoE$^{-/-}$ mice observed in this study, to our surprise we did not observe any effects of impaired FAK function on size or composition of collar aided carotid, brachiocephalic artery or aortic root atherosclerotic plaques. The lack of effect of FAK heterozygousity on atherogenesis was not due to the observed reduced hypercholesterolemic response to high fat diet feeding, which potentially could have masked FAK mediated adverse effects on leukocyte trafficking. This suggests that the marked immune effects of compromised FAK function per se do not influence atherogenesis. Apparently the complex interaction of immune cells in the arterial wall leads to a new equilibrium in the FAK$^{+/-}$ApoE$^{-/-}$ mice culminating into an unchanged development of atherosclerotic lesions. It might be concluded that therapeutical approaches to diminish atherosclerotic lesions must be focused upon targets which play a more unidirectional role in lesion formation.

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