Chapter 4

Annexin A5 Therapy Attenuates Vascular Inflammation and Remodeling and Improves Endothelial Function in Mice

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Arterioscler Thromb Vasc Biol. 2011;31:95-101

Abstract

Objective Annexin A5 (AnxA5) has antithrombotic, anti-apoptotic and anti-inflammatory properties; we investigated its effectiveness against vascular inflammation, remodeling and dysfunction in accelerated atherosclerosis.

Methods and Results AnxA5 (1 mg/kg/d or vehicle) was investigated in vascular injury models in hypercholesterolemic ApoE3*Leiden mice. AnxA5 treatment reduced adhesion and infiltration of leucocytes by 71-69% (p=0.015, p=0.031) and macrophages by 51-87% (p=0.014, p=0.018), as well as MCP-1 and TNF-α expression in a femoral artery inflammation model (perivascular cuff for 3d), indicating reduced vascular inflammation. In a vein graft model, 28d AnxA5 treatment reduced vein graft thickening (48%, p=0.006) and leukocyte infiltration (46%, p=0.003). In these mice, reduced plasma concentrations of IFNγ (-72%, p=0.040), G-CSF (-41%, p=0.010) and MIP1β (-66%, p=0.020) were measured, indicating reduced systemic inflammation. An in vitro endothelial cell model shows the importance of AnxA5's anticoagulant properties in reducing vascular inflammation.
Endothelium-mediated dilatation in hypercholesterolemic ApoE$^{-/-}$ mice was improved by 3d anxA5 treatment, shown by improved systolic and diastolic blood pressure reductions in response to metacholine, which could be abolished by L-NAME, indicating nitric oxide involvement.

Conclusions AnxA5 reduced local vascular and systemic inflammation, vascular remodeling and improved vascular function, indicating a therapeutic potential against atherosclerotic cardiovascular diseases.

Introduction

Accelerated atherosclerosis is one of the key features leading to vein graft failure next to acute thrombosis and occlusive vein graft thickening and is initiated by endothelial dysfunction, accompanied by an inflammatory activation of endothelial cells and smooth muscle cells (SMCs)[1, 2]. This activation promotes adhesion of activated platelets and leukocytes and increases endothelial permeability, after which low-density lipoprotein (LDL) cholesterol accumulates in the vessel wall and is taken up by macrophages, which become foam cells[3, 4]. Leukocytes produce pro-inflammatory cytokines such as monocyte chemotactic protein (MCP)-1, tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ). Leukocytes and cytokine expression are also observed in murine vein grafts[3, 5]. IFNγ can activate macrophages and stimulate apoptosis, leading to plaque instability which can result in plaque disruption and thrombosis[6, 7].

Viable cells expose phosphatidylcholine and sphingomyelin on their outer cell membrane leaflet and express phosphatidylserine (PS) on their inner membrane leaflet. The annexins are a family of phospholipid-binding proteins and annexin A5 (AnxA5) binds to PS. AnxA5 was originally identified as an anticoagulant and antithrombotic protein[8-11], but is now known to have several other additional properties[12, 13]. PS is highly polarised to the inner cell membrane in normal cells, but is externalized in the early phase of apoptosis and inflammatory cell activation. AnxA5 binds reversibly, specifically and with high affinity to these cells, as well as to aged erythrocytes, endothelial microparticles (EMP), activated platelets and oxidized (ox) LDL cholesterol, and is present in high concentrations in atherosclerotic plaques. The annexins are thought mainly to act on intracellular mechanisms, but AnxA5 also has extracellular functions[13-16].

Plasma levels of AnxA5 are reported to be inversely related to the severity of coronary stenosis and are an indication of the extent of atherosclerotic plaques[17], to be elevated in hypertensives with systolic dysfunction[18] and following acute myocardial infarction[19]. AnxA5 also has anti-inflammatory effects, e.g. by association with the IFNγ receptor and prevention of inflammatory cellular responses to secreted IFNγ5. Atherosclerosis is associated with a reduced capacity of the endothelium to produce vasodilating substances upon stimulation, such as nitric oxide (NO). A reduction in endothelium-mediated dilatation (EMD) is a risk factor for premature cardiovascular disease and is associated with increased atherosclerosis development[20]. Binding of AnxA5 to PS may prevent PS-mediated platelet and leukocyte adhesion and affect the level of systemic inflammation and circulating cytokines, resulting in improved local and systemic endothelial function.

The binding to oxLDL cholesterol, to apoptotic cells and the potential inhibitory effect on inflammation in combination with its antithrombotic effects make AnxA5 a promising therapeutic agent against atherosclerosis. By using three mouse models for accelerated atherosclerosis development that share a common basis of endothelial cell activation, followed by local vascular wall inflammation, eventually leading to vascular remodeling, the aim of the present study was to investigate if AnxA5 is therapeutically effective against vascular dysfunction, inflammation and remodeling, all of which are important hallmarks of cardiovascular disease.

Materials and Methods

We performed an in vivo intervention study in which Western-type diet fed ApoeE3*Leiden mice were injected intraperitoneally daily with 1 mg/kg AnxA5 or vehicle only and underwent femoral arterial cuff placement or vein graft surgery. In these vascular segments, inflammatory cell adhesion, infiltration, intimal thickening and lesion composition were assessed using histology, morphometry, and immunohistochemistry. Plasma AnxA5 and cytokine concentrations were determined using ELISA and a multiplex biometric immunoassay. For in vitro studies, TNFα-activated HUVEC, incubated with and without AnxA5, underwent whole blood perfusion, allowing assessment of platelet adhesion, aggregation and leukocyte adherence. Western-type diet fed ApoE$^{-/-}$ mice, receiving 3d AnxA5 treatment or vehicle only, were used for endothelial function assessment, performed by stimulating vasodilatation and aortic blood pressure measurements. All materials and methods are detailed in the supplement (available online at http://atvb.ahajournals.org).

Results

Annexin A5 effects

AnxA5 treatment did not affect body weight, total plasma cholesterol or triglyceride concentrations. In the ApoE$^{-/-}$ mice, human AnxA5 plasma concentration, measured with ELISA, was 35.3±1.5ng/ml 1h after 1mg/kg injection and fell to 3.2±1.1ng/ml 6h after injection, which is within the normal range in man (see online figures for plasma concentrations). In mice receiving AnxA5 no bleeding complications occurred and there was one case of vein graft thrombosis (in n=10 mice) leading to loss of graft patency after 28d, compared to three cases of graft thrombosis in mice receiving vehicle only (n=10), this was statistically not significantly different (vehicle: 3/10 cases, annexin A5: 1/10 cases, p=0.582).

Annexin A5 has systemic anti-inflammatory effects

To monitor effects of AnxA5 on systemic inflammation, plasma concentration of a set of cytokines and chemokines were defined before surgery, 3d after cuff placement and 28d after vein graft surgery, respectively, using a multiplex BioRad Luminex analysis. Before surgery, no statistical differences were observed. After 3d, plasma concentrations of inflammatory cytokines were increased in all groups due to the inflammatory response evoked by cuff placement. However, at 28d after vein graft surgery AnxA5 treatment significantly reduced the plasma concentration of G-CSF by 40.8% (p=0.010), of IFNγ by 72.2% (p=0.040) and of MIP-1β/CCL4 by 66.1% (p=0.020), when compared to vehicle. In addition, AnxA5 treatment gave a trend towards reduction of the plasma levels of the cytokines IL-12 (p40) (p=0.120) and MIP-1α /CCL3 (p=0.160), shown in table 1. The total dataset is represented in table 1 in the online data supplement.

Annexin A5 reduces inflammatory cell recruitment after vascular injury

AnxA5 treatment reduced the percentage of femoral arterial endothelial adhesion of leukocytes by 71.3% (vehicle: 34.2±3.0%, AnxA5: 9.7±8.9% of the sum of endothe

	Vehicle	Annexin A5
Cell type surface (%) area		
Macrophages	35.6±7.7%	32.5±7.4%
Smooth muscle cells	23.0±4.0%	28.4±3.6%
Collagen	35.8±5.5%	45.7±5.0%
Number of cells		
Leukocytes	53.1±6.1	28.8±2.3*
Apoptotic cells	6.2±2.2	4.3±0.8
Plasma cytokine concentration (pg/ml)		
Interferon γ	7.9±2.5	2.2±0.7*
G-CSF	14.2±2.6	8.4±1.0*
MIP-1β/CCL4	22.1±10.3	7.5±0.9*

Table 1. Percentage of cell type area out of total vein graft wall area, total cell numbers per vein graft wall cross-section and plasma cytokine concentration (pg/ml) in ApoE3*Leiden mice 28d after surgery and vehicle only or AnxA5 treatment (mean±SEM, n=10).

lial and intimal cells, p=0.015) and of macrophages by 51.4% (vehicle: 24.5±3.5%, AnxA5: 11.9±2.3%, p=0.014) after 3d compared to controls. Furthermore, within the media the percentage of leukocytes out of all cells in the media was reduced by 69.0% (vehicle: 19.6±4.6%, AnxA5: 6.1±2.6%, p=0.031) and that of macrophages by 87.3% (vehicle: 14.2±5.5%, AnxA5: 1.8±0.9% p=0.018). At this time point foam cells, recognisable by positive macrophage staining, intracellular lipid deposition and cell swelling, could not be detected.

Although plasma levels of MCP-1 and TNFα were not affected by AnxA5 treatment in the femoral artery cuff experiment, the arteries were stained for cells expressing MCP-1 and TNFα to investigate if AnxA5 reduced local vascular inflammation. It was found that AnxA5 treatment reduced the percentage of adherent and endothelial cells expressing MCP-1 by 31.0% (vehicle: 49.1±2.2%, AnxA5: 33.9±3.1%, p=0.003) and that of TNFα by 42.7% (vehicle: 39.6±8.0%, AnxA5: 22.7±2.2%, p=0.049) after 3d. Additionally, the percentage of cells in the media expressing MCP-1 dropped by 52.7% (vehicle: 31.1±1.8%, AnxA5: 14.7±2.8%, p=0.001), although no relative difference was observed for medial cells expressing TNFα (vehicle: 21.2±6.2%, AnxA5: 12.9±2.9%, p=0. 335) at this time point. The data, including photomicrographs, is shown in figure 1. A complete overview of all data is shown in table 2 of the online data supplement.

Annexin A5 reduces vascular remodeling and preserves vein graft patency

To investigate the therapeutic effectiveness of AnxA5 against vascular remodeling, vein graft thickening, measured as the area between the lumen and adventitia in cross-sections, was quantified after 28d. This revealed that AnxA5 significantly reduced vein graft thickening by 48.0% (vehicle: 0.25±0.05mm^2, AnxA5: 0.13±0.01mm^2, p=0.006), thus preserving graft patency, shown in figure 2. Although in the controls AnxA5 could be detected by immunohistochemistry, the staining in the vessel wall of the mice injected with human AnxA5 was much more intense. This suggests accumulation of human AnxA5 in the vein graft wall, although one should be careful with

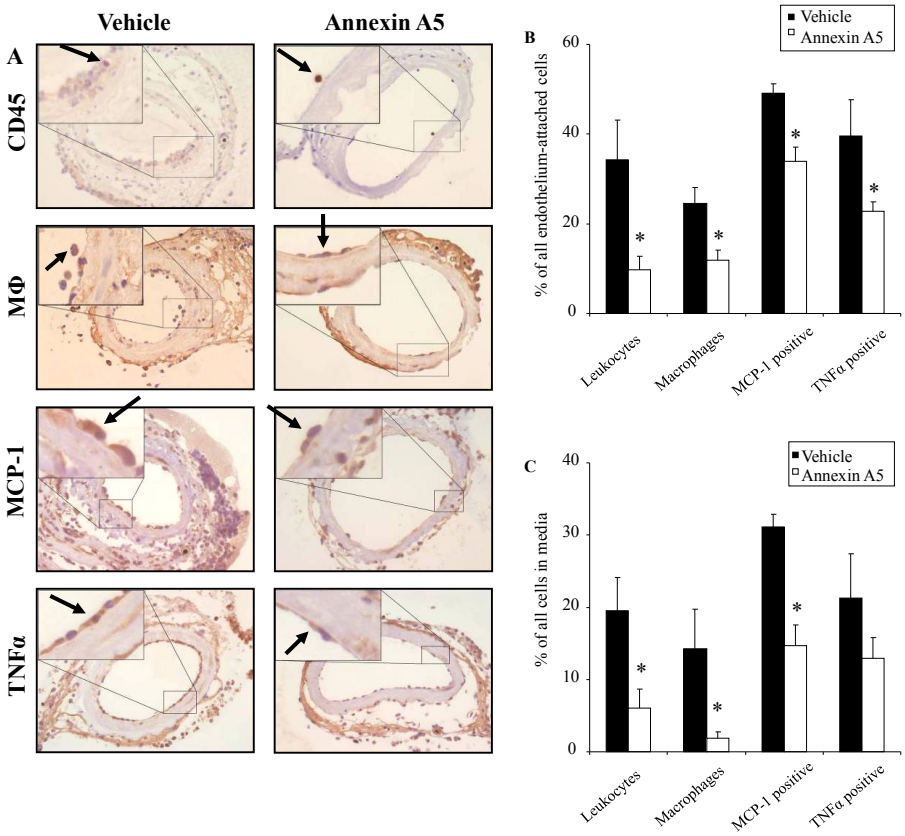

Figure 1. Panel A: representative cross-sections of cuffed-femoral arteries of ApoE3*Leiden mice treated with vehicle or AnxA5, 3d after cuff placement (leukocyte, macrophage, MCP-1 and TNFα staining, magnification 400x, arrows in inserts indicate positive staining). Panels B and C: quantification of cell types in cuffed femoral arteries attaching to the endothelium (panel B) or within the media (panel C), expressed as the percentage of all cells adhering to the endothelium or in the media, in vehicle and AnxA5 treated mice (mean±SEM, n=10).

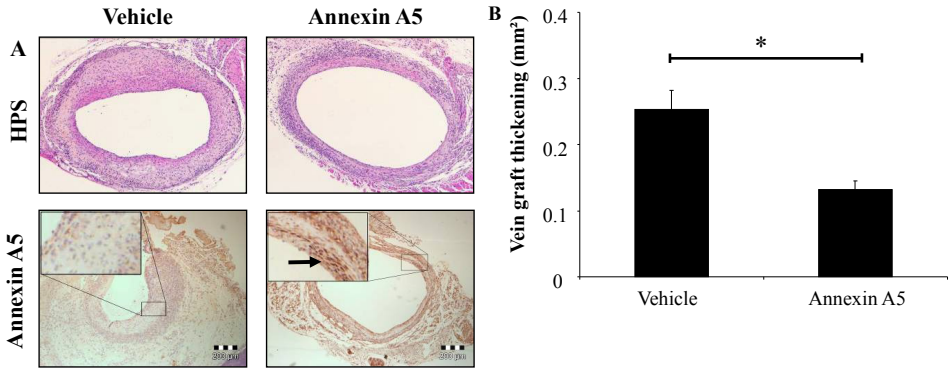

Figure 2. Effect of AnxA5 treatment on vein graft thickening in ApoE3*Leiden mice treated with vehicle or AnxA5, 28d after surgery. Panel A: representative cross-sections of vein grafts, (HPS and AnxA5 staining, magnification 80x, arrows in insert indicates positive staining). Panel B: quantification of vein graft thickening, expressed as mm^2 thickening in vehicle and AnxA5 treated mice (mean±SEM, n=10).

quantification of this immunohistochemistry data.
In order to identify if AnxA5 therapy altered atherosclerotic plaque composition, the presence of several important cell types and extracellular matrix was quantified, shown in table 1. AnxA5 treatment reduced the number of leukocytes in the vein graft wall per cross-section by 46% (vehicle: 53.1±6.1 cells, AnxA5: 28.8±2.3 cells, p=0.003). However, despite reduced wall thickening, there was no difference observed in contribution of cell types and collagen to the build-up of the vessel wall, observed in macrophage area (from total wall area) (vehicle: 35.6±7.7%, AnxA5: 32.5±7.4%, p=0.723), SMC area (vehicle: 23.0 ±4.0%, AnxA5: 28.4±3.6%, p=0.289) or collagen area (vehicle: 35.8±5.5%, AnxA5: 45.7±5.0%, p=0.289) between mice receiving daily AnxA5 or vehicle.
In order to evaluate the therapeutic effects of AnxA5 on plaque stability, apoptotic cell numbers and signs of plaque disruptions, characteristic for vein grafts, were quantified. AnxA5 treatment did not affect vein graft apoptotic cells numbers per cross-section (vehicle: 6.2±2.2 cells, AnxA5: 4.3±0.8 cells, p=0.796) after 28d, shown in table 1. Analysis of plaque morphology revealed the presence of disruption features and it was found that AnxA5 therapy did reduce the number, length and severity of endothelial erosions with fibrinogen lining the vessel wall (vehicle: 3/6 cases, AnxA5: 2/9 cases) and therefore characteristically distinct from post-mortem artifacts, leaky vessel formation with intramural erythrocytes (vehicle: 3/6 cases, AnxA5: 0/9 cases) and of severe plaque disruptions with erythrocytes and thrombi in a subendothelial space (vehicle: 1/6 cases, AnxA5: 0/9 cases), shown in figure 3. Although no firm conclusions about the possible protective functions of anxA5 on plaque stability can be drawn from these observations, the reported features are important to the plaque and vessel wall morphology and are known to be associated with increased plaque instability in human (coronary) atherosclerotic lesions.

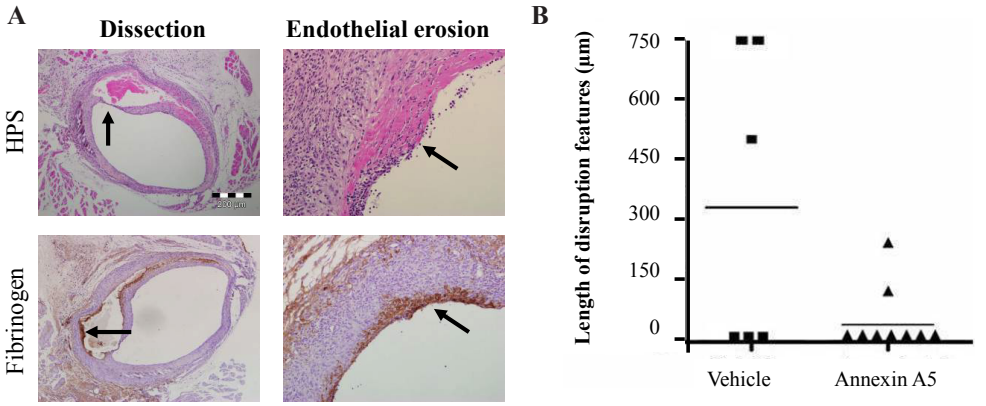

Figure 3. Panel A: representative cross-sections of spontaneous plaque disruption features in vein grafts of ApoE3*Leiden mice after 28d (HPS and fibrinogen staining, magnification 80x, arrows in inserts indicate features). Panel B: quantification of length (in µm) and number of plaque instability features (total of endothelial erosions, leaky vessel formation and plaque disruptions) in vehicle and AnxA5 treated mice (mean±SEM, n=10).

Annexin A5 reduced endothelial-platelet and leukocyte-endothelial adhesion

To investigate how AnxA5 led to a reduction of endothelial-leukocyte adhesion in injured vascular segments, human umbilical vein endothelial cells (HUVEC) were

stimulated with TNFα for 4h to induce endothelial cell activation in the presence and absence of AnxA5. AnxA5 (0.75 μg/mL) was added to HUVEC during TNFα-stimulation (4 hrs) and added to the whole blood prior (5 min) and during the perfusion. AnxA5 inhibited leukocyte adhesion by 58.8% (TNFα only: 47.8±16.5 leukocytes / microscopic field, TNFα + AnxA5: 19.7±11.4 leukocytes / microscopic field, n=25 frames, p<0.001) and platelet adhesion area by 48.6% (TNFα only: 21.7±7.8% coverage, TNFα + AnxA5: 11.1±6.6% coverage, n=25 frames, p<0.001), shown in figure 4.

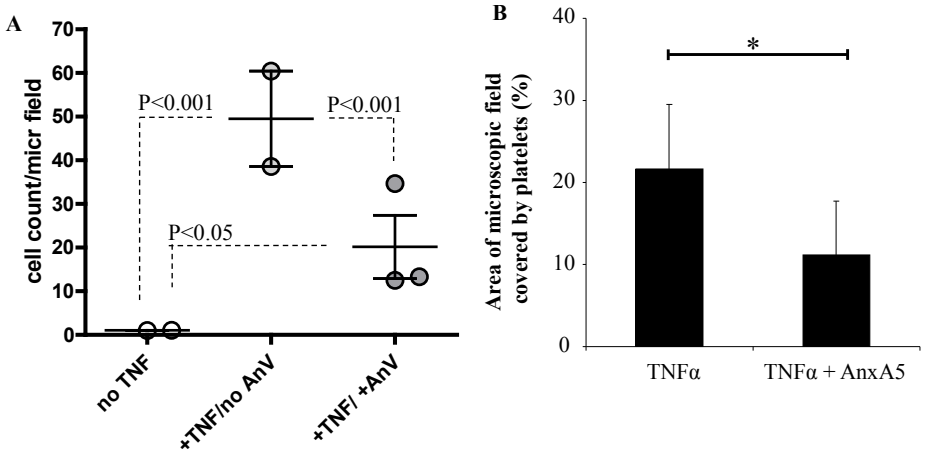

Figure 4. Whole blood was perfused over non-stimulated HUVEC or over TNFα-stimulated HUVEC in the presence of AnxA5 (0.75μg/mL) with LMWH-anticoagulated whole blood for 10 minutes at 1 dyne/cm2. After each perfusion, 25 images were made to quantify adhered leukocytes per microscopic field (Panel A). Panel B: in the presence of AnxA5, less platelet-adherence (area of microscopic field covered) aggregation are observed.

Non-stimulated HUVEC hardly supported adhesion of platelets and leukocytes, indicating that TNFα-induced tissue factor- and PS-expression was necessary for binding under flow of both platelets and leukocytes.

Annexin A5 improves endothelial function

To test the effect of AnxA5 on vascular function, endothelial dysfunction was induced by feeding ApoE$^{-/-}$ mice a Western-type diet for 16 to 17 weeks and was quantified by measuring the reduction of metacholine-induced NO-mediated vasodilatation. After this period, mice were given IP injections with 1mg/kg AnxA5 once daily for 3d. Mice were then anaesthetized and blood pressure measurements began. There were no differences in heart rate and systolic or diastolic blood pressure between groups receiving vehicle or AnxA5 at baseline.

In normal, non-atherosclerotic mice, 3μg/kg of metacholine lead to a transient reduction in blood pressure, the response was maximal after 1-3min and then returned towards baseline after approximately 5min. This effect was blunted in ApoE$^{-/-}$ mice on a Western-type diet. However, AnxA5 treatment restored EMD, leading to a larger systolic blood pressure reduction (vehicle: -5.0mmHg, AnxA5: -22.3mmgHg, p=0.027) and diastolic blood pressure reduction (vehicle: -0.8mmHg, AnxA5: -11.0mmHg, p=0.029) in the treated animals, shown in figure 5.

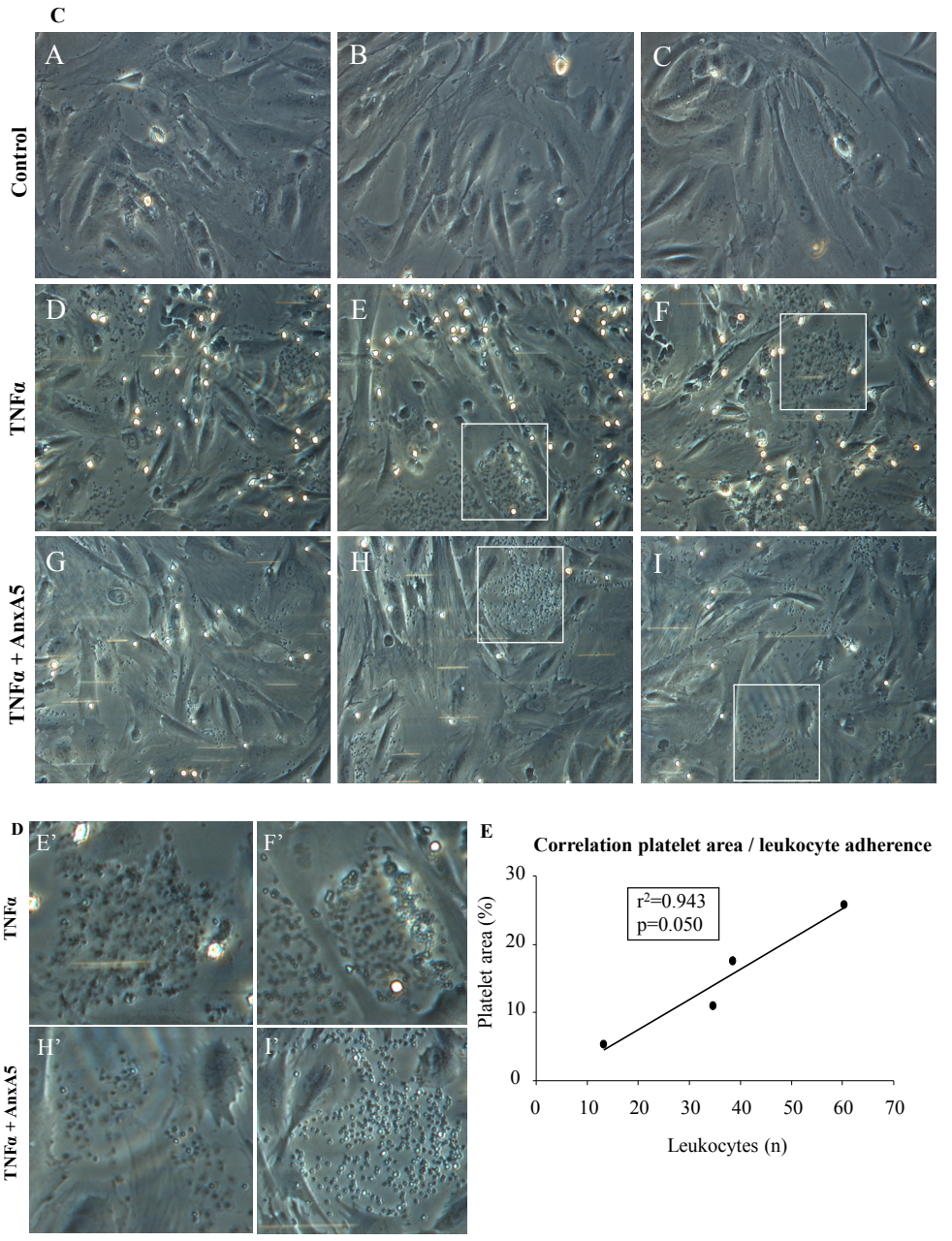

Figure 4. Whole blood was perfused over non-stimulated HUVEC or over TNFα-stimulated HUVEC in the presence of AnxA5 (0.75μg/mL) with LMWH-anticoagulated whole blood for 10 minutes at 1 dyne/cm^2. Panel C: three representative micrographs are shown of cell-adhesion to non-stimulated HUVEC (A, B and C), TNFα-stimulated HUVEC (D, E and F) and TNFα and AnxA5-stimulated HUVEC. The rectangles in panels E, F, H and I are magnified (panel D) and quantified (panel B) to show that platelet morphology is changed after AnxA5 treatment. Panel E: significant correlation exists between area of platelet adherence and leukocyte binding (all conditions, n=4, r^2=0.943, p=0.050).

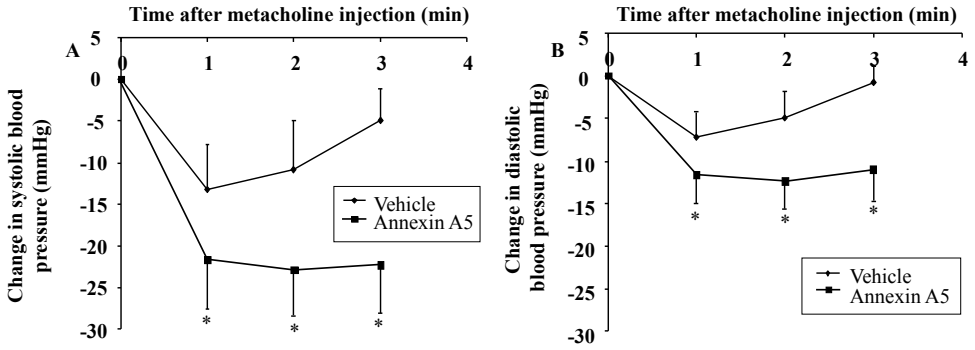

Figure 5. Effect of 3d AnxA5 treatment on impaired endothelial function in ApoE$^{-/-}$ mice, quantified as change in aortic systolic (panel A) and diastolic (panel B) blood pressure (mmHg) in time after IP injection of metacholine to induce vasodilatation (mean±SEM, n=10).

Blood pressures are reported in table 3 in the online data supplement. The reduction in systolic pressure was the largest, thus pulse pressure was reduced during EMD. Metacholine injection did not lead to changes in heart rate (vehicle: -1.5 beats per minute (bpm), AnxA5: -8.5bpm, p=0.379).
Injection of 50mg/kg L-NAME resulted in similar increases in systolic (vehicle: +27.6mmgHg, AnxA5: +29.7mmgHg, p=0.518) and diastolic (vehicle: +15.6mmgHg, AnxA5: +18.0mmHg, p=0.350) blood pressure in both groups, accompanied by an equal reduction in heart rate (vehicle: -40.0bpm, AnxA5: -37.8bpm, p=0.833). Subsequent IP injection of metacholine following L-NAME injection did not result in a significant change in heart rate (vehicle: -17.9bpm, AnxA5: -21.0bpm, p=0.559) or systolic (vehicle: -5.1mmHg, AnxA5: +1.0mmHg, p=0.060) and diastolic (vehicle: -1.3mmHg, AnxA5: +2.2mmHg, p=0.071) blood pressure in either group (online supplements, table 2), indicating that AnxA5 could affect vascular function through improved NO-signaling.

Discussion

Our study shows a therapeutic role for AnxA5 against vascular inflammation, remodeling and dysfunction, in mouse models for accelerated atherosclerosis development. It was demonstrated that injection of human AnxA5 had no adverse effects, resulted in a marked reduction in circulating plasma concentrations of IFNγ, G-CSF and MIP-1β/CCL4, early inflammatory cell recruitment, adhesion and infiltration in the vessel wall after vascular injury, eventually leading to decreased vein graft thickening with less plaque instability features. Furthermore, AnxA5 improved endothelial dysfunction by acting on NO-signaling. This study extends the role of AnxA5 as a regulator of inflammatory processes, and demonstrates its potential therapeutic use in inflammation-associated vascular disease in addition to its known functions in anticoagulation and its use as a marker of apoptosis.
Although these data can be of clinical significance and could potentially lead to new therapeutic strategies, at this point we do not have the complete insight in the underlying mechanism of how AnxA5 affects vessel wall pathology. It was previously

shown that AnxA5 could affect immune reactions on a systemic level. The injection of PS-exposing cells induced a delayed-type hypersensitivity reaction, which was critically affected by AnxA5[21]. This suggested that the PS receptor can act as a switch for immune reactions and AnxA5 will interfere with this function[22]. In vitro investigation using TNFα-stimulated HUVEC clearly indicated the importance of the anti-coagulant effects of AnxA5 in its ability to reduce leukocyte adhesion to activated endothelium, since platelet adherence is known to be essential for subsequent leukocyte adhesion.

A multiplex analysis of plasma cytokine levels revealed that the raise in levels of circulating cytokines observed 3d after femoral cuff implantation was not affected by AnxA5. However, prolonged exposure to AnxA5 reduced plasma levels of G-CSF, MIP-1β/CCL4 and IFNγ and certainly did have a systemic immunomodulatory effect. IFNγ production by inflammatory cells leads to improved antigen presentation and increases atherogenesis. In ApoE$^{-/-}$ mice lacking IFNγ or its receptor, atherogenesis was inhibited[3, 23]. In vitro research has shown that AnxA5 can prevent cellular responses to secreted IFNγ and these data suggest that AnxA5 influences the IFNγ production by circulating cells involved in the early inflammatory processes during atherosclerotic lesion formation and vascular remodeling[3-5].

Leukocyte, and specifically monocyte, adhesion, differentiation and activation is a key component in the onset and progression of atherosclerosis and post-interventional accelerated atherosclerosis[3]. To investigate effects of AnxA5 on local vascular inflammation, the ApoE3*Leiden femoral artery cuff model[24], characterized by inflammatory cell adhesion and infiltration in the vessel wall early after surgery, was used. Not only did AnxA5 reduce endothelial-adhesion and medial infiltration of both leukocytes and macrophages, it was also found that early local expression of the key inflammatory cytokines MCP-1 and TNFα was reduced[25, 26]. However, this local reduction of MCP-1 and TNFα levels was not reflected by an alteration of plasma levels after 3d, nor after prolonged AnxA5 exposure. Taken together, these findings suggest that AnxA5 can be used therapeutically to reduce local inflammatory responses after vascular injury. The exact mechanism by which AnxA5 led to these effects was not elucidated, but it has been shown that this is probably due to local AnxA5 binding to activated cells. AnxA5 reduces local adherence of platelets, especially their activation and subsequently adherence of leukocytes (figure 4), ultimately preventing their deleterious inflammatory effects.

To investigate if the reduced early vascular inflammation by AnxA5 could be extended to a beneficial clinical therapeutic effect on accelerated atherosclerosis in general, AnxA5 was studied additionally in an model for vein graft pathology[27]. Here, accelerated atherosclerosis forms due to vascular damage, endothelial cell loss and a pulsatile arterial pressure, similarly to human venous bypass graft disease in which rapid foam cells formation, intimal thickening, apoptotic cell exposure and plaque instability features are observed, processes that lead to loss of graft patency. AnxA5 treatment significantly reduced vein graft thickening after 28d by 48%, as well as leukocyte infiltration in the vein graft wall by 46%. Although AnxA5 did not alter plaque content, it nonetheless preserved graft patency greatly.

Complications of atherosclerosis are either the result of reduced blood flow due to luminal narrowing or from acute vessel occlusion due to rupture of the atherosclerotic plaque. Apoptosis is associated with decreased plaque stability and although AnxA5

can affect apoptosis[13], no differences in apoptotic cell numbers detected with TUNEL staining were observed at 28d after surgery. Since apoptosis is known to occur predominately within 14d after surgery in this model[28], effects on apoptosis by AnxA5 on earlier time points cannot be ruled out. Despite no change in apoptotic cells numbers after 28d by AnxA5 treatment, it did reduce vessel plaque instability features such as endothelial erosion, leaky vessel formation and severe plaque disruption in addition to vessel wall thickening, indicating that AnxA5 treatment is effective against both vascular inflammation and (the complications of) vascular remodeling.

Improvement of endothelial function is an important therapeutic goal in vascular diseases. In our study, short-term AnxA5 treatment improved EMD in response to metacholine injection to induce vasodilatation, displayed by a larger and longer-lasting reduction of systolic and diastolic blood pressure in ApoE$^{-/-}$ mice with impaired endothelial function. This larger blood pressure reduction was blunted by L-NAME administration prior to the metacholine injection, indicating this effect was mediated via NO. Since this is important for the central arterial compartment compliance[29], a large reduction of systolic blood pressure after AnxA5 treatment is suggestive for a positive effect on the endothelium in conduit arteries, often early affected by native atherosclerosis in humans[3, 4]. Both acute and chronic inflammation is known to hamper EMD, so the effects of AnxA5 on endothelial function could be a result of a reduced inflammatory burden (figures 1 and 2), but also from other mechanisms. For example, endothelial cells can release EMP which in turn can trigger endothelial cytokine release and leukocyte adhesion. AnxA5 can shield EMP through PS-binding and prevent their endothelial cell activation. Thus, although effectiveness was shown, the exact mechanism by which AnxA5 improved deteriorated endothelial function in vivo is not yet clear and awaits further research.

In normal humans, plasma AnxA5 concentration is normally well below 10 ng/ml, and is elevated in certain diseases[17-19]. In our experiments, 1mg AnxA5/kg/d only transiently elevated plasma AnxA5 above these levels (figure 1 online supplement). In spite of this, profound treatment effects were observed. This is probably explained by a rapid and strong binding to its target tissue, acting long after free AnxA5 is cleared from plasma. Clearance of AnxA5 is much slower from tissue compartments than from plasma, which is why it is useful as an apoptosis marker in vivo[30].

AnxA5 is already used safely in patients as a diagnostic tool for atherosclerosis[14]. Since the present study shows pronounced anti-inflammatory and anti-atherogenic effects of AnxA5 in vivo on accelerated atherosclerosis associated with post-interventional vascular remodeling and vein graft disease, as well as atherosclerosis-associated vascular dysfunction, it also indicates a possible therapeutic role for AnxA5 in the prevention of accelerated atherosclerosis after vascular surgery and coronary interventions.

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Supplement Material

Mice

All experiments were approved by the Institutional Committees for Animal Welfare. Transgenic male ApoE*3-Leiden mice (bred in our own laboratory), backcrossed for more than 20 generations on a C57BL/6J background and male ApoE$^{-/-}$ mice (Taconics, Lille Skensved, Denmark), aged 10-12 weeks at the start of a dietary run-in period, were used for this experiment.

Diets

Transgenic male ApoE*3-Leiden mice were fed a Western-type diet containing 1% cholesterol and 0.05% cholate to induce hypercholesterolemia (AB Diets, Woerden, The Netherlands). The diet was given three weeks prior to surgery and was continued throughout the experiment. Male ApoE$^{-/-}$ mice, 8-10 weeks old, received a Western-type diet (Harlan Teklad TD.88137) for 16-17 weeks to induce endothelial dysfunction. All animals received food and water ad libitum during the entire experiment.

Femoral artery cuff mouse model

To investigate the effect of annexin A5 on vascular inflammation, ApoE*3-Leiden mice underwent femoral arterial cuff placement[1] after three weeks of diet to induce vascular inflammation. Mice were anesthetized before surgery with a combination of intraperitoneally (IP) injected Midazolam (5mg/kg, Roche, Woerden, The Netherlands), Medetomidine (0.5mg/kg, Orion, Espoo, Finland) and Fentanyl (0.05mg/kg, Janssen, Berchem, Belgium). The right femoral artery was isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex, Kent, UK, 0.40mm inner diameter, 0.80mm outer diameter and an approximate length of 2.0mm). 3d after cuff placement, mice were anesthetized as before and euthanized.
The thorax was opened and mild pressure-perfusion (100mm Hg) with 3.7% formaldehyde in water (w/v) was performed for 5min by cardiac puncture in the left ventricle. After perfusion, the cuffed femoral artery was harvested, fixed overnight in 3.7% formaldehyde in water (w/v) and paraffin-embedded. Serial cross-sections (5µm thick) were taken from the entire length of the artery for analysis.
Mice received daily IP injections with 1mg/kg human recombinant annexin A5 (Bender Medsystems cat.no. BMS306/50mg. lot no.24926000) in a volume of 150µl, using 150µl 0.9% w/v NaCl (vehicle), injected IP daily as control.

Carotid vein graft model for accelerated atherosclerosis

To study the effect of annexin A5 on vascular remodeling, vein graft surgery[2] was done in ApoE*3-Leiden mice after three weeks of diet and performed by one surgeon, to exclude the possibility of inter-group variation due to different surgical skills. Mice were anesthetized and the right common carotid artery was dissected free from its surroundings from the bifurcation at the distal end towards the proximal end. The vessel was ligated twice with an 8.0 silk ligature and dissected between the middle ties. A cuff was placed over both ends after which these were everted over the cuffs and ligated with an 8.0 silk ligature. Littermates were used as donor for the inferior caval vein. The carefully harvested inferior caval vein was temporarily preserved in a

0.9% NaCl solution, containing 100U/ml of heparin and was interpositioned between the ends of the artery. The connections were ligated together with an 8.0 silk suture. Pulsations confirmed successful engraftment. Mice were sacrificed 28d after vein grafting as described before. Daily annexin A5 and vehicle treatments were as described above. Blood samples were taken from the tail vein for plasma annexin A5 analysis at 1, 3 and 6h after administration.

Biochemical analysis

Total plasma cholesterol (Boehringer Mannheim GmbH, kit 236691) and triglyceride (Sigma Diagnostics, kit 337-B) concentrations were measured enzymatically.
Annexin A5 plasma concentration was determined using a human annexin A5 ELISA kit (Bender MedSystems Products, Vienna, Austria).
To investigate effects of annexin A5 on systemic inflammation, a multiplex biometric immunoassay was used for cytokine and chemokine measurements according to the manufacturer's instructions (Bio-PlexTM Mouse Cytokine 23-Plex Panel; Bio-Rad., Hercules, CA, USA). The plasma concentrations of eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), IFNγ, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12(p70), IL-13, IL-17, keratinocyte chemoattractant (KC), MCP-1/CCL2, MIP-1α / CCL3, MIP-1β/CCL4, RANTES and TNFα were measured. Bio-PlexTM mouse cytokine standard values were provided by Bio-Rad Laboratories. Cytokine levels were determined using a Liquichip-200 multiplex array reader with Luminex x MAP™ technology (Bio-Rad Laboratories). The analyte concentration was calculated using software provided by the manufacturer (Bio-Plex Manager Software).

Quantification of cuffed femoral artery and vein graft lesions

At time of sacrifice, vessels were harvested after 5min in vivo perfusion-fixation with formaldehyde (4%), fixated overnight and embedded in paraffin. Immunohistochemical (IHC) staining was performed using positive and negative tissue-specific controls as indicated by the antibody manufacturer. All samples were stained with hematoxylin-phloxine-saffron (HPS) and specific vessel wall cellular composition was visualized using antibodies against leukocytes (anti-CD45 antibodies 1:200, Pharmingen, San Diego, CA, USA) and monocytes and macrophages (AIA 31240 1:3000, Accurate Chemical, Westbury, NY, USA). To evaluate if annexin A5 could affect the degree of inflammation within the arterial wall, cells expressing cytokines known to be important in the restenotic process were quantified using antibodies against TNFα (anti-TNFα 1:200, BioLegend, San Diego, CA, USA) and MCP-1 (anti-MCP-1 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The number of leukocytes, macrophages, foam cells and cells expressing MCP-1 and TNFα attached to the endothelium or in the media of the femoral arteries was quantified and is displayed as a percentage of the total number of present cells. All quantification in this study was performed on six equally spaced (150μm distance) serial stained perpendicular cross-sections throughout the entire length of the vessel and was performed by blinded observers.
Vein grafts were stained for collagen (Sirius Red staining) and with antibodies against vascular SMCs (α-SM-actin 1:800, Dako, Enschede, The Netherlands), annexin A5 (anti-annexin V 1:100, Biovision, Mountain View, CA, USA), fibrinogen and

fibrin (anti-fibrinogen and fibrin, 1:400[3]) and apoptotic cells (TUNEL staining, Roche, Applied Sciences, Almere, The Netherlands). The area containing SMCs, collagen, macrophages and foam cells in vein grafts was quantified using computer-assisted morphometric analysis (Qwin, Leica) and is expressed as a percentage of the total cross-sectional vein graft area. Since there are only a few cell layers within the media of murine veins and no clear morphological border exists between the media and neointima, the region between lumen and adventitia was used to define lesion area and vein graft thickening. To determine the therapeutic effect of annexin A5 on plaque instability features in vein grafts, the absolute number of apoptotic cells and leukocytes and signs of plaque erosion (endothelial disruption and fibrinogen deposition), leaky vessels (erythrocytes within newly formed vessels in the vessel wall) or plaque dissection (erythrocytes in a subendothelial space) were counted.

Effects of annexin A5 on endothelial-platelet and endothelial-leukocyte interaction using an ex vivo vascular injury perfusion model

To investigate if annexin A5 affects platelet-endothelium or leukocyte-endothelium interactions, human umbilical vein endothelial cells (HUVEC), isolated from umbilical cord according to the method of Jaffe et al[4], were cultured on gelatin-coated glass coverslips in endothelial specific medium (EGM-2, Clonetics, Lonza Verviers, S.p.r.l., Verviers, Belgium) supplemented with growth factors and cytokines. At confluence, HUVEC from passage 2 or 3 were stimulated for 4 hours with tumor necrosis factor-α (TNFα, 10 ng/mL, Boehringer, Ingelheim, Alkmaar, The Netherlands) to activate the endothelial cells and thus induce expression of TF and PS, in the presence or absence of annexin A5 (0.75 µg/ml, AbD Serotec, Düsseldorf, Germany). Non-stimulated HUVEC kept in EGM-2 medium were used as negative control. After TNF-stimulation, HUVEC were washed and kept in EGM-2 medium.

To evaluate the effect of annexin A5 on the adhesion of leukocytes, low-molecular weight heparin (LMWH-Pfizer, Capelle a/d IIssel, The Netherlands)-anticoagulated whole blood perfusions were performed, obtained from aspirin-free donors. Perfusions were performed for 10 minutes at 1 dyne/cm^2 according to de Boer et al[5]. When HUVEC were treated with annexin A5 during TNFα-stimulation, whole blood was also pre-incubated with annexin A5 for 5 min prior to the perfusion and during the perfusion, to inhibit PS, generated during flow. Non-bound blood components were removed by washing with HEPES buffer (20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L MgSO4, 1.2 mmol/L KH2PO4, 5 mmol/L glucose, 1.0 mmol/L CaCl2, 0.5% human serum albumin [CeAlb, Sanquin, Amsterdam, The Netherlands], pH 7.4). All perfusions were performed in duplicate/ triplicate runs. Afterwards, at least 25 photomicrographs were taken (Leica DMI-6000) per perfusion, allowing quantification of leukocyte adhesion to the endothelium.

Assessment of vascular function through endothelium-mediated dilatation

The determine therapeutic effects of annexin A5 on vascular function, EMD was evaluated in ApoE$^{-/-}$ mice after 16-17 weeks on a Western-type diet. Mice were treated with annexin A5 or vehicle as described above once daily for three days. Afterwards, mice were anaesthetized with Isofluran (Isoba®vet, Schering-Plough Animal Health, Denmark, 4.5% for induction and 1.5-2% for maintenance). A high fidelity pressure transducer (Samba Sensor preclin 420LP, Västra Frölunda, Sweden) was introdu-

ced into the left common carotid artery and transferred into the aortic arch. The Samba sensor was connected to a Samba 3200 unit, with 1000Hz as sample rate. Data was acquired in Powerlab (AD Instrument, v5) at 2KHz. Mice were allowed to stabilize for 15min after surgery.
Blood pressure measurements were performed at basal level and after IP injection of 3µg/kg metacholine to stimulate endothelial NO-release and vasodilatation (Acetyl-ß-methylcholine-chloride, 98%, Sigma-Aldrich, Stockholm, Sweden). The blood pressure was measured for up to 5min. When baseline pressure was again established, IP injection of 50mg/kg (10µl/g) Nw-Nitro-L-Arginine-methyl-ester-hydrochloride 98% (L-NAME), a NO-synthase inhibitor, (Sigma-Aldrich) was given. Blood pressure was then measured until a new plateau was reached (12-15min), after which an additional IP injection of 3µg/kg metacholine was given and blood pressure was measured again for 5min.

Statistical analysis
All data are presented as mean±standard error of the mean (SEM), unless otherwise indicated. Overall comparisons between data from groups were performed using the Kruskal-Wallis test. If a significant difference was found, groups were compared using a Mann-Whitney sum test. For the evaluation of cases of vein graft thrombosis, the Fisher's exact test was used (incidences in both groups were $n<5$). Comparison of endothelial-leukocyte adhesion between various groups was performed using a 2-way ANOVA group analysis with Bonferroni posttest. All statistical analyses were performed with SPSS 14.0 software for Windows. P-values ≤ 0.05 were regarded as statistically significant and are indicated with an asterisk (*).

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Supplemental figures

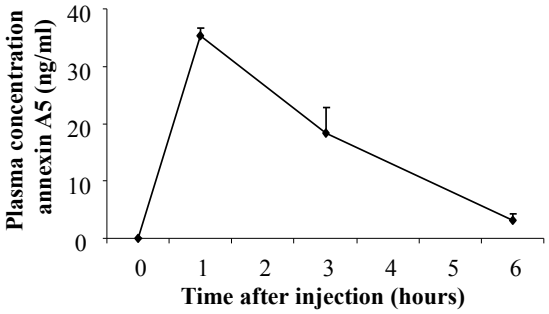

Figure I. Plasma concentration (ng/ml) of circulating human AnxA5 in ApoE3*Leiden mice after IP injection of 1mg/kg/d AnxA5, detected by ELISA, in time (mean±SEM, n=3).

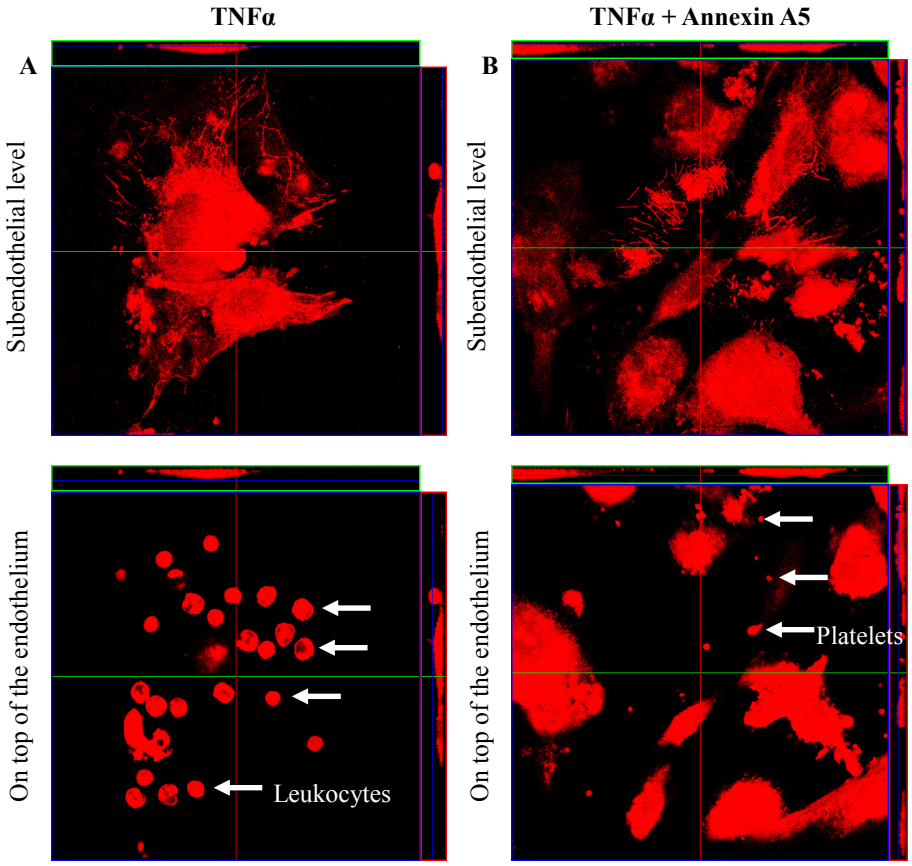

Figure II. Representative photomicrographs of cultured HUVECS, 4h after stimulation with TNFα with and without anxA5, taken using confocal laser scanning microscopy (original magnification 40x), in a three-dimensional area (1 and 8 µm cell height). Note the presence of platelets and fibrin at the endothelial cell level, with the presence of adhered leukocytes (n=21 in TNFα group, n=2 in the TNFα + AnxA5 group), restricted to the endothelial cell surface in the TNFα only group.

Cytokine (pg/ml)	Vehicle	Annexin A5
Eotaxin	76.5±38.7	118.5±24.2
G-CSF	14.2±2.6	8.4±1.0*
GM-CSF	9.9±4.4	3.3±1.2
Interferon γ	7.9±2.5	2.2±0.7*
IL-10	9.6±2.0	10.0±1.3
IL-12 (p40)	78.7±19.9	53.2±5.8
IL-12 (p70)	2.3±0.6	1.7±0.4
IL-13	56.1±18.2	102.3±21.3
IL-17	44.3±8.8	36.1±9.6
IL-1α	0.8±0.4	1.5±0.5
IL-1β	39.1±21.2	17.8±2.5
IL-2	13.6±3.6	14.2±2.6
IL-3	0.3±0.3	0.0±0.0†
IL-4	0.0±0.0†	0.0±0.0†
IL-5	2.9±1.7	1.3±0.5
IL-6	17.0±14.5	1.1±0.6
IL-9	74.4±21.8	102.4±13.8
KC	22.2±3.5	28.3±3.5
MCP-1	46.6±7.1	43.9±5.5
MIP-1α/CCL3	21.9±6.2	10.9±3.6
MIP-1β/CCL4	22.1±10.3	7.5±0.9*
RANTES	3.6±0.7	3.3±0.5
TNFα	204.2±48.2	158.4±37.3

Table I. Plasma cytokine concentration (pg/ml), determined using a multiplex biometric immunoassay in ApoE3*Leiden mice, 3 and 28 days after (femoral arterial cuff or vein graft) surgery and vehicle only or (1mg/kg/d) anxA5 treatment (mean±SEM, n=10). * $p<0.05$, † not detectable.

	Attached cells to endothelium		Cells in medial layer	
	Vehicle	AnxA5	Vehicle	AnxA5
Macrophages	24.5±3.5%	11.9±2.3%*	14.2±5.5%	1.8±0.9%*
Leukocytes	34.2±8.9%	9.7±3.0%*	19.6±4.6%	6.1±2.6%*
Cells expressing MCP-1	49.1±2.2%	33.9±3.1%*	31.1±1.8%	14.7±2.8%*
Cells expressing TNFα	39.6±8.0%	22.7±2.2%*	21.2±6.2%	12.9±2.9%

Table II. Quantification of inflammatory cell recruitment to the site of vascular injury in ApoE3*Leiden mice, 3d after femoral arterial cuff placement and treatment with vehicle or anxA5. Inflammatory cell types, expressed as a percentage of all cells present (stained with hematoxylin) in the arterial wall (mean±SEM, n=10).

	Systolic pressure (mmHg)		Diastolic pressure (mmHg)	
	Vehicle	Annexin A5	Vehicle	Annexin A5
Basal level	133.7±5.8	129.9±5.7	92.7±6.1	88.1±3.4
Metacholine	128.7±5.1	107.6±6.6*	91.9±5.5	77.1±5.1*
L-NAME	161.3±14.2	159.6±6.5	108.3±9.1	106.1±3.9
L-NAME + metacholine	155.9±11.8	160.6±8.3	107.0±7.8	108.3±9.4

Table III. Systolic and diastolic aortic blood pressure (mmHg) in ApoE$^{-/-}$ mice with endothelial dysfunction and 3d treatment with vehicle or annexin A5, shown at basal level, 3min after metacholine injection, after L-NAME injection and after a combination of L-NAME with subsequent metacholine injection (mean±SEM, n=10).