Annexin A1 Counteracts Chemokine-Induced Arterial Myeloid Cell Recruitment


Rationale: Chemokine-controlled arterial leukocyte recruitment is a crucial process in atherosclerosis. Formyl peptide receptor 2 (FPR2) is a chemoattractant receptor that recognizes proinflammatory and proresolving ligands. The contribution of FPR2 and its proresolving ligand annexin A1 to atherosclerotic lesion formation is largely undefined.

Objective: Because of the ambivalence of FPR2 ligands, we here investigate the role of FPR2 and its resolving ligand annexin A1 in atherogenesis.

Methods and Results: Deletion of FPR2 or its ligand annexin A1 enhances atherosclerotic lesion formation, arterial myeloid cell adhesion, and recruitment. Mechanistically, we identify annexin A1 as an endogenous inhibitor of integrin activation evoked by the chemokines CCL5, CCL2, and CXCL1. Specifically, the annexin A1 fragment Ac2-26 counteracts conformational activation and clustering of integrins on myeloid cells evoked by CCL5, CCL2, and CXCL1 through inhibiting activation of the small GTPase Rap1. In vivo administration of Ac2-26 largely diminishes arterial recruitment of myeloid cells in a FPR2-dependent fashion. This effect is also observed in the presence of selective antagonists to CCR5, CCR2, or CXCR2, whereas Ac2-26 was without effect when all 3 chemokine receptors were antagonized simultaneously. Finally, repeated treatment with Ac2-26 reduces atherosclerotic lesion sizes and lesional macrophage accumulation.

Conclusions: Instructing the annexin A1-FPR2 axis harbors a novel approach to target arterial leukocyte recruitment. With the ability of Ac2-26 to counteract integrin activation exerted by various chemokines, delivery of Ac2-26 may be superior in inhibition of arterial leukocyte recruitment when compared with blocking individual chemokine receptors. (Circ Res. 2015;116:827-835. DOI: 10.1161/CIRCRESAHA.116.305825.)

Key Words: annexin A1 ■ atherosclerosis ■ chemokine ■ leukocytes

Atherosclerosis is a chronic inflammation of the arterial vessel wall characterized by continuous leukocyte recruitment. Arterial leukocyte accumulation is controlled by various mechanisms, including apoptosis, egress, proliferation, and recruitment.1 The latter process is thought to be a dominant mechanism occurring at all stages of atherosclerosis.2 Indeed, inhibition of arterial myeloid cell recruitment was shown to reduce atherogenesis, atheroprogession, and plaque destabilization in mouse models of atherosclerosis.3,4 Recruitment of arterial leukocytes, predominantly myeloid cells, is regulated by interaction of leukocytic and endothelial cell adhesion molecules. The valency of leukocyte adhesion molecules, namely integrins, is essentially controlled by chemotactic molecules, which bind to G-protein–coupled chemokine receptors unleashing an intracellular signaling cascade ultimately fostering integrin activation. Although ligation of most chemokine receptors follows this activation pattern, other chemoattractant receptors bind ligands with opposing functions.

Formyl peptide receptor 2 (FPR2) is a G-protein–coupled receptor predominantly expressed by myeloid cells and to a lower degree by endothelial cells.5 In contrast to classical chemokine receptors, FPR2 is promiscuous and recognizes ligands...
Lack of FPR2 or its Ligand Annexin A1 Accelerates Atherogenesis

To investigate the role of FPR2 in early atherosclerosis, we fed Apoe−/− and Apoe−/−Fpr2−/− mice a HFD for 4 weeks and assessed atherosclerotic lesion sizes in aortic roots. Lack of FPR2 significantly increased lesion sizes (Figure 1A) characterized by increased cellularity and a matching increase in macrophage content (Figure 1B and 1C). Although infrequent in atherosclerotic lesions, neutrophils were also found in larger numbers in plaques of Apoe−/−Fpr2−/− mice (Figure 1D). In addition, no changes in the number of intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1)–expressing endothelial cells were found (Figure 1E), and the number of apoptotic cells as assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was not different between the strains (Online Figure IA). Finally, no differences were detected for counts of leukocyte subsets, plasma cholesterol, and triglyceride levels in Apoe−/− and Apoe−/−Fpr2−/− mice (Online Table 1).

These data are in striking contrast to the previously reported lesion phenotype of mice lacking the neutrophil-borne FPR2 ligand CRAMP (Cathelicidin-related Antimicrobial Peptide).7 Thus, we hypothesized that resolution-inducing FPR2 ligands may hold an important role during early atherosclerosis and consequently studied the development of atherosclerosis in mice lacking the proresolving FPR2 ligand AnxA1. Apoe−/−Anxa1−/− mice were fed a HFD for 4 weeks, and atherosclerosis development was compared with Apoe−/− mice. In parallel to what we observed in Apoe−/−Fpr2−/− mice, aortic root lesions of Apoe−/−Anxa1−/− mice were larger in size and contained more myeloid cells when compared with Apoe−/− mice (Figure 1).

Annexin A1-FPR2 Axis Prevents Arterial Myeloid Cell Recruitment

The similarity of atherosclerotic lesion characteristics in Apoe−/−Fpr2−/− and Apoe−/−Anxa1−/− mice points toward the importance of an endogenous AnxA1-FPR2 axis preventing atherogenesis. To study the presence of AnxA1 in atherosclerotic lesions, we stained murine plaques with an antibody to AnxA1. We could evidence abundant, specific staining of macrophage accumulation in absolute numbers (Figure 1A), Assessment of luminal expression of endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Displayed are representative images (top) and quantification of luminal coverage (bottom). Scale bar, 100 μm. *P<0.05 compared to Apoe−/− mice. All data are presented as mean±SD. Experiments were performed 3× independently with a total of 15 mice. Data were analyzed with 1-way ANOVA with Dunnett post test.
plasma levels showed no differences between Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup> mice (Figure 2C), and plasma AnxA1 levels were negatively correlated with lesion sizes in the aortic root of Apoe<sup>−/−</sup> mice, a relationship absent in Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup> mice (Figure 2D).

To assess the role of the AnxA1-FPR2 axis on arterial leukocyte recruitment, we performed intravital microscopy of the carotid artery. Herein, myeloid cells were visualized by administration of antibodies to Ly6G (neutrophils), Ly6C (classical monocytes), and CD11b (myeloid cells). Although tethering and rolling speed of myeloid cell subsets were not affected in mice lacking FPR2 or AnxA1 (Figure 3A and 3B; Online Figure IIIA and IIIB), rolling flux was reduced in both strains when compared with Apoe<sup>−/−</sup> mice (Figure 3C; Online Figure IIIC). Concomitantly, adhesion of myeloid cell subsets was significantly increased in both Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup> and Apoe<sup>−/−</sup>Anxa1<sup>−/−</sup> mice when compared with that in Apoe<sup>−/−</sup> mice (Figure 3D and 3E). Because endothelial adhesion molecule expression did not differ between the mouse strains and with the largely myeloid cell–restricted expression of FPR2, we suspected the adhesion defect to be leukocyte intrinsic.

and with antibodies toward macrophages (Mac2), neutrophils (Ly6G), smooth muscle cells (α-smooth muscle actin), or endothelial cells (von Willebrand Factor). Specific immunofluorescence was prominent in myeloid cells and in the endothelium, whereas scarce staining was found in smooth muscle cells (Figure 2B). Importantly, a similar distribution of AnxA1 staining was evidenced in human endarterectomy specimens (Online Figure IIA and IIB). In addition, AnxA1

Figure 2. Plasma annexin A1 (AnxA1) negatively correlates with atherosclerotic lesion sizes. A, Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Anxa1<sup>−/−</sup> mice were fed a high-fat diet for 4 weeks, and aortic root sections were stained with an antibody to AnxA1 or an isotype control antibody. B, Apoe<sup>−/−</sup> mice were fed a high-fat diet (HFD) for 4 weeks and AnxA1 was costained with markers for macrophages (anti-Mac2), smooth muscle cells (anti-αSMA), endothelial cells (anti-von Willebrand Factor [vWF]), or neutrophils (anti-Ly6G) in aortic root sections. Scale bar, 100 μm. C and D, Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup> mice were fed a HFD for 4 weeks and AnxA1 was quantified in the plasma (B). Correlation between aortic root lesion sizes and plasma AnxA1 levels in Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup> mice (Pearson correlation; D). DAPI indicates 4',6-diamidino-2-phenylindole.

Figure 3. Annexin A1-formyl peptide receptor 2 axis prevents arterial myeloid cell recruitment. Apoe<sup>−/−</sup>, Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup>, and Apoe<sup>−/−</sup>Anxa1<sup>−/−</sup> mice were fed a high-fat diet for 4 weeks, and intravital microscopy of the carotid artery was performed to assess luminal leukocyte endothelial interactions. Myeloid cell subsets were identified by intravenous injection of antibodies to Ly6G, Ly6C, and CD11b 10 minutes before recording. Displayed are quantification of tethering (A), rolling speed (B), and rolling flux (C) of CD11b<sup>+</sup> cells. Number of adherent cells (E) is displayed for CD11b<sup>+</sup> cells (left), Ly6G<sup>+</sup> cells (middle), and Ly6C<sup>+</sup> cells (right). Adhesion of CD11b<sup>+</sup> myeloid cells to carotid arteries of Apoe<sup>−/−</sup>, Apoe<sup>−/−</sup>Fpr2<sup>−/−</sup>, and Apoe<sup>−/−</sup>Anxa1<sup>−/−</sup> mice is depicted in D. Scale bar, 100 μm. *P < 0.05 compared with Apoe<sup>−/−</sup> mice. Experiments were performed 3× independently with a total of ≥15 mice. Data were analyzed using 1-way ANOVA with Dunnett post test.
Integrin activation was further studied in murine myeloid cells after stimulation with CCL2, CCL5, CXCL1, and leukotriene B4 (LTB4), all of which are chemotactic molecules with reported importance in arterial myeloid cell recruitment. Activation of β2 and β1 integrins was assayed by coimmunoprecipitation with their natural ligands VCAM-1 and ICAM-1, which were fused with a human Fc fragment allowing for detection of binding by flow cytometry. In this setting, CCL2 and CCL5 induced a robust activation of β2 and β1 integrins (Figure 4A and 4B), whereas CXCL1 and LTB4 exerted only moderate effects (Online Figure IVA and IVB). To assess the effect of AnxA1 on chemokine-evoked integrin activation, we made the use of the AnxA1 fragment Ac2-26, which was reported to act via FPR2. In homoligand competition binding experiments, we could demonstrate that Ac2-26 competed with [125I-Tyr]-Ac2-26 at mouse or human FPR2 overexpressed in HEK293 cells (Online Figure IVC and IVD). Furthermore, we used a biochemical approach to confirm the interaction of Ac2-26 and FPR2. Herein, we performed surface plasmon resonance experiments using FPR2 expressed in proteoliposomes, thus limiting further mechanistic studies to studying the effect of Ac2-26 on CCL5-evoked integrin activation. Here, we find Ac2-26 was immobilized on a CM4 chip and superfused with receptor-containing proteoliposomes (Online Figure IVE). With this approach, we were able to detect a clear binding of Ac2-26 to FPR2. Interestingly, pretreatment of myeloid cells with the FPR2-binding AnxA1 fragment Ac2-26 vastly reduced CCL5-inflicted activation of β2 and β1 integrins on neutrophils and classical monocytes (Figure 4A and 4B). In addition, the presence of Ac2-26 significantly lowered CCL2-induced binding of VCAM-1 on neutrophils, whereas there was only a trend toward reduced binding of ICAM-1 on neutrophils (P = 0.3) and VCAM-1 (P = 0.07) or ICAM-1 on classical monocytes (P = 0.2). Finally, CXC1L-mediated binding of ICAM-1 was significantly lowered by Ac2-26 (Online Figure IVA and IVB), whereas there was only a trend toward reduction for LTB4-stimulated binding of ICAM-1 (P = 0.12).

To study the effect of Ac2-26 on adhesion of human myeloid cells, isolated human monocytes and neutrophils were perfused over tumor necrosis factor–activated human umbilical vein endothelial cells in the absence or in the presence of increasing amounts of Ac2-26 (Online Figure IVF and IVG). In these experiments, the presence of Ac2-26 caused a significant, dose-dependent decrease in myeloid cell adhesion with significant reductions at doses of 10 and 50 µg/mL. With the robust effect of Ac2-26 on CCL5-evoked integrin activation (Figure 4A and 4B) and the reported importance of CCL5 in arterial recruitment of both neutrophils and monocytes,13,14 we limited further mechanistic studies to studying the effect of Ac2-26 on CCL5-evoked integrin activation. Here, we find
that Ac2-26 dose dependently reduces the affinity for ICAM-1 and VCAM-1 when neutrophils and classical monocytes are activated with CCL5 (Figure 4C and 4D). This response was annulled in myeloid cells harvested from Fpr2−/− mice (Figure 4C and 4D). Full integrin activation is accompanied by a shape change to the fully extended conformation that can be monitored in human cells by the use of antibodies recognizing the activation epitope. Here, Ac2-26 inhibited the CCL5-induced switch of β2 integrin conformation into its activated state in human neutrophils and monocytes (Figure 4E; Online Figure IVI). In addition, cell surface expression of β2 and β1 integrins was neither increased by CCL5 treatment (not shown) nor decreased by Ac2-26 treatment (Online Figure IVI). Likewise, Ac2-26 treatment did not affect the expression of the CCL5 receptors, CCR1, CCR3, or CCR5 (Online Figure IVK). Another well-described aspect of integrin activation is their clustering on the cell surface. Using confocal microscopy, we found that the relatively dispersed distribution of lymphocyte function-associated antigen 1 (LFA1), on mouse neutrophils, as well as on human neutrophils, and monocytes became much more clustered on treatment with CCL5 and that this clustering was strongly reduced by Ac2-26 (Figure 4F; Online Figure IVL and IVM). Thus, Ac2-26 inhibits the adhesiveness of myeloid cell β2 and β1 integrins by downmodulating their affinity and valency. Chemokine-triggered integrin activation is mediated by an early increase in cytosolic Ca2+, followed by a later activation of the small GTPase Rap1.15 To experiments, we performed dose–response and time–course injection. To define the optimal time and dose for such experiments, we performed dose–response and time–course analyses using the arterial adhesion of CD11b+ myeloid cells to the carotid artery of Apoe−/− mice fed a HFD for 4 weeks as read-out. In these experiments, Ac2-26 at 0.1 and 1 μg was without effect throughout the observation period of ≤30 minutes after Ac2-26 administration. In contrast, Ac2-26 at 10 and 50 μg significantly reduced the number of adherent cells at 10 and 30 minutes after injection (Online Figure VA). On the basis of this, we decided to study arterial leukocyte adhesion before and 30 minutes after a single dose of Ac2-26 (50 μg). Although Ac2-26 shifted the adherent CD11b+ cell fraction in Apoe−/− mice toward the rolling cell fraction, an effect observed even more pronounced in Apoe−/−Anxa1−/− mice, this effect was abolished in Apoe−/−Fpr2−/− mice (Figure 5A–5C).

Similar effects were also observed for Ly6G+ and Ly6C+ cells (Online Figure VB–VE). In addition, boiled Ac2-26 failed to affect adhesion of myeloid cells (Figure 5A–5C; Online Figure VB–VE).

With the potent effect of Ac2-26 to reduce arterial myeloid cell adhesion and to diminish integrin activation evoked by chemokines, we aimed at comparing the effect of Ac2-26 and chemokine receptor antagonists. Because Ac2-26 largely diminished CCL5-evoked integrin activation triggered by CCL2 and CXCL11 to a lower degree, we used specific antagonists to CCR5, CCR2, and CXCR2 to study the effect of Ac2-26 on arterial myeloid cell adhesion. Apoe−/− mice receiving a HFD for 4 weeks were treated with an antagonist to CCR5, CCR2, or CXCR2, a combination of all 3, or vehicle only 30 minutes before intravital microscopy. Adhesion of myeloid cell subsets along the carotid artery was studied before and 30 minutes after intravenous injection of Ac2-26. Although Ac2-26 treatment reduced adhesion of myeloid cell subsets when mice were treated with just 1 chemokine receptor antagonist, this was abrogated in the presence of the combination of antagonists to CCR2, CCR5, and CXCR2 (Figure 5D; Online Figure VF and VG).

These data indicate that Ac2-26 is able to counteract myeloid cell adhesion evoked by various chemokines, and hence we suspected that Ac2-26 should be a powerful tool in reducing early atherosclerotic lesion burden. Thus, we performed a therapeutic experiment with repeated injections of Ac2-26 (3× per week; 50 μg IP per injection) or vehicle control during 4 weeks of HFD. Ac2-26 did not have any obvious side effects and did not alter circulating leukocyte counts or plasma lipid levels (Online Table II). Histomorphometry of atherosclerotic lesions in aorto root sections, however, revealed a clear-cut reduction in mice receiving Ac2-26 (Figure 5E). In line, Ac2-26 administration lowered lesional macrophage counts, whereas neutrophil numbers were low in both groups and Ac2-26 treatment only exerted a trend toward reduction (Figure 5F and 5G).

**Discussion**

AnxA1 is a molecule that belongs to a large and structurally heterogeneous family of proresolving molecules. Other family members include resolving lipid mediators, proresolving cytokines, and proresolving hormones. Although these molecules are structurally different they share functional similarities. They terminate inflammatory leukocyte accumulation by
Reducing leukocyte recruitment and enhancing egress; they stimulate clearance of aged and apoptotic cells and actively reducing leukocyte recruitment and enhancing egress; they stimulate clearance of aged and apoptotic cells and actively promoting tissue repair. Here, we show that AnxA1 is an endogenous inhibitor of arterial leukocyte recruitment. Because this process is a mechanism essential to atherogenesis, it is not surprising that mice lacking AnxA1 or its receptor FPR2 have accelerated atherosclerosis. Mechanistically, we show that the AnxA1 fragment Ac2-26 counters chemokine-mediated integrin activation in neutrophils and monocytes and could hence be a potential tool for the treatment of atherosclerosis.

Direct interference with integrins, cell adhesion molecules, or chemokines to prevent arterial leukocyte recruitment has only achieved limited success in translational studies. Reasons for such limitations include the striking redundancy of cell adhesion molecules and chemokines during atherogenic recruitment, rendering interference with just 1 molecule insufficient.

Indeed, it has been shown that combined inhibition of chemokine signaling has an additive effect in the treatment of atherosclerosis in mice. By counteracting arterial myeloid cell adhesion instructed by a variety of chemokines, AnxA1-based therapeutic approaches may be superior to the use of individual chemokine receptor antagonists. However, systemic delivery of Ac2-26 may also affect myeloid cell recruitment in acute inflammation and thus impair host defense. Thus, delivery strategies with arterial tropism may be favorable for future translational approaches.

Beyond its ability to reduce myeloid cell adhesion, AnxA1 exerts several proresolving effects that may be beneficial during atheroprospective and plaque destabilization. In this context, AnxA1 was identified as a bridging molecule opsonizing apoptotic cells to mediate efferocytosis. In addition, AnxA1 induces a favorable macrophage M2a phenotype, which is

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**Figure 5. In vivo delivery of the annexin A1 fragment Ac2-26 reduces atherosclerosis.**

A-C, Apoe-/-, Apoe-/-Fpr2-/-, and Apoe-/-Anxa1-/- mice were fed a high-fat diet (HFD) for 4 weeks. Intravital microscopy of the carotid artery was used for assessment of luminal leukocyte endothelial interactions. Myeloid cells were identified by intravenous injection of an antibody to CD11b 10 minutes before recording. Myeloid cell adhesion (B) and rolling flux (C) in Apoe-/- (left), Apoe-/-Fpr2-/- (middle), and Apoe-/-Anxa1-/- (right) mice were assessed before and 30 minutes after injection of native (panels to the left) or boiled Ac2-26 (far right panel; 50 μg, IV). Representative images are shown in A. Scale bar, 100 μm. Each dot represents 1 mouse. *P<0.05 compared with read-out before Ac2-26 administration. Data were analyzed with paired t test. D, Apoe-/- mice were fed a HFD for 4 weeks and mice received a single dose of antagonist to CCR2 (RS504393, 5 mg/kg), CCR5 (DAPTA, 1 mg/kg), or CXCR2 (SB225002, 5 mg/kg), or a combination of all, or vehicle control. Thirty minutes later arterial adhesion of CD11b+ cells was studied (before). Immediately after recording, mice received Ac2-26 (50 μg IV) and adhesion was studied 30 minutes later (after). Each dot represents 1 mouse. *P<0.05 compared with read-out before Ac2-26 administration. Data were analyzed with Wilcoxon matched-pairs signed rank test. E-G, Ac2-26 reduces atherogenesis. Apoe-/- mice (n=6 per group) were repeatedly injected with Ac2-26 (3x per week; 50 μg IP per injection) or vehicle control during 4 weeks of HFD. E, Quantification of atherosclerotic lesion sizes in Oil-Red-O–stained aortic root sections. Representative images are shown aside. F, Quantification of Mac2+ macrophages. G, Quantification of lesional Ly6G+ neutrophils. *P<0.05 compared with vehicle treatment. Data in F and G are presented as mean±SD. Data in E-G were analyzed with Mann-Whitney test.
characterized by the release of the cytokines interleukin-10 and TGFβ. Thus, AnxA1 and its fragments counteract inflammatory processes of central importance during advanced stages of atherosclerosis, and delivery of AnxA1 fragments may promote plaque stability. Of note, analyses of human atherosclerotic plaques revealed a correlation between lesional AnxA1 and signs of plaque stability. Interestingly, treatment with the annexin family member annexin A5 has been shown to reduce inflammation during advanced stages of atherosclerosis and in models of arterial injury. These indications point toward the possible importance of AnxA1 in plaque stabilization. However, a recent study indicates that mice lacking FPR2 and FPR3 are partially protected from atherosclerosis at a later stage. Thus, subsequent studies are required to define the role of AnxA1 and its therapeutic potential during athero-progression and plaque destabilization clearly.

An additional point of interest is the origin of AnxA1 in the context of hypercholesterolemia-induced atherosclerosis. Although neutrophils release AnxA1 after establishing interactions with the endothelium, it appears unlikely that neutrophils are a major source during atherogenesis because they have been shown to promote early lesion formation. Instead, we here show that the arterial endothelium expresses large amounts of AnxA1. Given that the interaction between AnxA1 and myeloid cells is likely to occur in the blood or at the interface between blood and the atherosclerotic lesion, the endothelium could be an important source of AnxA1 in atherosclerosis. In fact, it has been shown that endothelial expression of AnxA1 decreases during endothelial dysfunction and vascular inflammation. Interestingly, AnxA1 is primarily a cytosolic protein lacking signal sequences that could direct it into the classical secretory pathway. Nevertheless, AnxA1 can be detected in the plasma under conditions of inflammation or in the supernatant of activated endothelial cells and hence alternative pathways for the secretion of AnxA1 have been proposed. One of these pathways includes the externalization via ABCA1 and endothelial expression of this transporter is proposed. Additionally, AnxA1 could localize at intercellular junctions and may be released upon microvascular endothelial cell apoptosis. Overall, AnxA1 exerts antiatherogenic effects. Such opposing effects are not just shown in disease models but have also been reported on a cellular level. For example, acute-phase protein serum amyloid A and cathelicidin mediate FPR2-dependent proinflammatory leukocyte activation, including leukocyte trafficking, cytokine secretion, and inhibition of neutrophil apoptosis.

In contrast, AnxA1, Ac2-26, and lipoxin A4 also signal through FPR2 to inhibit leukocyte recruitment, enhance neutrophil apoptosis, and macrophage effectorosis. Key events in inflammatory resolution. Lipid and peptide ligands act with different affinities and bind to distinct pockets on the receptor, thus making a direct competition unlikely. One mechanistic explanation for divergent effects of FPR2 ligands may reside in the ability of FPR2 to form homo- and heterodimers. Although dimerization of G-protein–coupled receptors may not generally be required for ligand recognition, emerging evidence indicates that dimers of G-protein–coupled receptors may affect signaling functions and that ligand selectivity may be directly related to different receptor conformation. FPR2 homodimers and FPR2/FPR1 heterodimers constitutively occur in human leukocytes. In addition, dimer formation and change in receptor conformation can be induced in a ligand-specific fashion, and this does not seem to be simply a consequence of ligand–receptor interaction. As an example, Ac2-26 was found to activate the JNK/caspase-3 pathway, leading to apoptosis in neutrophils. Importantly, this pathway over-rides the apoptosis suppressing action of serum amyloid A and cathelicidin.

Recent reports have suggested the tight control of leukocyte recruitment by endogenous inhibitors. In this context, leukocyte-derived pentraxin-3 binds to endothelial P-selectin thereby abrogating leukocyte rolling. Del-1 directly inhibits the interaction of leukocytic LFA1 and ICAM-1. Finally, GDF-15 (growth differentiation factor 15) counteracts chemokine-induced activation of β3 integrins by interfering with the activity of Rap1-GTPase. Hence, in the context of leukocyte recruitment, the latter molecule seems to share much of its functionality with AnxA1. However, the role of these molecules in atherosclerosis has not been investigated yet. Thus, the presented study is the first report on the role of an endogenous inhibitor of leukocyte recruitment in atherosclerosis. Local delivery of AnxA1, its Ac2-26 fragment, or other endogenous inhibitors of leukocyte recruitment, may harbor valuable opportunities for the prevention and treatment of atherosclerosis.

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Disclosures
None.

References


17. AnxA5 reduces plaque inflammation of advanced atherosclerosis.


15. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice.


13. Neutrophile and resistance to glucocorticoids in annexin 1-/- mice.


11. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis.


7. Chemokine receptor antagonists: overcoming developmental hurdles.


5. Cell adhesion molecules as pharmaceutically useful targets in atherosclerosis.


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What Is Known?

- Neutrophils and monocytes enter atherosclerotic lesions in a process involving chemokine-mediated integrin activation.
- Annexin A1 is an endogenous inhibitor of leukocyte recruitment preventing firm adhesion of neutrophils in the microcirculation.

What New Information Does This Article Contribute?

- Annexin A1 and its receptor formyl peptide receptor 2 are protective during early stages of atherosclerosis in Apoe null mice.
- The annexin A1 fragment Ac2-26 inhibits chemokine-evoked integrin activation and with this arterial adhesion of neutrophils and monocytes.
- Repeated treatment with Ac2-26 reduces early atherosclerosis.

Endogenous inhibitors of leukocyte recruitment have been identified as a set of breaks to fine-tune inflammatory leukocyte trafficking. However, their role in atherosclerosis is largely unknown. Here, we show that annexin A1, a protein known to inhibit leukocyte adhesion in microvascular inflammation, and its receptor formyl peptide receptor 2, are protective during early stages of atherosclerosis in Apoe null mice. Mechanistically, the annexin A1 fragment Ac2-26 counteracts chemokine-induced integrin activation, a process important in arterial leukocyte recruitment. Notably, Ac2-26 overrides the chemokine signaling exerted by several chemokines. Hence, Ac2-26 fragment of annexin1 might be of clinical importance in settings where redundant chemokine signaling guides leukocytes to sites of inflammation.
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Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment

Drechsler: Annexin A1 prevents atherogenesis

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- Data Supplement –


Supplementary Materials and Methods

Immunohistochemistry
The extent of atherosclerosis was assessed in aortic roots by staining for lipid depositions with oil-red-O. Aortic roots were stained with antibodies to Ly6G (1A8, BD Biosciences), Mac2 (Cedarlane), VCAM-1 (429, BD Biosciences), or ICAM-1 (3E2, BD Biosciences). Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). Furthermore, TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) to assess the number of apoptotic/necrotic cells within aortic root sections. Annexin A1 was stained after de-paraffinization of human atherosclerotic plaques or Methanol permeabilisation of aortic root sections from mouse using a rabbit polyclonal anti-Annexin A1 antibody (Life Technologies) and a FITC-conjugated anti rabbit antibody (Sigma-Aldrich). Subsequently, tissue-specific antibodies were used to co-stain for Mac2 (Cedarlane), CD68 (eBioscience), endothelial vWF (Abcam), smooth muscle actin (Dako) or Ly6G (BD Biosciences), CD177 (Abnova) and detected by use of Cy3-conjugated anti-rat (Abcam), anti-mouse (Jackson Immuno Research) or anti-sheep (Abcam) antibodies. A Leica DM4000 microscope with a 25×/0.95 water emersion objective (Leica Microsystems) and a Leica DFC 365FX camera were used to capture images. Leica Qwin Imaging software (Leica Ltd.) was employed for image analysis.

The Ethics Committee of the Medical Faculty (RWTH Aachen University) approved the study protocol for the collection of human atherosclerotic plaque samples, and written informed consent was obtained from all participating subjects.

Intravital microscopy
Leukocyte-endothelial interactions along the carotid artery were analyzed in mice having received high fat diet for 4 weeks. Mice were placed in supine position and the right jugular vein was cannulated with a catheter (PE10, Becton Dickinson) for antibody injection. Antibodies to Ly6G (0.5 µg, PE, Biolegend), Ly6C (0.5 µg, AF488, eBioscience), and CD11b (4 µl, 650NC, ebioscience) were administered to label myeloid cell subsets. Antibodies were allowed to circulate for 10 minutes. The left external carotid artery was surgically exposed. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell software was used. Rolling flux was determined as the number of cells passing a reference line perpendicular to blood flow within 30 seconds. Neutrophils were considered adherent when no rolling was observed for at least 30 seconds. For experiments employing Ac2-26 treatment, leukocyte endothelial interactions were recorded prior and 30 minutes after administration of native or boiled Ac2-26 (50 µg via jugular vein catheter). In experiments employing chemokine receptor antagonists, mice received antagonists to CCR2 (RS504393, 5 mg/kg), CCR5 (DAPTA, 1 mg/kg), CXCR2 (SB225002, 5 mg/kg, all Tocris Bioscience) alone or in combination (i.p., in 200 µl vehicle) 30 minutes prior to exposure of the carotid artery. Then, adhesion of myeloid cell subsets was recorded and mice received a bolus of Ac2-26 (50 µg via jugular vein catheter). A second recording was made 30 minutes after Ac2-26 treatment.

Integrin activation assay
Murine peripheral blood cells were drawn retro-orbitally from wild type or Fpr2 KO mice, erythrocytes were lysed (lysis buffer: 150 mM NH4Cl; 10 mM KHCO3; 0.1 mM diNaEDTA, pH 7.4) and leukocytes were suspended in Hanks Balanced Salt Solution containing 1 mM CaCl and MgCl (Invitrogen) and 0.5 % BSA (Sigma). Cells were exposed to CCL2 (5 µg/ml), CCL5 (5 µg/ml), CXCL1 (5 µg/ml; all Peprotech), and LTB4 (1 µg/ml, Santa Cruz) or an equal volume of buffer, in the presence of ICAM-1/Fc (5 µg/ml, R&D Systems) or of VCAM-1/Fc (5 µg/ml, R&D Systems) and PE-conjugated anti-human IgG1 (1 µg/ml; Fc-specific, Southern Biotechnology) for 5 minutes at 37 °C. After washing, cells were labeled with antibodies to CD45, CD115, Ly6G and Gr1 to identify classical monocytes and neutrophils. Binding of ICAM-1 or VCAM-1 was measured by flow cytometry.

Measurements of chemokine receptor and integrin expression
Murine peripheral blood cells were drawn retro-orbitally from wild type mice, erythrocytes were lysed and leukocytes were suspended in HBSS. Cells were stained with antibodies to CCR1 (FITC, R&D systems), CCR3 (APC, eBioscience), and CCR5 (PE, eBioscience). After washing, cells were labeled with antibodies to CD45, CD115, Ly6G and Gr1 to identify classical monocytes and neutrophils.
Chemokine receptor expression was measured by flow cytometry. For measurement of total surface \( \beta_1 \) and \( \beta_2 \) integrins leukocytes were exposed to CCL5 (5 µg/ml, Peprotech) or an equal volume of buffer. After washing, cells were labeled with antibodies to CD18 (eBioscience) and CD29 (eBioscience). Integrin expression was assessed by flow cytometry.

**Cholesterol measurements**

Cholesterol and triglyceride levels in mouse serum were quantified using enzymatic assays (Roche and BioTrend) according to the manufacturer’s protocol.

**Annexin A1 ELISA**

Commercially available ELISAs for mouse Annexin A1 were performed in accordance with the manufacturer’s protocol (Cloud Clone Corp.).

**LFA-1 clustering**

Clustering of the integrin \( \alpha L \beta_2 \) was investigated as described before (Kempf et al., 2011). Briefly, isolated murine or human neutrophils and monocytes were incubated with Ac2-26 at 37°C for 30 minutes and consecutively stimulated with CCL5 for 5 minutes. After fixation with 4% PFA/PBS, cells were immobilized on Poly-L-Lysine-coated glass slides (Lab-Tek 2 chamber slides, Thermo Scientific) and incubated with an Alexa488-labeled anti-LFA1 antibody (clone M17/4, Biolegend). Human neutrophils or monocytes were stained with and uncoupled anti-LFA1 antibody (clone TS2/4, Biolegend) and secondary staining was performed with an Alexa488-conjugated antiouse antibody (Life Technologies). Images were acquired using a confocal spinning disc microscope (CellObserver SD, Zeiss).

**Assessment of \( \beta_2 \) integrin conformation**

We incubated human neutrophils and monocytes (5x10⁵) with monoclonal antibody clone 24 (mAb24) or mouse IgG1 (clone NCG01, Dianova) for 15 min at 37 °C in 50 µL HBSS (pH 7.4) containing 1 mM Ca²⁺, 1 mM Mg²⁺, 0.1% glucose, and 10 mM HEPES in the presence or absence of CCL5 and Ac2-26. We detected antibody binding by flow cytometry using FITC-conjugated goat antibody to mouse IgG (Dianova). In some experiments, isolated human neutrophils or monocytes were preincubated with 1 µM Rap1-WT or Rap1-CA Tat peptides at 37°C for 30 minutes prior to stimulation.

**Rap1 activation assay**

Rap1 activation in neutrophils following chemokine stimulation was investigated as described previously, with some modifications (Kempf et al. 2011). Briefly, isolated murine or human neutrophils and monocytes were incubated with Ac2-26 at 37 °C for 30 minutes and consecutively stimulated with CCL5 for 5 minutes. After lysis with ice-cold lysis buffer (50 mM TrisHCl pH 7.4: 500 mM NaCl; 1% NP40; 10% glycerol; 2.5 mM MgCl₂; 10µg/ml PEFA-Block; 25 mM NaF; 1 mM Vanadate and 1 mM PMSF, 1% Roche HALT protease inhibitor cocktail), GTP-bound Rap1 (Rap1-GTP) was precipitated from whole cell lysates using GST-Ral beads. Precipitated proteins were separated using SDS-PAGE, transferred to a PVDF membrane and immunoblotted against Rap1. To determine the level of total Rap1, a small portion of the whole cell lysate was mixed with SDS sample buffer and separated by SDS-PAGE. Rap1 was detected using a rabbit polyclonal antibody (Santa Cruz).

**Ca²⁺ mobilization**

Mouse neutrophils were loaded with FLIPR Calcium 5 dye (Molecular Devices) for 1 h at 37 °C and suspendend at a final concentration of 10⁶ cells/ml. Neutrophils were allowed to sediment on a Nunc™ Lab-Tek™ chambered coverglass and then treated with CCL5 (5 µg/ml). In same conditions cells were pre-incubated for 30 minutes with Ac2-26. Using a live cell imaging microscope (Olympus MT10 and Cell²™ Imaging software), fluorescence images (at 488 nm) were obtained at 1-s intervals for up to 40 s after stimulation. Regions-of-interest, ROIs, were drawn around individual cells for measurement of fluorescence intensities (one ROI/cell). Fluorescence intensities of ROIs were determined using Image J software.
**Flow adhesion assay**

Human umbilical vein endothelial cells (HUVEC) (PromoCell) seeded on collagen-coated dishes and grown in endothelial growth medium (PromoCell) were activated with TNF (10 ng/ml, 12 h, Peprotech) prior to experimentation. Neutrophils were isolated by density gradient centrifugation using polymorphprep (Axis Shield) and monocytes were isolated by magnetic bead isolation (Monocytes Isolation Kit II, MACS Miltenyi Biotec Inc.). Isolated cells were labeled with Calcein AM (Invitrogen) and incubated with vehicle or Ac2-26 at indicated concentrations prior to performance of the flow chamber assay. Cells were perfused at 2.5 dyne/cm2 and adherent cells were quantified in 15 randomly chosen fields.

**Surface plasmon resonance**

Interaction between Ac2-26 and FPR2 were studied by Surface Plasmon Resonance on a Biacore X100 system (GE Healthcare). Ac2-26 was immobilized on a CM4 sensor chip at a level of 400 response unit (RU) by amine coupling. Human FPR2 incorporated into liposomes (Abnova) was used as analyte and diluted in a HBS-N running buffer (0.1M HEPES, 1.5M NaCl pH 7.4). Each experiment was performed with a flow of 10µl/min with running buffer.

**Competitive Ac2-26 binding assay**

Human embryonic endothelial (HEK) 293 cells overexpressing either mouse FPR2 or human FPR2 were incubated with increasing concentrations of unlabeled Ac2-26 (0.1 nM to 10 μM) and [125I]-Tyr–Ac2-26 (50 nM, 1 h, 4 °C) (Phoenix Pharmaceuticals, Karlsruhe Germany). Cells were then transferred on to Whatman GF/C glass microfiber filters (Kent, U.K.) and unbound tracer was washed off using ice-cold PBS (pH 7.45). Filters were then transferred into tubes and radioactivity measured using a gamma counter.

**Statistics**

All data are expressed as mean±SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). After calculating for normality by D’Agostino Pearson omnibus test, unpaired Student’s t-test, paired Student’s t-test or nonparametric Mann-Whitney U-test, One-way ANOVA, Friedman-test, Wilcoxon matched-pairs signed rank test or Kruskal-Wallis test with post-hoc Dunn test were used as appropriate. p-values < 0.05 were considered significant.
### Blood cell counts

<table>
<thead>
<tr>
<th></th>
<th>Apoe&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Apoe&lt;sup&gt;-/-&lt;/sup&gt;Fpr2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Apoe&lt;sup&gt;-/-&lt;/sup&gt;Anxa1&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Neutrophils</td>
<td>12.83 +/- 6.08</td>
<td>14.10 +/- 6.09</td>
<td>15.47 +/- 4.55</td>
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<td>Total monocytes</td>
<td>4.55 +/- 2.11</td>
<td>3.98 +/- 1.88</td>
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<td>Classical monocytes</td>
<td>3.51 +/- 1.71</td>
<td>3.72 +/- 2.04</td>
<td>4.13 +/- 1.23</td>
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<tr>
<td>Non-classical monocytes</td>
<td>0.80 +/- 0.46</td>
<td>0.51 +/- 0.25</td>
<td>0.70 +/- 0.47</td>
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<td>T-cells</td>
<td>7.75 +/- 2.39</td>
<td>5.86 +/- 1.31</td>
<td>9.19 +/- 1.54</td>
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### Lipid levels

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>839.50 +/- 98.40</td>
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<tr>
<td>Triglyceride</td>
<td>143.20 +/- 54.61</td>
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**Online Table I:** White blood cell counts and plasma lipid levels in Apoe<sup>-/-</sup>, Apoe<sup>-/-</sup>Fpr2<sup>-/-</sup>, and Apoe<sup>-/-</sup>Anxa1<sup>-/-</sup> mice. Absolute counts of indicated circulating leukocyte subsets, serum cholesterol, and triglyceride levels were measured after four weeks of high fat diet. All data are presented as mean ± SD. Experiments were performed three times independently with a total of 15 mice. Data were analyzed with one way ANOVA with Dunnett post test.
Online Table II: White blood cell counts and plasma lipid levels in Apoe<sup>−/−</sup> mice treated with Ac2-26 or vehicle. Absolute counts of indicated circulating leukocyte subsets, serum cholesterol, and triglyceride levels in Apoe<sup>−/−</sup> mice receiving Ac2-26 (3x/week, 50 µg i.p./injection) or vehicle control during 4 weeks of HFD. Data were analyzed with Mann-Whitney test.

<table>
<thead>
<tr>
<th>Blood cell counts</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt; vehicle</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt; Ac2-26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>10.44 +/- 4.55</td>
<td>8.02 +/- 4.30</td>
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<tr>
<td>Total monocytes</td>
<td>2.67 +/- 0.75</td>
<td>2.65 +/- 1.43</td>
</tr>
<tr>
<td>Classical monocytes</td>
<td>2.26 +/- 0.59</td>
<td>2.07 +/- 1.08</td>
</tr>
<tr>
<td>Non-classical monocytes</td>
<td>0.44 +/- 0.28</td>
<td>0.58 +/- 0.46</td>
</tr>
<tr>
<td>T-cells</td>
<td>5.49 +/- 1.58</td>
<td>4.87 +/- 2.45</td>
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<table>
<thead>
<tr>
<th>Lipid levels</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>936.00 +/- 219.99</td>
<td>748.66 +/- 92.91</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>123.19 +/- 46.28</td>
<td>129.49 +/- 48.05</td>
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</table>
Online Figure I: Lack of FPR2 or its ligand Annexin A1 does not affect accumulation of apoptotic cells in early atherosclerosis. A: Apoe<sup>-/-</sup>, Apoe<sup>-/-</sup>Fpr2<sup>-/-</sup>, and Apoe<sup>-/-</sup>Anxa1<sup>-/-</sup> mice were fed a high fat diet for 4 weeks. Apoptotic cells were quantified by TUNEL staining. Displayed are representative images and quantification. All data are presented as mean ± SD. Experiments were performed three times independently with a total of 15 mice. Data were analyzed with one way ANOVA with Dunnett post test. B: Display of gating strategy for identification of circulating myeloid cell subsets. NCM, non-classical monocytes; CM, classical monocytes.
Online Figure II: Annexin A1 is present in lesional myeloid cells and the endothelium. Human atherosclerotic specimens were stained with an isotype control antibody (A), or an Annexin A1 antibody together with antibodies identifying macrophages (anti-CD68), smooth muscle cells (anti-αSMA), endothelial cells (anti-vWF), or neutrophils (anti-CD177) (B). Scale bar represents 100 µm.
Online Figure III: Annexin A1-FPR2 axis does not impact on early interactions between myeloid and endothelial cells. A: Apoe−/−, Apoe−/−Fpr2−/−, and Apoe−/−Anxa1−/− mice were fed a high fat diet for 4 weeks. Intravital fluorescence microscopy of the carotid artery for assessment of luminal leukocyte endothelial interactions. Myeloid cell subsets were identified by i.v. injection of antibodies to Ly6G (PE, 0.5 µg) or Ly6C (AF488, 0.5 µg). Displayed are quantification of tethering (A), rolling speed (B), and rolling flux (C) of Ly6G+ (left in each panel) and Ly6C+ (right in each panel) cells. All data are presented as mean ± SD. Experiments were performed three times independently with a total of at least 15 mice. Data were analyzed with one way ANOVA with Dunnett post test.
Online Figure IV: Ac2-26-inflicted reduction of integrin activation is independent of integrin expression and chemokine receptor expression but depends on inhibition of Rap1 activation.

A/B: Neutrophils (A) and classical monocytes (B) from C57Bl/6 mice were treated with murine CXCL1 (5 µg/ml) or LTB4 (1 µg/ml), and binding of soluble VCAM-1-Fc or ICAM-1-Fc was assayed by flow cytometry. Effect of Ac2-26 was determined by pretreatment for 30 min prior to chemokine stimulation. C/D: Binding affinity of Ac2-26 was investigated by incubation of Ac2-26 (0.1 nM to 10 µM) with human FPR2 (C) or mouse FPR2 (D) overexpressing HEK293 cells. E: Representative surface plasmon resonance sensorgrams of binding of FPR2 expressed in proteoliposomes to immobilized Ac2-26 on a CM4-sensorchip. Empty proteoliposomes were used as control. F/G: Flow adhesion of human neutrophils (F) or monocytes (G) to TNF-activated endothelial cells in presence of indicated concentrations of Ac2-26. H: β2 integrin activation assessed by mAb24 binding and flow cytometry in human monocytes incubated with CCL5 and preincubated with Ac2-26. Where indicated, neutrophils were pretreated with wild type (WT) or constitutively active (CA) Rap1-Tat peptides. I: Dose-dependent effect of Ac2-26 on surface expression of CD29 and CD18 on neutrophils and classical monocytes in presence of CCL5. K: Dose-dependent effect of Ac2-26 on surface expression of CCL5 receptors CCR1, CCR3, and CCR5 on neutrophils and classical monocytes. L/M: Redistribution of surface LFA-1 on human neutrophils (L) or classical monocytes (M) stimulated for 90 s with CCL5 and pretreated for 30 min with Ac2-26. N: GTP-bound Rap1 protein in mouse neutrophils 30 s after stimulation with CCL5 and prestimulation for 30 min with Ac2-26. Representative blots are shown. O/P: GTP-bound Rap1 protein in human neutrophils (L) or monocytes (M) 30 s after stimulation with CCL5 and prestimulation for 30 min with Ac2-26. Representative blots and densitometric quantification of one out of four independent experiments is displayed. All data are presented as mean ± SD. Experiments were performed at least 6 times independently for A/B/F/G/H, three times independently for C/D/I/K, and four times independently for L/M. * indicates p < 0.05, in F and G * indicates significant difference compared to adhesion in absence of Ac2-26. Data were analyzed using Kruskal-Wallis test with Dunn post test.
Online Figure V: In vivo delivery of the Annexin A1 fragment Ac2-26 inhibits arterial leukocyte accumulation. A: Apoe−/− mice were fed a high-fat diet for 4 weeks and myeloid cell adhesion was quantified before and at indicated time points after Ac2-26 injection. B-E: Apoe−/−, Apoe−/−Fpr2−/−, and Apoe−/−Anxa1−/− mice were fed a high-fat diet for 4 weeks. Intravital fluorescence microscopy of the carotid artery for assessment of luminal leukocyte endothelial interactions. Myeloid cell subsets were identified by i.v. injection of antibodies to Ly6G (PE, 0.5 µg) or Ly6C (AF488, 0.5 µg). Adhesion (B and C) and rolling flux (D and E) of Ly6G+ cells (B and D) and Ly6C+ cells (C and E) were assessed before and 30 minutes after injection of native or boiled Ac2-26 (50 µg, i.v.). F/G: All data points represent individual mice. * indicates p < 0.05 compared to read-out before Ac2-26 administration. Data were analyzed with either Friedman test with Dunn post-test (A), paired t-test or Wilcoxon matched-pairs signed rank test.