Vascular Inflammation

1-Type Calcium Channel Inhibitor Diltiazem Prevents Aneurysm Formation by Blood Pressure–Independent Anti-Inflammatory Effects

Anja Mieth, Marc Revermann, Andrea Babelova, Andreas Weigert, Ralph T. Schermuly, Ralf P. Brandes

Abstract—Formation of abdominal aortic aneurysms is a progressive inflammatory process that involves infiltration and differentiation of monocytes in the vessel wall, proliferation and migration of smooth muscle cells, and eventually the degradation of the internal elastic lamina, which leads to outward vascular remodeling and distension of the vessel. Because calcium channel blockers exert multiple beneficial effects on the vascular system, we investigated the effect of the benzothiazepine-type calcium channel blocker diltiazem on aneurysm formation in a mouse model. Angiotensin II infusion induced massive suprarenal aortic aneurysm formation in male apolipoprotein E–deficient mice that was blocked by cotreatment with diltiazem even if the blood pressure was controlled by coinfusion of phentolamine. Diltiazem prevented the angiotensin II–mediated induction of proinflammatory cytokines after 7 days of angiotensin II treatment in the aortic arch attributable to a reduction in the amount of locally infiltrating macrophages. To identify the underlying mechanism, vascular segments and cultured vascular cells as well as monocytes were studied. Diltiazem failed to reduce the angiotensin II–induced expression of proinflammatory chemokines and cytokines in isolated mouse thoracic aortic segments in organ culture. Furthermore, diltiazem did not affect the recruitment of proinflammatory Ly6C+ monocytes in vivo pointing toward an effect of the compound on gene expression in monocytes/macrophages. Indeed, diltiazem prevented the interleukin-6–induced mRNA expression of interleukin-1β and the monocyte chemotaxant protein CCL12 in peritoneal macrophages and RAW264.7 cells independent of the intracellular calcium concentration. Thus, diltiazem limits aortic aneurysm formation in mice by a blood pressure–independent anti-inflammatory effect on mononuclear cells. (Hypertension. 2013;62:1098–1104.) • Online Data Supplement

Key Words: aneurysm • calcium channels, 1-type • diltiazem • interleukin-6

The pathogenesis of aortic aneurysm formation is not fully understood, but chronic inflammation, atherosclerosis, and genetic and environmental factors contribute to this process.1,2 Levels of proinflammatory cytokines like tumor necrosis factor α (TNF), interleukin-1β (IL1B), interleukin-6 (IL6), transforming growth factor β-1 (TGFβ1), and chemokines like monocyte chemotactic protein 1 (CCL2) are markedly elevated in aneurysms as compared with the healthy vessel wall,3 and reactive oxygen species generated in response to mechanical stress and cytokines accelerate the process by activation and induction of proteases and promotion of the inflammation.4 The subsequent disintegration of the extracellular matrix leads to vascular distension, which may eventually result in vessel rupture. The complexity of the aneurysmal process has led to the development of several mouse models with the infusion of angiotensin II (ATII) into apolipoprotein E–deficient (ApoE−/−) mice being a frequently used and particularly well-characterized one. Although the main site of aneurysm formation in the suprarenal aorta is different from the human disease, the model covers several important pathogenetic aspects such as atherogenesis and vascular inflammation.5,6 Although ATII increases blood pressure, the model is mainly driven by the aortic inflammation in response to the peptide,7 a reason why blocking infiltration of inflammatory cells, inhibition of cytokine production,8 or reduction of oxidative stress9 limits aneurysm formation in this mouse model.

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Voltage-dependent calcium channels transduce depolarization into mechanical or excitation activity, leading to contraction of muscle tissue, secretion of neurotransmitters, or gating of other channels. Calcium channels activated by high changes in voltage (HVA-type) are further classified into \( L \)-type calcium channels (LTCC) mainly expressed in smooth muscle cells (SMCs) or cardiac muscle cells and neuronal type calcium channels (NTCC). Blockade of LTCC leads to negative inotropic, chronotropic, dromotropic effects, and peripheral vasodilatation. Three types of organic LTCC inhibitors have been developed: benzothiazepines (eg, diltiazem, DIL), dihydropyridines (eg, nifedipine, NIF), and phenylalkylamines (eg, verapamil). Clinically, these are used to treat a variety of diseases including hypertension, angina pectoris, certain forms of cardiac arrhythmias, congestive heart failure, and hypertrophic cardiomyopathy.10,11

Because aneurysm formation is a chronic inflammatory process, and several studies have linked LTCC blockers to anti-inflammatory effects in vitro12 and in vivo,13 we hypothesized that DIL attenuates aneurysm formation and tested this aspect in the ATII-infusion model of \( \text{ApoE}^{-/-} \) mice.

### Methods

Aneurysms were induced by infusion of ATII (1.44 mg/kg bw/d) into male \( \text{ApoE}^{-/-} \) mice. Subgroups received 100 mg/kg bw/d DIL with the drinking water. An additional subgroup received coinfusion of 18 mg/kg bw/d phenylephrine. Vascular distension was measured from calibrated photographs, and the severity of aneurysm formation was classified according to Daugherty et al.14 Gene expression was determined by quantitative RT-PCR, protein expression by Western blot analysis. For further details, please see the expanded Material and Methods section in the online-only Data Supplement.

### Results

DIL Prevents Aortic Aneurysm Formation in a Blood Pressure–Independent Manner

Infusion of ATII for 4 weeks in \( \text{ApoE}^{-/-} \) mice was sufficient to induce hypertension (Figure 1A). Macroscopically, ATII-infusion induced suprarenal aneurysm formation and structural changes of the whole aorta, covering all stages of aneurysm formation (Figure 1B–1E). Additional treatment with the \( L \)-type calcium channel inhibitor DIL strongly reduced the vascular remodeling but also lowered the blood pressure. To counteract this antihypertensive effect, we coinfused phenylephrine together with ATII in a subgroup of animals receiving DIL. This adjusted the blood pressure to the level of the ATII-only treated group. Phenylephrine-infused, hypertensive \( \text{ApoE}^{-/-} \) mice receiving DIL still showed almost no structural changes of the vessel wall or aneurysm formation despite the high blood pressure. Elastica van Gieson staining of the suprarenal part of the abdominal aorta directly cranial to the right renal artery revealed local destruction of the aortic elastic fibers and the infiltration of inflammatory cells into and around the vessel in the ATII group, which was reduced in mice treated with DIL irrespective of their blood pressure. As quantitative parameters, DIL significantly reduced the weight and the suprarenal diameter of the abdominal aorta. The classification of ATII-induced aneurysms in mice according to Daugherty et al14 demonstrated a significantly greater aneurysm severity in ATII-treated animals compared with the DIL-treated group as DIL abrogated aneurysm formation.

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**Figure 1.** Effect of diltiazem (DIL) on aortic aneurysm formation and blood pressure. **A**, Systolic blood pressure of apolipoprotein E–deficient \( \text{ApoE}^{-/-} \) mice treated for 4 weeks with saline control (CTL), angiotensin II (ATII; 1.44 mg/kg bw/d), or ATII and DIL (100 mg/kg bw/d) with or without phenylephrine coinfusion (P; 18 mg/kg bw/d; n=5–8). **B**, Aneurysm severity score (n=8–9) and (C) suprarenal diameter of aortas from mice treated as indicated (n=5–13; \( *P<0.05 \)). **D**, Representative macro images of the whole aorta, and (E) Elastica van Gieson stainings of the suprarenal part of the abdominal aorta.
DIL Attenuates ATII-Induced Macrophage Accumulation and Inflammation

In a second set of experiments, ApoE−/− mice were treated with ATII or ATII, DIL, and phenylephrine for 6 days only to allow a molecular analysis of the processes before the occurrence of structural changes or alterations of the tissue composition. No macroscopic effect of ATII was visible in the aortic arch after 6 days of infusion. Quantitative RT-PCR for mRNA expression of smooth muscle-α actin (Acta2; Figure 2A) and fibroblast-specific protein 1 (S100a4; Figure 2B) did not reveal any alterations in the cellular composition of the aortic arch at this early time point. Although aneurysms induced by this model are most frequently developing in the suprarenal part of the abdominal aorta, we used the aortic arch for an mRNA cytokine profile. Not only is the yield of mRNA from the suprarenal region of the healthy aorta exceedingly low, but also this region had to be used for histology. Despite this limitation, also in the aortic arch, 6-day ATII treatment induced a massive elevation of the macrophage marker (Emr1; Figure 2C), indicating that vascular inflammation was induced. Cotreatment of ApoE−/− mice with DIL significantly reduced aortic macrophage content independently of the treatment with phenylephrine. DIL also reduced the elevated levels of proinflammatory cytokines produced preferably by macrophages, like Tnf, Il1b, Il6, Ccl2, Tgfβ1, as well as those of matrix metalloproteinase 9 (Mmp9; Figure 2D–2I). Interestingly, the ATII-mediated induction of matrix metallopeptidase 2 (Mmp2), which is mainly expressed in SMCs and fibroblasts, was prevented by DIL in a blood pressure–dependent manner. Although DIL lowered Mmp2 mRNA expression, this effect was lost after readjustment of the blood pressure with phenylephrine (Figure 2J). Despite ATII-mediated aortic cytokine induction, no alterations in the serum concentration of most of these proinflammatory cytokines were detected (data not shown). However, in response to ATII, a significant increase in CCL12 plasma level was observed, which was absent in the plasma of mice treated with DIL and phenylephrine (Figure 2K). Similar results were obtained for IL1B and IL18 (data not shown). Thus, DIL is able to reduce the ATII-induced vascular inflammation, and this effect is probably because of a reduction of vascular macrophage infiltration. The data also illustrate that the effect of ATII is not restricted to the abdominal aorta but throughout the vasculature, suggesting that additional factors, like embryonic origin and biochemical aspects (higher pulse pressure), are responsible for the fact that aneurysms most frequently form in this region.

DIL Suppresses Chemokine Expression in Macrophages but not in SMCs

To identify the mechanism of the anti-inflammatory effect of DIL, we studied ATII-induced gene expression in different cell and tissue types. In aortic tissue of ApoE−/− mice stimulated ex vivo, ATII (100 nmol/L for 1 hour) increased the mRNA expression of monocyte-attracting chemokine Ccl2 and proinflammatory cytokine Il6. However, neither coinubcation nor 14-hour preincubation with DIL (10 μmol/L) affected this process (Figure 3A and 3B). To exclude potential interfering effects of the endothelium, we performed a similar experiment in cultured rat aortic SMCs; however again, DIL had no effect on the ATII-induced cytokine induction, although the dihydropyridine-type LTCC inhibitor NIF attenuated the ATII-mediated induction of Il6 and Ccl2 mRNA in SMCs (Figure 3C and 3D). Next, we determined whether DIL affects the activation or migration of inflammatory cells. The latter was studied by an in vivo matrigel macrophage recruitment assay in ApoE−/− mice. Matrigel containing recombinant CCL2 or CCL2 in combination with DIL was injected

Figure 2. Effect of diltiazem (DIL) on angiotensin II (ATII)–induced macrophage accumulation and inflammation. A–J, Quantitative RT-PCR analysis of proinflammatory cytokines and cellular markers in the aortic arch of apolipoprotein E-deficient (ApoE−/−) mice treated for 6 days with saline control (CTL), ATII (1.44 mg/kg bw/d), ATII and DIL (100 mg/kg bw/d), or ATII (1.44 mg/kg bw/d) and DIL (100 mg/kg bw/d) and phenylephrine (P; 18 mg/kg bw/d; n=4–5; *P<0.05). The following genes were used as cellular markers: Acta2 (smooth muscle-α actin) for smooth muscle cells (SMCs), S100a4 (fibroblast-specific protein) for fibroblasts, Emr1 for macrophages. K, Plasma protein levels of CCL12 (n=3–5; *P<0.05).
subcutaneously into the back of ApoE−/− mice, and the number of inflammatory cells infiltrating was determined after 3 days by flow cytometry. CCL2 alone led to a significant accumulation of proinflammatory Ly6C+ monocytes within the matrigel compared with the saline-mixed control. DIL added to CCL2-containing matrigel, however, did not alter the amount of infiltrated proinflammatory Ly6C+ monocytes or the entity of CD45+ leukocytes compared with matrigel mixed with CCL2 alone (Figure 3E and 3F). Because DIL was neither able to reduce the proinflammatory cytokine release from the vessel wall nor able to affect the migration of proinflammatory monocytes toward these substances, we analyzed the effect of DIL on gene expression of macrophages harvested from the peritoneum of ApoE−/− mice 5 days after injection of 2 mL Brewer’s thioglycollate. Although numerous cytokines and chemokines contribute to the process of aneurysm formation, a mechanistic analysis can only cover a limited number of stimuli. The proinflammatory cytokine IL6, which is generated in the vasculature in response to ATII and which is significantly elevated in the serum of human patients with abdominal aortic aneurysms, was therefore used in further experiments. Importantly, both acute coincubation with DIL (data not shown) as well as 14-hour preincubation with DIL in the same concentration attenuated the IL6-stimulated induction of Ccl12 and Il1b mRNA in peritoneal macrophages (Figure 3G and 3H). In contrast, ATII stimulation for the same duration did not elevate cytokine or chemokine levels. According to these findings, the vascular protective effects of DIL are most likely a consequence of the direct anti-inflammatory effects of the compound on IL6-induced signaling in macrophages.

**DIL Does Not Interfere With the Calcium Homeostasis in the RAW264.7 Cell Line**

To gain further insights into the mechanism of action of DIL, the murine leukemic macrophage cell line RAW264.7 was studied. Similar to the previously examined murine macrophages, RAW264.7 cells responded to IL6 stimulation with an increased Il1b mRNA expression, which was sensitive to DIL (Figure 4A). A potential mechanism of action could be that the calcium channel blocker DIL may lower the intracellular calcium concentration of macrophages, which should elicit an anti-inflammatory effect. As a benzothiazepine-type inhibitor of LTCCs, DIL blocks members of the NTCC family. By quantitative PCR, we identified the α1c subunit (Cacna1c) of voltage-dependent calcium channels as the highest expressed form of the HVA-type in the vessel wall, which was not expressed in RAW264.7 cells. The second
highest expression in aortic tissue was detected for α1d subunit (Cacna1d). This LTCC was present in the aorta as well as in RAW264.7 cells but reached only a small part of the mRNA expression level of Cacna1c. The neuronal P/Q-type calcium channel–specific subunit α1a (Cacna1a) was found in RAW264.7 cells but only to an insignificant extent in aortic tissue (Figure 4B). Other HVA calcium channels were found only to be expressed to a negligible extent in aortic tissue as well as in RAW264.7 cells. Furthermore, no major difference was seen between the calcium channel expression in intact whole aortic tissue and endothelium-free preparations. Calcium measurements in RAW264.7 with Fura-2/AM revealed that DIL, despite reducing the inflammatory response to IL6, did not affect the intracellular calcium concentration in RAW264.7 cells (Figure 4C). This indicates that the antianeurysmal effect of DIL is unrelated to its effect on calcium channels.

**DIL Inhibits IL6-Induced AP-1 Promoter Activity in RAW264.7 Cells**

To determine the mechanism of action of DIL in macrophages, we focused on transcription factor activity measured by luciferase reporter assays in response to stimulation with IL6 and LPS (lipopolysaccharide). IL6 (10 ng/mL) had no effect on NF-κB activity but doubled the AP-1 promoter activity in RAW264.7 cells. Importantly, the latter effect was blocked by DIL and NIF preincubation (Figure 5A and 5B). The mechanism of action of DIL seems to be specific for IL6 stimulation because even low concentrations of LPS induced AP-1 and NF-κB promoter activity in a way that was not sensitive to DIL or NIF (Figure 5C and 5D). IL6 also increased the phosphorylation of the signal transducer and activator of transcription 3 (STAT3); however, this process was not sensitive to DIL or NIF. Interestingly, LPS was not capable of inducing STAT3 phosphorylation (Figure 5E). As expected, LPS treatment resulted in a nuclear accumulation of NF-κB subunit p65. As already suggested by the reporter gene assays, this effect could not be blocked with neither DIL nor NIF and was not observed in response to IL6 stimulation (Figure 5F). Phosphorylation and nuclear accumulation of the mitogen-activated protein kinase 1 (MAPK1) was increased by IL6, but this effect was independent of DIL or NIF (Figure 5G).

Collectively, these data suggest that DIL interferes with AP-1 activation via a MAPK-independent pathway.

**Discussion**

In the present study, we report that DIL attenuates aortic aneurysm formation in the model of ATII-infused ApoE−/− mice in a pressure-independent manner. DIL also prevented the ATII-stimulated accumulation of macrophages in the aortic arch and the induction of chemokines locally. Cell culture experiments suggest that the anti-inflammatory effect of DIL is mediated by an inhibition of chemokine signaling in macrophages.

Aneurysm formation is a complex process, in part driven by inflammation. The model used here is particularly inflammatory because it combines hypercholesterolemia and elevated ATII levels and activates a broad spectrum of cells. Numerous cytokines therefore contribute to the process, but the best characterized effect is certainly the direct ATII-induced expression of IL6 and CCL2 by vascular SMCs, which is why we focused on these factors.

Among these different cell types mediating ATII signaling, vascular SMCs were considered the main target of DIL therapy because these cells highly express LTCCs, the major, but not sole, effector of DIL. Although ATII-induced aneurysm formation is not blood pressure dependent, ATII increases intracellular calcium in SMCs by the inositol trisphosphate pathway and by a diacylglycerol-mediated activation of LTCCs. It was therefore unexpected that only NIF, but not DIL, altered the proinflammatory stimulation of vascular SMCs. DIL and NIF act on different sites on the main subunit α1 of the channel and although NIF is a more potent inhibitor for LTCCs than DIL, the latter additionally blocks P/Q-, N-, and R-type calcium channels within the group of NTCCs.

Because we also found no evidence for endothelial cells being a target of DIL, we focused on macrophages. Although stationary vascular cells release proteases like MMP2 and MMP9, the proteolytic capacity of macrophages is much greater.
reduced amount of macrophages reduces aneurysm progression, and macrophage-derived cytokines are required to maintain vascular inflammation in the ApoE−/− ATII-infusion model. Macrophages are attracted into the vessel wall by an IL6 and CCL2 autocrine-paracrine loop,22 and we observed that the macrophage content and the mRNA expression of macrophage-derived cytokines were largely reduced by DIL. Although it was previously suggested that LTCC inhibitors impair cell migration in vitro,23 we failed to detect any in vivo relevance of these findings and therefore propose that the attenuation of macrophage inflammatory signaling is the most probable effect of DIL.

IL6 is of major importance for the aneurysmal process in humans.15 Although ATII directly amplifies macrophage migration,24 and ATII receptor type 1 (AT1) blockade is beneficial in an ATII-infusion atherosclerosis model,25 our data suggest that direct ATII stimulation is not sufficient for macrophage-derived cytokine production. Following the concept that IL6 released by fibroblasts and SMCs promotes aneurysm formation,7 the effect of DIL on macrophage activation was determined. IL6 stimulated the AP-1 but not the NF-κB promoter and induced nuclear accumulation of p-STAT3. Of these, the AP-1 activation was blocked in response to DIL. Unfortunately, further attempts to specifically identify the site of inhibition failed because DIL had no impact on LPS-induced signaling, MAP3K1 kinase activity, PPAR transcription factor activity (data not shown), or intracellular calcium. Obviously, there are numerous other potential targets of DIL; however, an impairment of AP-1 promoter activity in macrophages will have a strong inhibitory effect on the aneurysmal process because MMP9 is under the control of this transcription factor.26 Indeed, we observed a differential response between MMP9 and MMP2 expression in the vessel wall: Whereas MMP9 was suppressed by DIL, MMP2 expression seemed to be rather pressure dependent. Treatment with phenylephrine increased the expression of this protease to the same level as in the absence of DIL. This observation not only indicates that pressure and inflammation differentially contribute to the aneurysmal process but also demonstrates that our approach to circumvent the hypotensive effect of DIL by phenylephrine was successful.2 Given that DIL also has a negative inotropic effect and thus should lower pulse pressure, it was not self-evident that an α1-adrenoanogist like phenylephrine would be suited to counteract the effects of DIL.

Surprisingly, few studies have been performed on the effect of calcium channel blockers on experimental model and clinical aneurysm development. This is probably a consequence of the fact that these drugs have seen their height before the introduction of inhibitors of the angiotensin-converting enzyme (ACE) into general practice and before the development of mouse models of aneurysm formation.

In humans, DIL is most commonly used at a dose of 240 mg/d to treat angina pectoris although the dose for hypertension treatment is usually described with 360 mg/d, and doses of 720 mg/d have been administered successfully to patients having pulmonary hypertension.27,28 Potential side effects range from edema formation, nausea, and fatigue to hypotension, dysrhythmias, and sinoatrial and atrioventricular nodal depression.29 In our ApoE−/− ATII-infusion model, the dose of DIL was ≈10-fold higher compared with human patients to compensate the high murine metabolism rate. Although blood pressure was reduced, suggesting that an appropriate concentration was used, we did not observe a significant effect of DIL on the heart rate. Calcium channel blockers are highly effective drugs, and their current neglect is probably predominantly a consequence of the intense research and marketing activity on newer targets. The few studies performed on antianeurysmal effects of calcium channel blockers suggest that their protective effect is not necessarily class dependent. Verapamil failed to block neutrophil elastase release,30 and amloidipine even enhanced elastin degradation ex vivo and potentiated MMP9 activity in a pig model.31 In a solely blood pressure–dependent model of collagen degradation, amloidipine blocked aneurysm formation in hypertensive mice treated with a lysyl oxidase inhibitor to reduce collagen cross-linking.32 In contrast, in a rat model of inflammatory aneurysm formation, inhibition of the ACE as well as the AT1 receptor, but not amloidipine, prevented aneurysm formation.23 In a rat model of ATII-infusion, NIF reduced aneurysm formation and exerted an anti-inflammatory effect, which was mediated by inhibition of NF-κB.33 Indeed, an interaction of NIF and NF-κB has been suggested previously, but because the study did not include a blood pressure control, it remained open whether or not the effect of NIF was truly blood pressure independent.

Although different mouse aneurysm models have been developed, there is none that covers all aspects of the human disease. In the ATII-infused ApoE−/− mice, particularly the inflammatory component is predominant, which is present in the whole aorta, as we also observed in the present study. Why in this model despite a general vascular inflammation aneurysms develop preferentially in the suprarenal region is unclear and different to the human situation with the infrarenal region being the main predilection site. Altered embryonic origin, different matrix composition, and diverging hemodynamic forces may all impact on the complex process, eventually leading to local establishment of the aneurysm.3 Nevertheless, it is a clear limitation of our study that we involved other parts of the aorta in our mechanistic analysis and did not focus on the abdominal aorta. Despite all these consideration, because both, the human disease and the mouse model, depend on macrophages driving aortic inflammation and remodeling, the macrophage-dependent antianeurysmal effect of DIL reported in the present study may be of clinical importance.

Perspectives

The present data demonstrate the antianeurysmal effect of diltiazem in the in vivo angiotensin II infusion mouse model even during hypertension. Clinical studies to determine whether this effect is also operative in human aneurysm development seem justified.

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Disclosures

None.
References


Novelty and Significance

**What Is New?**

- The L-type calcium channel inhibitor diltiazem prevents aneurysm formation in a blood pressure–dependent manner in the ApoE-/- mouse infused with Angiotensin II.

**What Is Relevant?**

- Diltiazem suppresses vascular inflammation by blocking interleukin-6 cytokine–induced AP-1 promoter activity and cytokine production in macrophages.

**Summary**

Diltiazem suppresses vascular inflammation accompanying atherosclerosis and abdominal aortic aneurysm formation in vivo in an angiotensin II infusion mouse model independent of the blood pressure. The antianeurysmal effect is maintained even if the blood pressure lowering effect of diltiazem is counteracted with phenylephrine. Diltiazem inhibits the interleukin-6–induced cytokine expression and thereby macrophage-mediated proinflammatory priming and chemotaxis of inflammatory cells into the vessel wall.
Supplemental Material

The L-type calcium channel inhibitor diltiazem prevents aortic aneurysm formation by blood pressure-independent anti-inflammatory effects

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Expanded Material and Methods Section

Animal experiments. All experiments were performed in compliance with the German animal protection law (TierSchG) and only male animals were used. The mice were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. The animal welfare committees of the Goethe-Universität Frankfurt am Main as well as the local authorities (Regierungspräsidium Darmstadt) approved all animal experiments.

ATII infusion AAA mouse model. 8-week-old male ApoE−/− mice were infused with 1.44 mg/kg bw/d ATII (Bachem, Bubendorf, Switzerland) via osmotic mini-pumps (Alzet, Cupertino, CA, USA) for 6 days or 4 weeks and blood pressure was measured by tail-cuff technique. Subgroups received 100 mg/kg bw/d DIL (Sigma-Aldrich, St. Louis, MO, USA) with the drinking water. An additional subgroup received co-infusion of 18 mg/kg bw/d phenylephrine (Sigma-Aldrich, St. Louis, MO, USA) via the implanted minipumps. At the end of the experiment, aortas were removed, photographed and used for histological or molecular analyses. Vascular distension was measured from calibrated photographs with ImageJ (NIH, Bethesda, MD, USA) and severity of aneurysm formation was classified according to Daugerty et al.2

Elastica van Gieson's stain. Murine abdominal aortas including suprarenal and infrarenal area were perfused with PBS (Life Technologies, Carlsbad, CA, USA) and fixed with Roti-Histofix 4% (Carl Roth, Karlsruhe, Germany). After 6 h the tissues were transferred into 70% ethanol followed by an ascending ethanol series, ending with xylol and paraffin embedding. 3 µm slices were incubated successively with resorcinol-fuchsin-solution according to Weigert for 45 min, 80% ethanol, aqua dest., hematoxylin solution according to Weigert for 5 min, aqua for 10 min, aqua dest. Van Gieson's picrofuchsin solution for 2 min (all from Carl Roth, Karlsruhe, Germany), aqua dest. for 10 min, 70% ethanol for 1 min and embedded in Entellan (Merck, Darmstadt, Germany). Nuclei will appear in black, elastic fibers in dark violet.

Induction and isolation of peritoneal macrophages. 40.5 g/L Brewer thioglycollate medium (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally into 8-week-old male ApoE−/− mice. After 5 days the mice were sacrificed and the peritoneum washed twice with RPMI medium (Life Technologies, Carlsbad, CA, USA) containing 5 mmol/L HEPES, 50 U/mL penicillin and 0.05 mg/mL streptomycin (PAA, Pasching, Austria) for isolation of macrophages. Cells were incubated for 14 h with either 10 μmol/L diltiazem (DIL) or nifedipine (NIF, both from Sigma-Aldrich, St. Louis, MO, USA) and further stimulated with 10 ng/mL murine interleukin-6 (IL6, from PeproTech, Hamburg, Germany) for 4 h.

In vivo matrigel plug recruitment assay. Matrigel (growth factor reduced basement membrane matrix from BD Biosciences, Franklin Lakes NJ, USA) was mixed with 10 IU/mL heparin-natrium (Ratiopharm, Ulm, Germany) and either aqua ad injectabilia (B. Braun, Melsungen, Germany), 600 µg/L recombinant mouse CCL2 (R&D Systems, Minneapolis, MN, USA) or CCL2 in combination with 30 µmol/L DIL. 8-week-old male ApoE−/− mice were anesthetized by intraperitoneal injection of chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) and 400 µL liquid matrigel mix was implanted subcutaneously into the left and right flank at the lower part of the back. Plugs were removed after 3 days, washed in PBS, weighted, digested by dispase II (Roche, Basel, Switzerland) and collagenase II (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C and filtered through a 70 µm cell strainer. Cells were collected by 800 xg centrifugation for 10 min at 4°C, washed twice with PBS containing 0.5% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 2 mmol/L EDTA and resuspended in 100 µL PBS containing 2% BSA. Polychromatic flow cytometry was performed as previously described by Weigert et al.3 In brief, non-antigen-specific antibody binding to FC-γ receptors was blocked with rat-anti-mouse Fc Block (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C. Cells were mixed with Flow-Count Fluospheres (Beckman Coulter, Brea, CA, USA) and incubated for 30 min with a set of antibodies containing CD45-VioBlue (Clone 30-F11.1 from Miltenyi Biotec, Bergisch Gladbach, Germany) and Ly6C-PerCP-Cy5.5 (Clone AL-21 from BD
Biosciences, Heidelberg, Germany). Samples were acquired with a LSRII/Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed by FlowJo software 7.6.5 (Treestar, Ashland, OR, USA).

**Semi-quantitative real time PCR.** Frozen aortic samples were homogenized by stainless steel bead-based disruption in a TissueLyser (Qiagen, Hilden, Germany). Total RNA from tissues or cells was isolated by RNA-Mini Kit (Bio&Sell, Feucht, Germany), transcribed with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and semi-quantitative real-time PCR was performed with Fast-Plus EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA) in a Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Expression was calculated using the delta-delta-Ct-method standardized to eukaryotic translation elongation factor 2 (Eef2) or to DNA directed RNA polymerase II polypeptide A (Polr2a) and standardizing control conditions to a relative expression of 1. The oligonucleotide primers (BioSpring, Frankfurt am Main, Germany) were used with an annealing temperature of 60°C and are listed in supplemental table S2 below.

**ELISA.** Chemokine expression in murine sera was assessed by RodentMAP v2.0 Antigens assay (Myriad RBM, Austin, TX, USA) creating a 58 biomarker-containing protein expression profile.

**Ex vivo aortic ring assay.** The thoracic part of the aorta was extracted from 8-week-old ApoE<sup>-/-</sup> mice, cut into rings and maintained in MCDB131 (Life Technologies, Carlsbad, CA, USA) containing 0.1% BSA for 4 h at 37°C. Afterwards, the aortic rings were transferred into medium ± 10 µmol/L DIL, incubated for 14 h and further stimulated with 100 nmol/L ATII. After the stimulation, the aortic rings were washed twice in cold Hanks' balanced salt solution and flash frozen in liquid nitrogen.

**Cell culture.** Cells of the murine macrophage RAW264.7 cell line (ATCC, Manassas, VA, USA) were cultured in MEM Alpha Modification (GE Healthcare, Little Chalfont, UK) containing 8% FCS, 50 U/mL penicillin and 0.05 mg/ml streptomycin and maintained at 0.1% BSA ± 10 µmol/L DIL for 14 h before stimulation. Vascular smooth muscle cells isolated from rat aorta were cultured in MEM (Sigma-Aldrich, St. Louis, MO, USA) containing 8% FCS and 0.05 mg/mL gentamycin. Plates were coated with 50 µg/mL collagen type I (BD Biosciences, Franklin Lakes NJ, USA) and cells were starved for 14 h at 0.1% BSA in the presence of either 10 µmol/L DIL or NIF before stimulation.

**Western blot.** RAW264.7 cells were harvested directly after stimulation and nuclear fractions were isolated based on the method initially described by Dignam et al.<sup>4</sup> In brief, cells were washed with Hanks' Balanced Salt Solution, scraped off the culture plate and lysed for 15 min in buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 0.5 mmol/L PMSF (all from Sigma-Aldrich, St. Louis, MO, USA), 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT (all from AppliChem, Darmstadt, Germany) and protease inhibitors. 0.75% Nonidet P-40 (AppliChem, Darmstadt, Germany) was added and the cytosolic fraction removed after centrifugation. The remaining pellet was resuspended and incubated for 15 min in buffer containing 0.4 mol/L NaCl, 20 mmol/L HEPES, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF and protease inhibitors. After centrifugation the nuclear fraction was taken, protein concentrations were measured according to Bradford et al.<sup>5</sup> and SDS-PAGE followed by Western blot was performed. Proteins were identified with primary antibodies (please see table S1 below) and detected by near-infrared labeled secondary antibodies in an Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog Nr.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit-anti-phospho-Stat3</td>
<td>Cell Signaling</td>
<td>9131</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit-anti-phospho-MAPK3/1</td>
<td>Cell Signaling</td>
<td>9101</td>
<td>1:1000</td>
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<tr>
<td>goat-anti-Topoisomerase I</td>
<td>Santa Cruz</td>
<td>sc-5342</td>
<td>1:500</td>
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<tr>
<td>rabbit-anti-NF-xB</td>
<td>Santa Cruz</td>
<td>sc-109</td>
<td>1:500</td>
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</table>

**Transfection and luciferase activity assay.** RAW264.7 cells were transfected by Lipofectamine2000 (Life Technologies, Carlsbad, CA, USA) with plasmids coding for luciferase of Photinus pyralis under the control of NF-xB or AP-1 promoter and a control plasmid coding for luciferase of Renilla reniformis in a pGL4 backbone vector. The NF-xB promoter plasmid was kindly provided by Prof. Dr. Gerhard Fritz (Institute of Toxicology, Mainz, Germany) whereas the AP-1 promoter plasmid was purchased (Agilent Technologies, Santa Clara, CA, USA). Enzymatic activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI, USA) 24 h after transfection and 4 h after stimulation.

**Intracellular calcium measurement.** RAW264.7 cells were seeded into 96 well microplates, washed with Heps-buffered Tyrode's solution and loaded with 1 µmol/L Fura-2/AM (Merck, Darmstadt, Germany) in the presence of 2 mmol/L probenecid (Life Technologies, Carlsbad, CA, USA). Fluorescence was measured at 340 nm and 380 nm in an Envision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA, USA) and quantified using FlowJo software 7.6.5 (Treestar, Ashland, OR, USA).
1 µmol/L ionomycin or 10 mmol/L EGTA to obtain maximal or minimal intracellular calcium concentration according to standard methodology.6

**Statistical Analysis.** Data are expressed as mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) by Kruskal-Wallis-Test followed by Dunns correction for the comparison of more than two groups or the Mann-Whitney-Test. For paired comparisons of two dependent groups the Mann-Whitney-Wilcoxon-Test was used. A probability value < 0.05 was considered significant and indicated with an asterisk.

**Method references**

**Table S2: Real time PCR primers**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequences</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acta2 (alpha 2 aortic smooth muscle actin)</td>
<td>fw: 5'-ACAGAGGCACCACCTGAACCTAAGG-3'  rv: 5'-ACAATCTACGCTCGCAGTATGC-3'</td>
<td>311 bp</td>
</tr>
<tr>
<td>S100a4 (fibroblast-specific protein-1)</td>
<td>fw: 5'-TACTCAAGGAAAGAGgtGTGACAG-3'  rv: 5'-CTGGTTGCTGTCCTCAAGTTTCCTAC-3'</td>
<td>143 bp</td>
</tr>
<tr>
<td>Emr1 (F4/80 macrophage marker)</td>
<td>fw: 5'-TGGATGCTGATGAGAAGTGAGTG-3'  rv: 5'-GCAATGCTGAGGCTGAGCAGAC-3'</td>
<td>362 bp</td>
</tr>
<tr>
<td>Tnf (tumor necrosis factor alpha)</td>
<td>fw: 5'-CCCCGACTACGTCCTCACC-3'  rv: 5'-CTCCAGCTGGAAGACCTAGT-3'</td>
<td>171 bp</td>
</tr>
<tr>
<td>Il1b (interleukin-1 beta)</td>
<td>fw: 5'-GACCTTCCAGGAGAGATGACATGAG-3'  rv: 5'-GGTGTTGCTGCTCTTACCA-3'</td>
<td>343 bp</td>
</tr>
<tr>
<td>Il6 (interleukin-6)</td>
<td>fw: 5'-CCAGTTGCTCCTCTTGGGAGCTAGT-3'  rv: 5'-CTCAGGGACTGTAAGTTAC-3'</td>
<td>128 bp</td>
</tr>
<tr>
<td>Ccl2 (monocyte chemoattractant protein-1)</td>
<td>fw: 5'-CCACCTCACGCTGACTTACC-3'  rv: 5'-GTCACACTCCAGAAGTTGCTAAG-3'</td>
<td>338 bp</td>
</tr>
<tr>
<td>Ccl12 (monocyte chemoattractant protein-12)</td>
<td>fw: 5'-CTCAGGGATGTTGAGAACAGTATG-3'  rv: 5'-GGACACTGCTGCTTTGATTCAT-3'</td>
<td>124 bp</td>
</tr>
<tr>
<td>Tgfb1 (transforming growth factor beta 1)</td>
<td>fw: 5'-TGACATGCTACGTTGAGTACGG-3'  rv: 5'-GGTTTCATCAGTTGAGTTGTC-3'</td>
<td>170 bp</td>
</tr>
<tr>
<td>Mmp2 (matrix metallopeptidase 2)</td>
<td>fw: 5'-GGCACCACCTGACTTCTGCC-3'  rv: 5'-GTCACGTACATCAGTACATC-3'</td>
<td>321 bp</td>
</tr>
<tr>
<td>Mmp9 (matrix metallopeptidase 9)</td>
<td>fw: 5'-GAGCCAGGCTGATCCTGTTAC-3'  rv: 5'-GGCAGCCAGTGAAGTCTAATG-3'</td>
<td>78 bp</td>
</tr>
<tr>
<td>Ef2 (eukaryotic translation elongation factor 2)</td>
<td>fw: 5'-GACATCACAAGGGTGTTGCG-3'  rv: 5'-GCGGTACACACACTGCGCATA-3'</td>
<td>218 bp</td>
</tr>
<tr>
<td>Pol2a (DNA directed RNA polymerase II polypeptide A)</td>
<td>fw: 5'-CTCGAAAACAAGGAGATATGTCGACT-3'  rv: 5'-CAACACCCACCTTGTCTACGATTAGT-3'</td>
<td>332 bp</td>
</tr>
<tr>
<td>Cacna1a (P/Q type calcium channel, alpha 1A subunit)</td>
<td>fw: 5'-GGCCCTGCTGCTGCTGCTGCA-3'  rv: 5'-GGCCGCTGCTGCTGCTGCTGCA-3'</td>
<td>101 bp</td>
</tr>
<tr>
<td>Cacna1c (L type calcium channel, alpha 1C subunit)</td>
<td>fw: 5'-CAACCTGAAAGGAGAGATGACAGTAC-3'  rv: 5'-CTCCTCTGCTGCTGCTGCTGCA-3'</td>
<td>243 bp</td>
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<tr>
<td>Cacna1d (L type calcium channel, alpha 1D subunit)</td>
<td>fw: 5'-ACTCATTTGGCAACAGGATCTG-3'  rv: 5'-TTGGCTTCTACGTTGAGATG-3'</td>
<td>412 bp</td>
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</tbody>
</table>