Thiopurines and inhibition of Rac1 in vascular disease

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CHAPTER 3

Immunosuppressive drug Azathioprine reduces aneurysm progression through inhibition of Rac1 and JNK in endothelial cells

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ABSTRACT

Objective: In aortic aneurysms the arterial vessel wall is dilated due to destruction of its integrity, which may lead to lethal vessel rupture. Chronic infiltration of inflammatory cells into the vessel wall is fundamental to aneurysm pathology. We aim to limit aneurysm growth by inhibition of inflammation and reducing endothelial cell activation with immunosuppressive drug Azathioprine (Aza).

Approach and Results: Aza and its metabolite 6-mercaptopurine (6-MP) have anti-inflammatory effects on leukocytes. We here demonstrate that treatment of endothelial cells with 6-MP inhibits cell activation as illustrated by reduced expression of IL-12, CCL5, CCL2 and VCAM1 and inhibition of monocyte-endothelial cell adhesion. The underlying mechanism of 6-MP involves suppression of GTPase Rac1 activation, resulting in reduced phosphorylation of c-Jun-terminal-N-Kinase (JNK) and c-Jun. Subsequently, the effect of Aza was investigated in aneurysm formation in the Angiotensin-II (AngII) aneurysm mouse model in Apolipoprotein E-deficient mice. We demonstrated that Aza decreases *de novo* aortic aneurysm formation from an average aneurysm severity score of 2.1 (control group) to 0.6 (Aza-group) and that Aza effectively delays aorta pathology in a progression experiment, resulting in a reduced severity score from 2.8 to 1.7 in Aza-treated mice. In line with the *in vitro* observations, Aza-treated mice showed less JNK activation in endothelial cells and reduced leukocyte influx in the aortic wall.

Conclusions: The immunosuppressive drug Aza has an anti-inflammatory effect and in endothelial cells inhibits Rac1 and JNK activation, which may explain the protective effect of Aza in aneurysm development and, most importantly for clinical implications, aneurysm severity.

INTRODUCTION

Abdominal aortic aneurysm (AAA) formation is characterized by progressive degradation of the vessel wall, which may cause the formation of a balloon-like dilatation of the artery. Aneurysms are usually asymptomatic, however, these enlarged arteries are vulnerable to local rupture with high mortality rates. Due to decreased smoking habits in the Western society, the AAA incidence has declined recently from approximately 5% to 2% of the elderly male population[1]. However, as the number of elderly people in the Western society increases, AAA remains a serious health problem. In addition, a larger number of elderly will be diagnosed by coincidence with a small aneurysm due to the use of more sophisticated imaging techniques. Small aneurysms are screened regularly upon discovery to monitor the growth rate. When the size exceeds 5.5 cm, open or endovascular repair surgery is the only form of treatment. So far, commonly used medication such as angiotensin-converting enzyme (ACE) inhibitors, β-blockers, statins and antibiotics have been explored for treatment of AAA growth with variable success[2-7].

Chronic inflammation of the aorta is fundamental in AAA pathology, with infiltrating leukocytes producing cytokines and proteases that cause progressive deterioration of the vessel wall[8]. Furthermore, analyses of gene expression profiles and various imaging techniques of murine aneurysm models underscored the role of inflammatory processes in AAA[9-11]. So far, many animal studies using anti-inflammatory drugs were successful in inhibition of initiation of aneurysm formation. For example, celecoxib, a non-steroidal anti-inflammatory drug inhibiting cyclooxygenase-2, has been shown to prevent aneurysm formation very potently in the mouse angiotensin II (AngII) induced aneurysm model[12]. Multiple immunosuppressive drugs have been investigated in various aneurysm models. For example, sirolimus has been shown to prevent aneurysm formation in rats in the elastase model[13]. Furthermore, cyclosporine A has been described as a potent inhibitor of *de novo* aneurysm formation in mice[14], whereas hydrocortisone causes increased aortic rupture and aneurysm formation in mice[15]. The inconclusive observations with immunosuppressive therapy to treat aneurysm progression in humans recently raised a relevant discussion[16]. It seemed obvious that anti-inflammatory therapy would be beneficial to stop aneurysm progression, however, it does not appear that simple in clinical practice[16]. Each immunosuppressive drug has its specific targets and underlying mechanism, which makes the impact of a specific drug on the vessel wall different, but maybe highly relevant to AAA progression. It should be emphasized that AAA patients by definition already have an aneurysm at the moment when treatment is initiated, whereas most animal aneurysm experiments study prevention at the onset of aneurysm formation.

We propose that it is necessary to explore which anti-inflammatory drugs affect the vessel wall directly in a beneficial way. Azathioprine (Aza) caught our attention because of its described beneficial effects on endothelial cells (ECs). Aza increases EC survival[17], in contrast to sirolimus, which induces apoptosis, causing the thrombotic problems in sirolimus-coated coronary stents[18], or cyclosporine A which is toxic to ECs and provokes vasculopathy[19]. We and others have previously shown that Aza maintains the contractile phenotype of smooth muscle cells[20, 21] and has an anti-inflammatory effect in macrophages[22] and T-cells[23]. Thus Aza seems a good candidate drug to treat inflammation *and* protect the vessel wall.

Here, we show that Aza, and its active metabolite 6-MP, potently modulate activation of small GTPase Rac1 and downstream c-Jun terminal kinase (JNK) in ECs. JNK is a kinase with a pivotal role in aneurysm pathology[24]. We demonstrate that the JNK activation status in ECs is also reduced *in vivo* during mouse aneurysm formation by Aza and that Aza prevents aneurysm formation and inhibits aneurysm progression.

MATERIALS AND METHODS

Human endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in M199 (GIBCO Invitrogen) with 100 U/ml Penicillin/Streptomycin (P/S), 20% heat-inactivated FCS, 0.05μg/ml of heparin (Sigma), 2mM L-Glutamine (Invitrogen) and 25μg/ml Endothelial Cell Growth Supplement (ECGS, Sigma). When the cells reached 60% confluency ECGS was lowered to 12.5μg/ml. One day prior to experiments with 6-MP, medium was changed to M199 medium without the purine-based compounds adenosine sulphate, ATP disodium salt and guanine hydrochloride. HUVECs were stimulated for 24h with TNFα [10ng/ml] with or without overnight pretreatment with 6-MP [10 μM]. HUVECS were used between passage 1 and 3 for our experiments.

BRdU Incorporation Assay

Cultured endothelial cells were seeded in 96-well plates at a density of $2x10^3$ cells/well and incubated overnight in full medium. Cells were made quiescent by incubation in previously described special medium without FCS for 24h. Later the same day 6-MP was added or not. The following day FCS was added (20% v/v) to the cells and TNFα (10ng/ml) stimulation was applied to the appropriate wells. Cells were left for another 24h. The third day DNA synthesis was measured by the BRdU incorporation assay (Roche) according to manufacturer's instructions.

In vitro scratch-wound healing

Endothelial cells were seeded in a 6-well plate and cultured as previously described. They were pretreated with or without 6-MP, followed by TNFα stimulation for 24h on the next day. Live imaging started immediately after TNFα stimulation. A scratch was made with a pipet tip throughout the middle of the well. Three areas were chosen randomly to capture images every 10 min under the Leica live-cell microscope (DMIRBE) and relative closure speed of the scratch was measured. Images were captured with a digital camera (Apogee) and movies were generated. Quantification was performed using custom made software.

Monocyte-endothelial cell interaction

Human monocytic THP-1 cells were cultured at a density of $1\text{-}2x10^5$ cells/ml in RPMI1640 medium, supplemented with 10% FCS and 100 U/ml P/S. To visualize cell adhesion THP1 cells were fluorescently labeled with the Cell Trace™ CFSE cell Proliferation Kit (Invitrogen™, Molecular probes) prior to adding the cells to HUVECs. ECs were activated with TNFα [10ng/ml] with or without 6-MP [10μM] pretreatment, whereas monocytes were only pre-incubated or not with 6-MP for 24h. Undifferentiated THP1 monocytes from suspension at $2x10^5$ cells/ml were added to a confluent monolayer of HUVECs. After 4h the wells were gently washed and adherent cells were photographed by fluorescence microscopy (Zeiss).

RNA isolation and real-time RT-PCR

RNA was isolated from lymph nodes of control and Aza treated mice in the *de novo* aneurysm experiment or from *in vitro* cell culture EC experiments using the Aurum™ Total RNA Mini Kit (Biorad) and cDNA was generated by reverse transcription of RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). Real-time

RT-PCR was performed using an iCycler thermal cycler system (Bio-Rad Laboratories) with the cDNA samples with specific forward and reverse primers and SYBR Green Supermix (Bio-Rad Laboratories). The primer sequences for the PCR are listed in the table below. mRNA levels were normalized for the average values of two housekeeping genes, namely hypoxanthine phosphoribosyltransferase (HPRT) and large ribosomal phosphoprotein P0.

Table I- List of primers

	Forward primer sequence	**Reverse primer sequence**
IL12A (p35) *Homo Sapiens*	5'-AGGAATGTTCCCATGCCTTCAC-3'	5'-GCAACTCTCATTCTTGGTTAATTC-3'
CCL2 *Homo Sapiens*	5'-CCTAGCTTTCCCCAGACACC-3'	5'-CCCAGGGGTAGAACTGTGG-3'
CCL5 *Homo Sapiens*	5'-CGCTGTCATCCTCATTGC-3'	5'-CCACTGGTGTAGAAATACTCC-3'
ICAM-1 *Homo Sapiens*	5'-CAGAGGTTGAACCCCACAGT-3'	5'-CCTCTGGCTTCGTCAGAATC-3'
VCAM-1 *Homo Sapiens*	5'-GCAAAGGGAGCACTGGGTTGACT-3'	5'-GCCACATTGGGAAAGTTGCACAGG-3'
P0 *Homo Sapiens*	5'-TCGACAATGGCAGCATCTAC-3'	5'-ATCCGTCTCCACAGACAAGG-3'
P0 *Mus Musculus*	5'-GGACCCGAGAAGACCTCCTT-3'	5'-GCACATCACTCAGAATTTCAATGG-3'
IFN-γ *Mus Musculus*	5'-CCAAGCGGCTGACTGAACTC-3'	5'-GCCTGTTACTACCTGACACATTCG-3'
HPRT *Homo Sapiens*	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCAGCAGCAAGCA-3'
CD3 *Mus Musculus*	5'-GCTGGTGAGTAGGGGGAATGA-3'	5'-AACGGTATCGCCAACAGTTC-3'
CD19 *Mus Musculus*	5'-AAGAGGGAGGCAATGTTGTG-3'	5'-CAGGAAGGGTGTTGACTGGT-3'

Active Rac1 pulldown

Endothelial cells were treated with or without TNFα [10ng/ml], in the presence or absence of 6-MP overnight. Cells were washed with ice cold PBS (+ 1 mM CaCl2; 0.5 mM MgCl2) and lysed in 50 mM Tris, pH 7.4, 0.5 mM MgCl2, 500 mM NaCl, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) deoxycholic acid, and 0.1% (wt/vol) SDS supplemented with protease inhibitors. Lysates were cleared at 14,000 g for 5 min. GTP-bound Rac1 was isolated with biotinylated Pak1-Crib peptide coupled to streptavidin agarose beads[25]. Beads were washed four times in 50 mM Tris, pH 7.4, 0.5 mM MgCl2, 150 mM NaCl, 1% (vol/vol) Triton X-100, protease inhibitors, and pull-downs or total lysates were then immunoblotted with monoclonal Rac1 Ab. Rac1 (clone 102) mAb antibody was purchased from BD Biosciences (Breda, The Netherlands). Rac1 GTP levels were quantified and corrected for total Rac1 levels with ImageJ. Five experiments were performed.

Western blotting

SDS–PAGE samples were analyzed on 12% (wt/vol) polyacrylamide gels, depending on the size of the proteins of interest, and transferred onto PVDF membrane (Millipore; Immobilion™-P). Following blocking 1h in Odyssey blocking buffer (*Li-cor*®; Biosciences) mixed 1:1 with TBST, the blots were incubated with the primary antibody and addition of 0,1% Tween-20 O/N at 4°C, washed 3 times for 5 min in Tris-buffered saline–Tween-20

(TBST), and subsequently incubated with Odyssey IRDye® labeled secondary antibodies (dilution: 1:15000) in TBST and 0,01% SDS for 1 h at room temperature; this was followed by 3 washes with TBST for 5 min each. Infrared signal was detected and analyzed with the Odyssey infrared detection system (LI-COR Biosciences, Lincoln, NE).

JNK activation

Endothelial cells were treated overnight with 6-MP (10μM) or Rac-1 inhibitor (ITX3; Sigma-Aldrich) 100μM, followed by the next day 15 min. treatment with 10 ng/ml of TNF alpha. Cells were then washed with ice cold PBS and lysed with NP-40 lysis buffer supplemented with protease inhibitors. Lysate was cleared at 14.000 rpm for 5 min. Samples were blotted for Active JNK (p-SAPK/JNK Rabbit mAb; Cell Signaling), Total JNK (JNK1 2C6 Mouse mAb; Cell Signaling) and GAPDH (rabbit monoclonal; Cell Signaling). Five experiments were performed.

c-Jun activity assay

c-Jun TransAM™ Transkription Factor Assay Kit was purchased from Acive Motif® (Carlsbad, CA). The experiment was performed as described previously. Nuclear extracts were made and used according to manufacturer's protocol. DNA binding capacity of active nuclear p-c-Jun was measured using TransAM™ AP-1 quantitative transcription factor ELISA, following manufacturer's instruction. Quantification was performed using ELISA reader at 450 nm. Three experiments were performed.

Mice

Animal care and experimental procedures were approved by the animal experimental committee for Animal Welfare of the Amsterdam Medical Center. For the AngII-aneurysm model, Apolipoprotein E deficient (ApoE-/-; strain B6.129P2-ApoE tm1Unc/Crl) mice (Charles River, The Netherlands) were obtained and bred in our local animal facility for the *de novo* experiment. ApoE-/- mice were used for the Aza dose finding study (N= at least 3 mice per group). Food intake of Western type diet (WTD; containing 15% cacao butter, 1% corn oil and 0.25% cholesterol (Arie Blok, Woerden, the Netherlands)) was similar with and without medication. Consequently, the weight increase and the total serum cholesterol levels between control and Aza-treated mice did not differ (data not shown). Plasma cholesterol levels were measured with a colorimetric assay (bioMérieux®, Marcy l'Etoile, France). None of the mice died during this dose finding experiment.

In the *de novo* aneurysm formation and aneurysm progression experiments 16-week old male mice were studied. The mice were fed a WTD, which was initiated 4 week before AngII-osmotic minipump placement and continued until the end of the experiment. Osmotic minipumps were placed under inhalation isoflurane anesthesia (Baxter, Illinois, United States). Carprofen (5mg/kg mouse) was administered locally in the neck to provide pain relief. The osmotic minipumps (Alzet, DURECT Corporation, Cupertino, United States) released AngII (Sigma-Aldrich, St. Louis, United States) with a pump-rate of 1.44mg/kg/day.

The Aza-treated groups switched from WTD to WTD containing Aza (0.1 mg/kg/day) one day after osmotic minipump placement in the *de novo* aneurysm formation experiment. In the progression experiment the mice switched after 10 days of AngII infusion.

In the *de novo* aneurysm formation experiment, mice were harvested after 4 weeks (n=14 Co, n=13 Aza). In the progression experiment (N=14 per group), mice were harvested 3 weeks after switch of the diet, thus after 31 days of AngII infusion. Selection of the Aza-

treated and control mice was based on non-significant differences between weight and cholesterol levels of the mice one week prior to Aza treatment. Before and after harvest of the mice, no significant differences were observed in weight and total serum cholesterol between control and Aza treated mice (data not shown). In the progression experiment 4 mice died during the first ten days of AngII infusion due to aortic rupture, therefore when the switch to the Aza diet was introduced after ten days, both groups contained 10 mice, on which the analysis were performed.
The mice were harvested under anesthesia and perfused under slight pressure by hand slowly, to prevent loss of EC due to perfusion pressure via the heart with 10 ml ice cold PBS (1 min). Then lymph nodes were removed for RNA extraction (1 min), upon which the mouse was perfusion fixed via the heart with 5 ml 4% paraformaldehyde (1 min). Incisions in the liver facilitate perfusion.
To assess severity of aneurysm formation, the updated classification index for murine aneurysms was used[26]. In short, mice that did not develop an aneurysm were typed 0, aneurysms with a maximal diameter between 1.5 and 2 mm are type I, aneurysms of 2 mm and bigger are type II, multiple aneurysms and/or dissections are type III and mice that died during the experiment of aortic rupture are typed IV. Maximal thoracic and abdominal diameters were quantified macroscopically on photographs of the aorta using Adobe Photoshop CS4 by a researcher blinded to the treatment groups. When adding up all the aneurysm types, the severity score per group is divided by the number of mice in that group.

Immunohistochemistry
Murine descending thoracic aortic ring specimens (free of aneurysms) were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Seven µm aortic sections of all surviving mice in the control and Aza treated group (*de novo* experiment) were mounted on glass slides and immunohistochemical staining was performed. Macrophages (MAC-3; Pharmingen), T cells (anti-CD3; Labvision) and matrixmetalloproteinase-2 (MMP2) and MMP9 were detected (anti-MMP2; R&D and anti-MMP9; Abcam). Phosphorylated (active) JNK was detected using anti-p-JNK antibody (Cell Signaling). HRP-conjugated secondary antibodies were used and the stainings were developed with DAB chromagen as substrate for detection.
Immunopositive area was quantified in three sections per mouse using Leica QWin V3 software. T cells, macrophages and endothelial cells were counted manually in each aortic ring and divided by the surface area of the vessel wall (Tcells and macrophages) or by the total number of endothelial cells in the aortic rings, in the *de novo* experiment (Control n=11, Aza n=12).
To estimate the number of macrophages incorporated into the medial layer of the outer curvature of the ascending aorta, longitudinal sections of the aortic arches were generated from all mice of the progression experiment (n=10 per group). MAC-3 staining was performed as described and positive area was quantified by using Leica QWin V3 software. P-JNK positive endothelial cells were traced and divided by the total endothelial cell surface (as detemined with haematoxylin staining) lining the aortic arch, using Leica QWin software.
The AAA of the progression experiment were also sectioned and cross-sections of the area where most luminal dilation/media pathology was observed were used for quantification of external AAA diameter and luminal diameter by calculating the diameter from the measured circumference with Leica QWin software. Sirius red (collagen), Lawson (elastin), smooth muscle alpha-actin (SMC), Mac-3 (macrophages), CD3 (T-cell) and p-JNK stainings (N=10 per group) were performed and analyzed. All staining were analyzed by the QWin software, except CD3 positive cells, which were calculated as number of positive cells per area.

Statistics

To measure a 50% difference in the total aneurysm severity score in the treatment group (on average 2.4 in control group) with a power of 80% and a significance of 95%, 14 mice per group are needed. For the murine aneurysm data, statistical analyses were established by non-parametric Mann Whitney U-test. For all cell culture experiments students t-tests were performed. P-values below 0.05 are considered significant. Data are presented as mean value +/- SEM. Outliers were calculated by Grubbs outlier test: http://www.graphpad.com/quickcalcs/Grubbs1.cfm

RESULTS

6-MP decreases endothelial cell activation

To study the effect of Aza on proliferation and migration of ECs, the cells were treated with 6-MP and subsequently serum-induced BrdU incorporation was measured or a migration assay was performed. Treatment of ECs with 6-MP did not result in changes in DNA synthesis (Supplemental Fig IA) and also the migratory capability in the scratch assay was not affected (Supplemental Fig IB).

To investigate EC activation, we determined THP-1 monocyte adherence to quiescent and TNFα-activated ECs in the presence or absence of 6-MP. Fluorescently labeled THP-1 cells predominantly adhered to TNFα-stimulated cells (Fig.1A). Pretreatment of either ECs or monocytes or both cell types with 6-MP, resulted in reduced attachment of THP-1 cells.

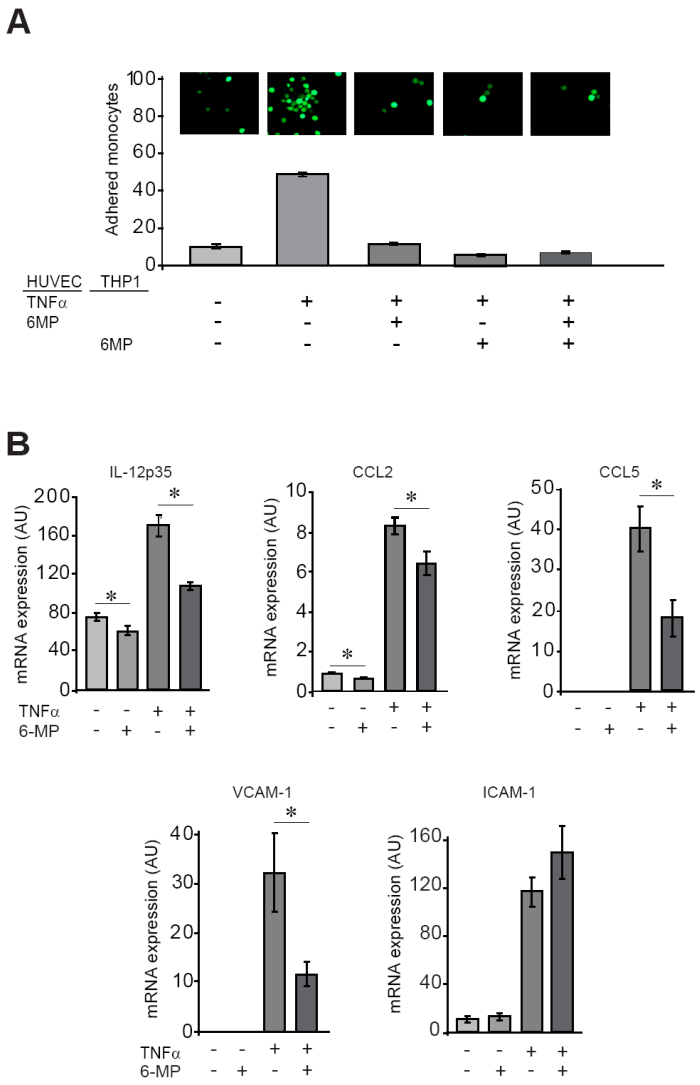

Figure 1. *6-MP reduces the inflammatory response of endothelial cells.*
(A) Fluorescently labeled THP1 cells adhere to TNFα-treated ECs. Pre-incubation of either ECs, monocytes or both cells types with 6-MP reduces the number of adherent THP1 cells significantly (compared to TNFα only, all *P<0.0002). (B) 6-MP inhibits the pro-inflammatory response of ECs by reducing mRNA expression of IL12p35, CCL2, CCL5 and VCAM-1, while ICAM-1 was not affected. (*P<0.05)

Because treatment of just the ECs revealed reduced monocyte adherence, we analyzed the effect of low dose 6-MP on the expression of a number of characteristic cytokines and adhesion molecules in ECs. ECs were pretreated overnight with or without 6-MP and then incubated with or without TNFα for 24 hrs. TNFα significantly induced mRNA expression of Rantes (CCL5), monocyte chemoattractant protein-1 (CCL2), interleukin-12 (IL-12), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Fig.1B). 6-MP strongly inhibited expression of IL-12, CCL2, CCL5 and VCAM-1, whereas mRNA levels of ICAM-1 were not changed.
Together these data demonstrate that 6-MP inhibits the inflammatory response of ECs selectively and reduced the adherence of monocytes, which is a crucial process in inflammatory diseases.

6-MP inhibits Rac-1 activation and Rac-1 mediated signaling
In activated CD4+ T-lymphocytes, it has been established that the small GTPase Rac1 is a target of Aza/6-MP, which becomes permanently inactivated and thereby limits Tcell activation[23,27]. Rac1 is a small G-coupled protein and member of the Rho family of GTPases involved in multiple cellular processes. We anticipated that the 6-MP dependent disturbance of Rac1-mediated processes may not be limited to T-cells. Therefore, we set out to determine the amount of activated Rac1 after 6-MP treatment in cultured ECs. In active Rac1 pulldown assays, the amount of GTP-bound Rac1 was increased upon TNFα stimulation (Fig.2).

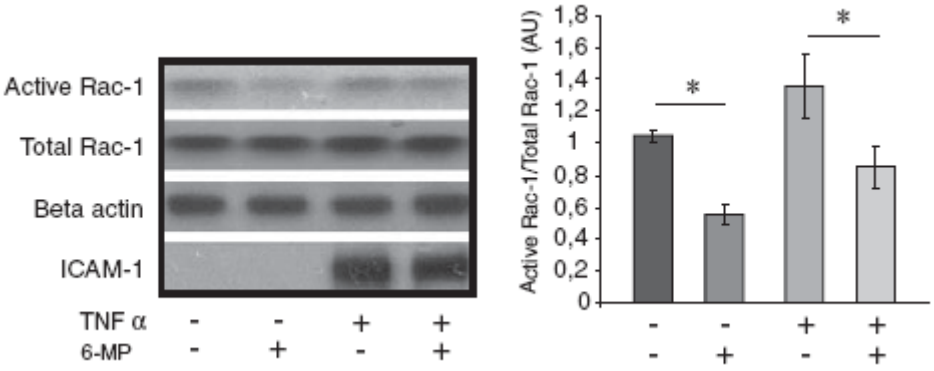

Figure 2. *6-MP reduces activation of Rac-1 in endothelial cells.*
Western blot showing pull down of active GTP-bound Rac1, revealing increased active Rac1 in response to TNFα stimulation and a lower level of active Rac-1 upon 6-MP pre-treatment. Levels of active Rac-1 are also reduced only upon 6-MP. Quantification of active Rac-1 was normalized for total Rac1. ICAM-1 protein served as the readout of EC activation by TNFα and the ICAM-1 expression level was not influenced by 6-MP. Data are means ± SEM (n=5). *P<0.03

Moreover, the extent of Rac1 activation was decreased in the presence of 6-MP, both in unstimulated and TNFα-activated ECs. As a control for TNFα activation the amount of ICAM-1 protein was assayed and shown to be equal under control and 6-MP conditions (Fig.2), similarly independent of 6-MP as on RNA expression levels (Fig.1B).
A pro-inflammatory pathway in ECs that is dependent on Rac1 activation involves JNK[28]. We show that in TNFα-stimulated ECs, 6-MP decreases the amount of JNK phosphorylation (p-JNK) and thus reduced JNK activation (Fig.3A,B). A similar reduction in p-JNK was also

observed when a Rac-1 inhibitor was used, suggesting that indeed inactivation of Rac-1 by 6-MP could be responsible for reduced levels of p-JNK.
Since JNK is an activator of the transcription factor partner c-Jun, a crucial component of the early response transcription factor AP-1, activation of c-Jun was determined. The amount of nuclear, active c-Jun was significantly abrogated in the presence of 6-MP or the Rac-1 inhibitor (Fig.3C). These results suggest that pro-inflammatory responses in the presence of TNFα can be successfully dampened in ECs by 6-MP via inactivation of Rac-1 (Fig.3D).

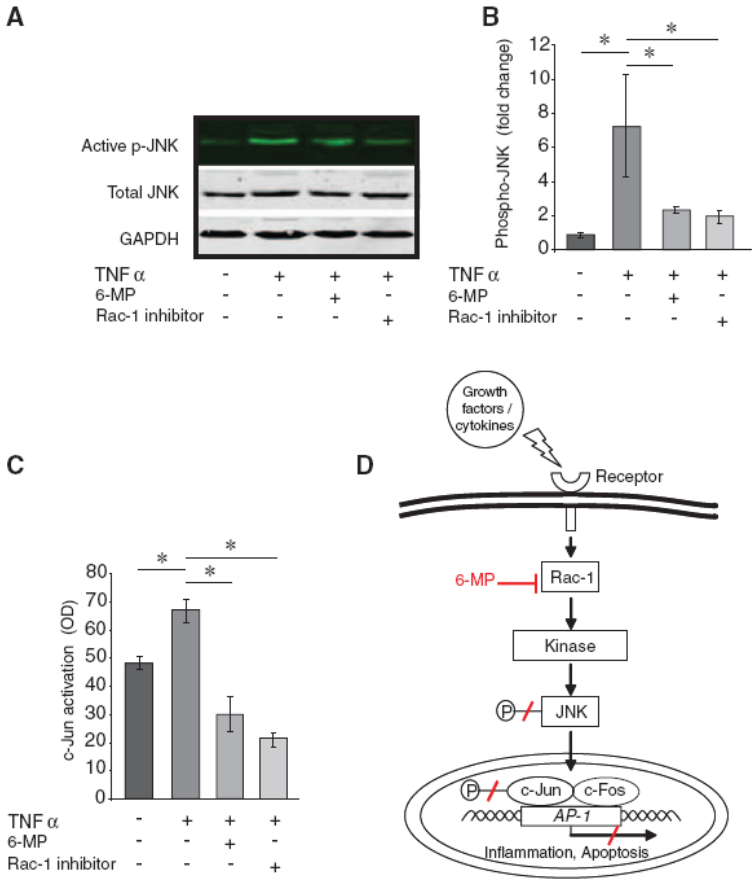

Figure 3. *6-MP reduces activation of the Rac-1 dependent JNK signaling pathway.*
(A) TNFα activated ECs show reduced levels of phosphorylated (p)-JNK in the presence of either 6-MP or Rac-1 inhibitor. (B) Quantification of active p-JNK is normalized for GAPDH (n=5). The amount of active p-JNK increases upon TNFα stimulation and is reduced by 6-MP or Rac1 inhibitor to baseline levels. *P<0.003 (C) Activated ECs show a similar reduction in nuclear c-Jun transcriptional activity in the presence of either 6-MP or Rac-1 inhibitor (n=3). *P<0.01 (D) A model of 6-MP interference in the JNK signaling cascade. 6-MP inhibits Rac-1 activation and thus subsequent phosphorylation of JNK, which results in decreased c-Jun activation, which together with c-Fos forms activator protein 1 transcription factor (AP-1). Consequently transcription of downstream genes is inhibited, resulting in a decreased inflammatory response.

Azathioprine reduces *de novo* aneurysm formation

Inflammatory processes and JNK activation play key roles in aneurysm formation and both processes are inhibited by Aza; therefore we studied the effect of Aza on aneurysm formation. First, a dose finding study was performed to determine the optimal oral Aza dose for mice. Chronic immunosuppression of humans is maintained at a dose of 1-4 mg/kg/day Aza. Therefore, ApoE-/- mice were exposed to 10, 5, 1 and 0.1 mg Aza/kg/day, which was

administered orally in a Western Type Diet (WTD). Circulating cell numbers were determined after 4 weeks of treatment and we observed that only the low dose of 0.1 mg Aza/kg/day did not affect red blood cell counts and that these mice showed only limited reduction in white blood cell numbers (Supplemental Fig.II). Subsequent aneurysm experiments were performed with this low dose.

The effect of Aza on *de novo* aneurysm formation was investigated in the AngII-infusion model and the mice were treated with Aza from the onset of aneurysm induction. Three mice (3/14) died in the control group, whereas only one mouse (1/13) in the Aza-treated group died due to aortic rupture within the first week of the experiment. After 4 weeks aortas were isolated and reveal that aneurysm formation is strongly inhibited by Aza, since only 17% (2/12) of the Aza-treated mice develop an aneurysm (Fig.4A), whereas in the control group 73% (8/11) show aneurysm formation.

Aneurysm severity is scored and mice in the control group show a more severe aortic phenotype with mostly type-III aneurysms, compared to the Aza-treated mice (Fig.4B) with predominantly healthy aortas. The type-III aneurysm score indicates that thoracic aneurysms are present in addition to AAA. Thoracic aneurysms of the descending aorta are clearly visible, whereas the so-called saccular aneurysms that develop in the ascending aorta (pink star in Fig.4A, enlarged in Supplemental Fig.III) are less obvious by macroscopic inspection, but can be identified by microscopic analyses of sections of aortic arches. All together Aza strongly reduces the number and the severity of aneurysm formation from a total score of 2.1 to 0.6 (Fig.4C, $P<0.01$).

Azathioprine reduces JNK activation in endothelial cells and inhibits inflammation

To confirm the immunosuppressive effect of Aza, we isolated draining lymph nodes of the thoracic aorta and compare expression levels of Interferon-γ (IFN-γ). Application of Aza indeed results in decreased mRNA expression of IFN-γ in lymph nodes, as well as decreased Tcell (CD3) and Bcell (CD19) content, which represents the reduced inflammatory status of the mouse upon Aza treatment (Supplemental Fig.IV). To understand the protective effect of Aza on aneurysm formation we aimed to study JNK activation and inflammation in the aortic vessel wall[29]. Only two aneurysms were formed in the Aza-treated mice, and taking into account that the cellular composition of aneurysms is extremely variable, we rationalized that analysis of the vessel wall within the aneurysm lesions may limit quantitative analyses. To circumvent this problem, we analyzed the descending thoracic aorta of all mice, which is a region of the aorta that did not show overt pathology (N=11 control and N=12 Aza-treated mice), yet systemic effects of AngII and Aza can be measured. JNK phosphorylation is low in medial smooth muscle cells in these sections. However, JNK phosphorylation is abundantly present in ECs upon AngII infusion and significantly decreased by Aza in endothelium lining the lumen of the aorta segment (Fig.4D). On average 64% of the ECs show p-JNK in AngII infused mice, whereas only 47% is p-JNK positive in the Aza-treated AngII mice ($P<0.0004$).

Subsequently, the presence of leukocytes in the media and adventitia is determined and we observe decreased numbers of macrophages and T-cells in the vessel wall of Aza-treated mice (Fig.4E,F). It is of importance to mention that in the AngII model, there is fibroblast hypertrophy, which causes thickening of the adventitia, which was not significantly different between control and Aza treated aorta's, with regard to the aortic ring sections that were quantified here (data not shown), therefore the decreased inflammation upon Aza was not just a reflection of adventitial area. In line with reduced leukocyte recruitment to the vessel wall, the expression of MMP2 and -9 protein is also reduced after Aza treatment

(Supplemental Fig.V). We conclude that Aza reduces endothelial JNK activation, recruitment of inflammatory cells to the aortic vessel wall and attenuates protease expression.

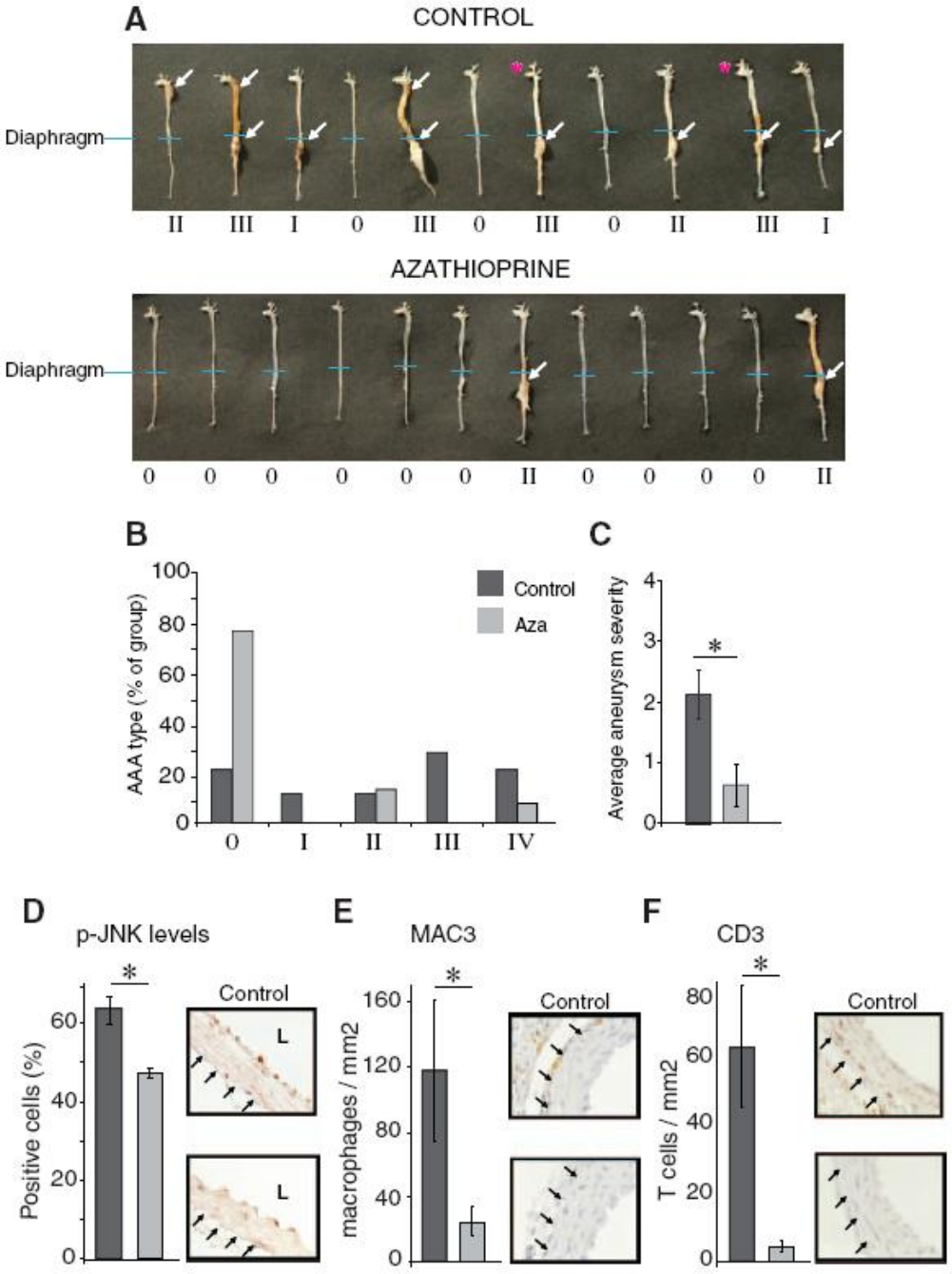

Figure 4. *Aza decreases de novo aneurysm formation.*
(A) Macroscopic overview of aortas from control (n=11) and Aza-treated (n=12) mice. Arrows point at aneurysms/dissections and 2 aortas contain a saccular aneurysm (pink star). The interrupted blue line marks the diaphragm location. (B) Analysis of the aortas according to the aneurysm severity score reveal prevention of aneurysm development upon Aza treatment; healthy aortas of non-responders are indicated with '0' and the four types of aneurysms in the control and Aza-treated groups are shown (percentage within group). (C) The average aneurysm severity score per group is decreased in the Aza-treated mice. *P<0.01 (D) A decrease in active phosphorylated (p)-JNK is observed in ECs in the aorta wall (*P<0.0004) in response to Aza treatment. Aza also causes reduction in the number of macrophages (Mac3; *P<0.05; E) and T cells (CD3; *P<0.003; F) in the vessel wall. Number of infiltrated macrophages and T cells is expressed per mm^2 of the aortic tissue. L indicates lumen.

Azathioprine inhibits aneurysm progression

After demonstrating that Aza inhibits *de novo* aneurysm formation, we aimed to assess whether Aza also stabilizes the growth of existing aneurysms. In the AngII-model, aortic dilation is already induced after one week[30,31]. We started the Aza intervention 10 days after the onset of AngII infusion to allow initiation of aneurysm formation. In this experiment, all mice that died because of aortic rupture within the first 10 days (N=4 per group), before Aza treatment was initiated, were excluded from further analyses. One mouse in the control group was euthanized one day before harvest (day 30) because of immobility due to aortic rupture (this mouse was typed as IV). Again, mice from the control group develop more severe aneurysms than the Aza-treated mice. According to the severity score, the most prevalent aneurysm types in the control group are type II and III, while Aza-treated mice develop predominantly type I and II aorta pathology (Fig.5A,B). Aza attenuates the progression of aneurysm formation, resulting in a significantly lower severity score of 1.8 compared to 2.7 in controls (Fig.5C; $P<0.02$). The discrepancy between aneurysm incidence in the *de novo* and progression experiment is a feature of the AngII model, where incidence can vary between 50% and 100%. However, in our case the *de novo* experiment was performed in mice that were bred in our facility for a year and in the progression experiment the mice were delivered from Charles River directly.

A similar pattern of p-JNK reduction is observed as in the *de novo* experiment. In the AngII infused aortas 62% of aortic ECs are positive for p-JNK in comparison to 47% in the delayed Aza-treated mice (Fig.5D; $P<0.0001$). Decreased EC activation is reflected by decreased inflammation in the vessel wall. In the control mice, one saccular aneurysm is detected in the outer wall of the ascending aorta (pink star in Fig.5A). Macrophage influx in the media is damaging and therefore quantified to locally monitor the effect of Aza on inflammation in the vessel wall. Medial macrophage influx in the thoracic aorta has not been observed in early timepoints[31], however we find it in 9/10 mice after 31 days of AngII infusion at this location. Interestingly, the Aza-treated mice show a strongly reduced macrophage influx into the media (Fig.5E), demonstrating the anti-inflammatory effect of Aza-treatment, even when the onset of treatment was delayed in the disease process.

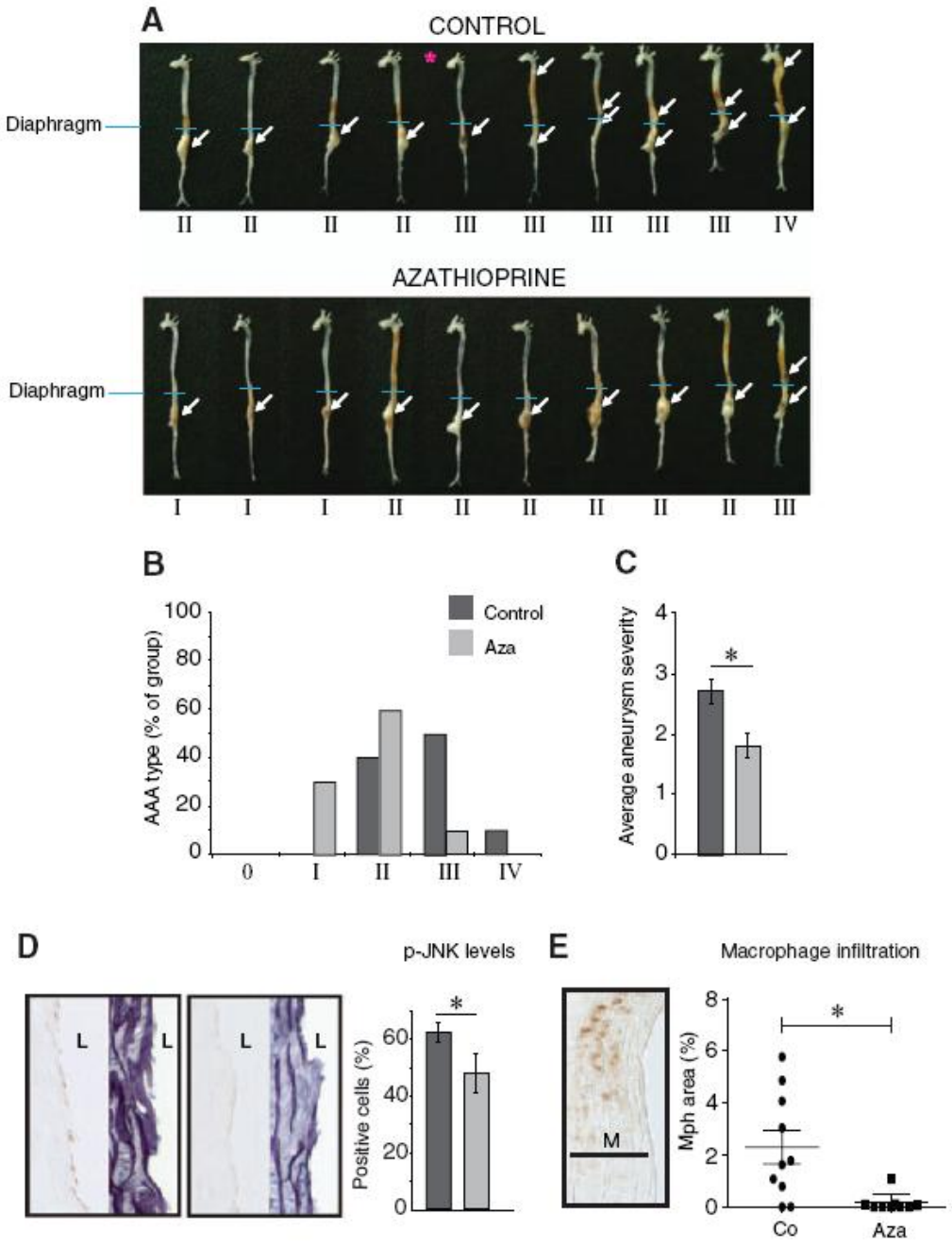

Figure 5. *Aza inhibits aneurysm progression*.
(A) Macroscopic overview of aortas of control Aza treated mice (n=10 per group). Arrows point at aneurysms/dissections and 1 mouse shows a saccular aneurysm (pink star). The interrupted blue line marks the diaphragm location. (B) A shift in aneurysm severity score (%) towards a milder phenotype is observed upon Aza treatment. (C) The average aneurysm severity score per group is reduced in Aza-treated mice. *P<0.02 (D) Activated phosphorylated (p)-JNK in the aorta is shown next to elastic lamellae staining for orientation purposes and quantified, revealing a decrease in the percentage of p-JNK positive ECs in Aza-treated mice.*P<0.0001 (E) Macrophage infiltration into the media is shown and quantified. A decrease in the amount of macrophage positive area is measured after late onset of Aza treatment. *P<0.01

Azathioprine inhibits inflammation within AAA

While the pathology score of the entire aorta is decreased by Aza in the progression experiment, the effect of Aza within the AAA can be analyzed as well, since all mice (N=10 per group) had developed an AAA. The outer (maximal) diameter is measured macroscopically (Supplemental Fig.VI A) and microscopically (Fig.6A) and is equal in control and Aza treated mice, revealing that both methods are valid to determine aneurysm size. Also the luminal maximal diameter is measured and did not differ significantly between the groups. In addition, the matrix components collagen (Fig.6B) and elastin (Fig.6C) co-localize mostly with the smooth muscle cell area (Fig.6D), but are similar between the two groups. While the macrophages show only a modest trend towards a decrease in the Aza treated group (Fig.6E), the T cell (Supplemental Fig.VI B) content of the AAA is significantly lower in the Aza treated mice, indicative of reduced inflammation within the AAA. Yet, the p-JNK in luminal endothelial cells is not different within the AAA (Supplemental Fig.VI C), but a significant decrease in total AAA p-JNK is observed in the Aza group (Fig.6F).

P-JNK is abundantly present in different cell types within the AAA and overlapped in part with macrophage positive area (compare Fig.6E,F). As a readout of the inflammatory state of the AAA, we analyzed proteases MMP2 and -9 positive area. Indeed MMP2 and -9 area is significantly lower in the Aza treated mice (Supplemental Fig.VI D,E).

In conclusion, p-JNK is decreased in Aza treated mice and may result in the decreased inflammatory status within these AAA. This site specific decrease of p-JNK in the suprarenal aorta has been shown to be important to achieve improvement of aneurysm pathology[32].

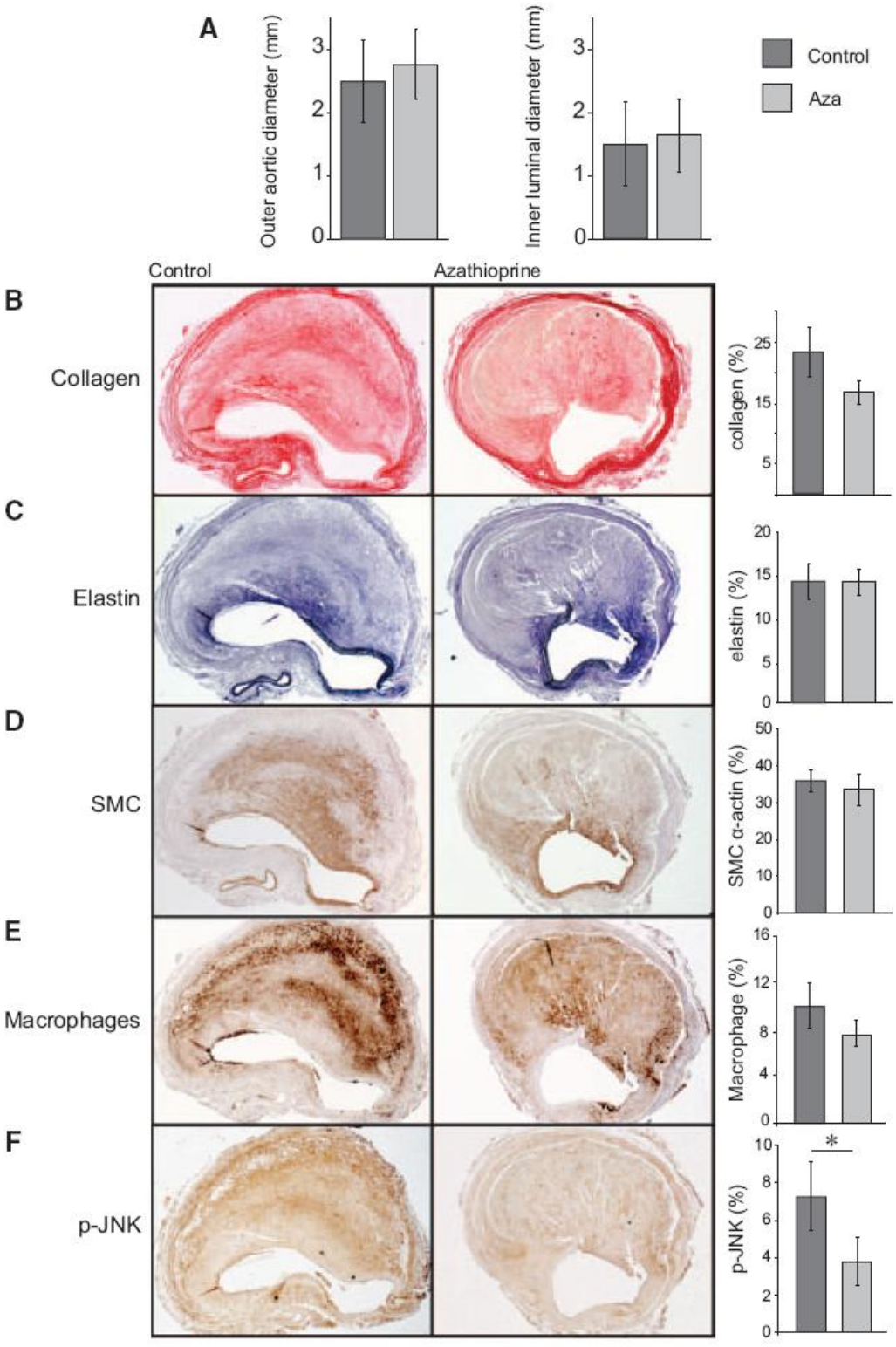

Figure 6. *Histological assessment of Aza effect on pre-existing aneurysms*.
(A) Aortic aneurysms from control (n=10) and Aza (n=10) treated mice were assessed for differences in outer and luminal diameter which do not show any differences between two groups. Application of Aza did not influence collagen deposition (B) or the amount of elastin (C) present in the aneurysms. Aza did not alter smooth muscle cell (SMC) content (D) or macrophage presence (E). On the other hand, Aza administration led to less inflamed aneurysms reflected in reduced levels of active p-JNK (F).*P<0.04

DISCUSSION

In this study, we examined the effect of Aza on EC function and aneurysm development. Aza is a well-known immunosuppressive drug, yet not much is known about its cellular working mechanism. We reveal that 6-MP has an anti-inflammatory effect on endothelium, resulting in reduced monocyte adhesion. The underlying mechanism involves inhibition of Rac1, similarly as decreased Rac1-GTP levels have previously been observed in T-cells treated with 6-MP[23, 27]. In ECs, active Rac1 is essential to activate signal transduction via JNK and subsequent activation of c-Jun, a constituent of the pro-inflammatory transcription factor AP-1. We show decreased Rac-1 activation upon 6-MP treatment in ECs and a strong reduction of p-JNK and c-Jun activation. Since the Rac1-JNK signaling pathway is one of the main pro-inflammatory pathways downstream of the AngII-receptor type 1 and because JNK has been shown to play an important role in aneurysm development[24, 33-35], we performed aneurysm experiments using the AngII-mediated model. We demonstrate that Aza has a protective effect on *de novo* aneurysm formation in mice, where the incidence as well as aneurysm severity is reduced. Most meaningfully, Aza-treatment inhibited aneurysm progression in mice and mediated its effect through inhibition of macrophage infiltration and reduced JNK activation in the vessel wall. In human surgical AAA and cerebral aneurysm material, JNK levels are strikingly elevated[24, 36] and even correlate with increased size and rupture risk in the latter[37], suggesting that JNK inhibition could be a relevant target of intervention in the clinic. The protective effect of JNK inactivation can be explained by reducing the levels of the (active) proteases in the vessel wall and thus better preserved extracellular matrix[24, 38]. Along this line, we observe a reduction of MMP2 and -9 protein in the vessel wall and AAA of Aza-treated mice.

Furthermore, we show that the anti-inflammatory effect of 6-MP in ECs involves downregulation of expression of hallmark activation markers IL-12, CCL2/MCP1, CCL5/Rantes, and VCAM-1, which are involved in different steps of vascular inflammation. Cytokine IL-12 is secreted and can activate surrounding cells, chemokines CCL2 and CCL5 attracts specific leukocytes and VCAM-1 will capture leukocytes to penetrate the vessel wall, which are all downregulated by Aza. Chemokine CCL2 is one of the most prominent chemokines found in aneurysms, yet its function is inconsistent. In mice, MCP1 is important for the attraction of monocytes, which clearly aggravates aneurysm pathology, but it also plays a role in tissue repair, which alleviates aneurysm development[39-41]. Interestingly, the role of CCL5 is more apparent, since recently, Iida *et al* showed in two different AAA mouse models that inhibition of CCL5 prevented AAA development and progression[42]. In addition, a polymorphism has been described for the receptor of CCL5 (CCR5 Delta 32 deletion) that correlates with aneurysm formation in two out of three human studies[43-45], which suggests an active role for CCL5 in the human aneurysm.

Attraction and penetration of macrophages to sites of activated aorta is key to achieve continuous aortic expansion from as early as 1 day after AngII infusion up to 56 days[31, 46]. The markedly reduced leukocyte influx that is observed after Aza treatment in both mouse aneurysm experiments involves reduced endothelial activation, but may also partly be explained by a direct immunosuppressive effect of 6-MP on macrophages[22] and T-cells[23, 27]. This may also explain our reduced p-JNK found in the AAA upon Aza treatment. Since Rac1-mediated pro-inflammatory signaling cascades are not limited to ECs, macrophages or T-cells, it is likely that this pathway will also be inhibited by Aza in other cell types, including vascular smooth muscle cells and fibroblasts. Similarly, the effective chronic treatment of inflammatory bowel disease (IBD) patients with Aza[47-49], may involve Rac1 inhibition in

inflammatory cells as well as in gut endothelial, smooth muscle and epithelial cells. Other functions of Rac1, apart from JNK activation, will also be affected by Aza. In most cell types Rac1 stimulates actin polymerization resulting in rearrangement of the cytoskeleton, which is necessary to change cell shape when migrating or for cell adhesion[50], which we have not studied here.

We believe that the lack of success to translate anti-inflammatory drug regimes that are successful in mouse models to the clinic is in part due to the limited progression studies that have been performed in mice. An informative example is the antibiotic doxycycline that has been demonstrated to very effectively prevent aneurysm development in multiple aortic aneurysm mouse models[51-53], yet the first human clinical trial of 18 months doxycycline therapy versus placebo (NTR 1345, http://www.trialregister.nl/trialreg/index.asp) failed to show a beneficial effect of doxycycline therapy on aneurysm progression or repair[55]. This clinical observation is, however, in line with a recent progression study in mice, in which doxycycline did not show any effect on aneurysm progression[56], suggesting that aneurysm initiation and progression involve more and different processes. The mouse JNK study by Yoshimura *et al.* does comprise a progression study in which JNK-inhibitors indeed provoke aneurysm regression[24]. Here, we performed an aneurysm prevention and progression study, which both show beneficial effects on aneurysm severity. Analysis of the AAA in our progression study reveals decreased inflammation and protease activity presumably because of the decreased level of p-JNK. Yet, no decrease in AAA size was observed. Probably, the timeframe of 3 weeks Aza treatment was too short to expect a significant effect, when comparing it to the 6 weeks treatment in the $CaCl_2$ aneurysm model by Yoshimura *et al.*[24]. Obviously, aneurysm prevention is less difficult than inhibition of the growth of existing aneurysms, as can be observed in our AAA, whereas the challenge in humans is to cure existing aneurysms, involving vessel wall repair. In our view, the compound-specific impact of distinct anti-inflammatory drugs, not only on immune cells, but also on endothelial and smooth muscle cells, has been overlooked and needs to be assessed. We demonstrate here that Aza reduces vascular inflammation, at least in part via an anti-inflammatory function in ECs and thus inhibits aneurysm pathology. Although Aza has its side effects and may therefore not be the ideal drug for use in elderly patients with existing aneurysms, our data is highly relevant to eventually define the necessary characteristics of the ideal drug to control aneurysm growth in humans.

SIGNIFICANCE

Abdominal aortic aneurysm (AAA) formation is characterized by progressive degradation of the vessel wall by inflammation. Apart from vascular repair surgery when the aorta diameter becomes to large, there is no standardized pharmaceutical treatment available to stabilize AAA growth.
A candidate drug to treat AAA should reduce inflammation without having a detrimental effect on the vascular cell types within the aorta wall. The study presented here reports on the anti-inflammatory function of immunosuppressive drug Azathioprine (Aza) on endothelial cells via inhibition of GTPase Rac1 and thereby reducing downstream Jun terminal N-Kinase (JNK) signaling. Aza inhibited de novo AAA and progression of aneurysm development in the Angiotensin II mouse model. We show that Aza has the capacity to decrease activation in

other cell types than immune cells alone, which may explain its potency as anti-inflammatory drug in chronic inflammatory diseases of which AAA may be next.

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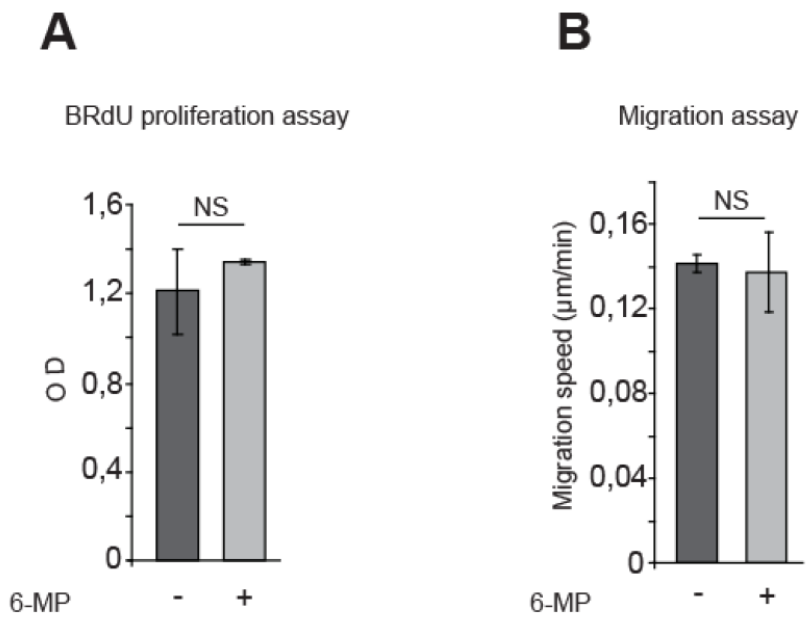
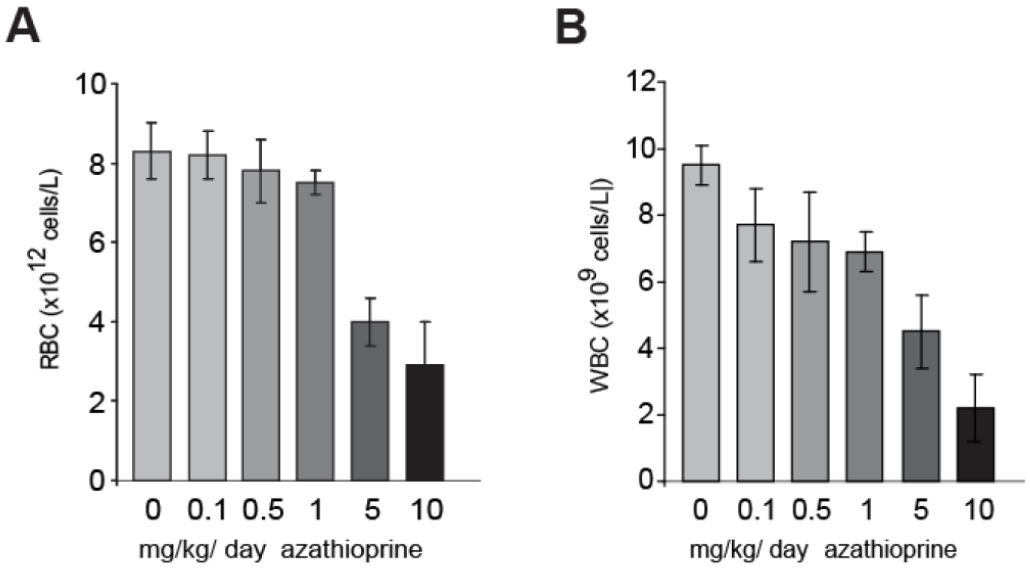
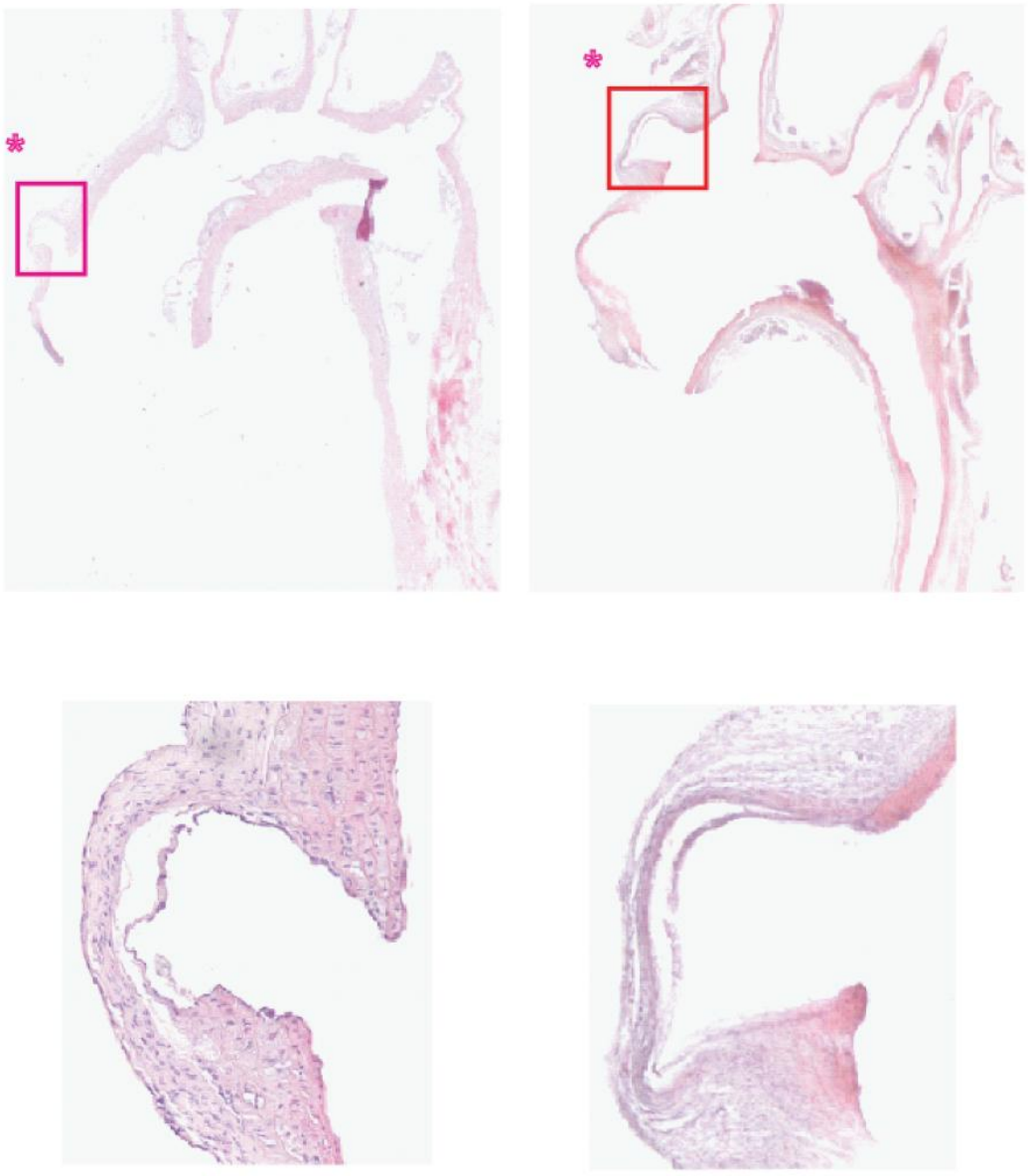
SUPPLEMENTARY INFORMATION



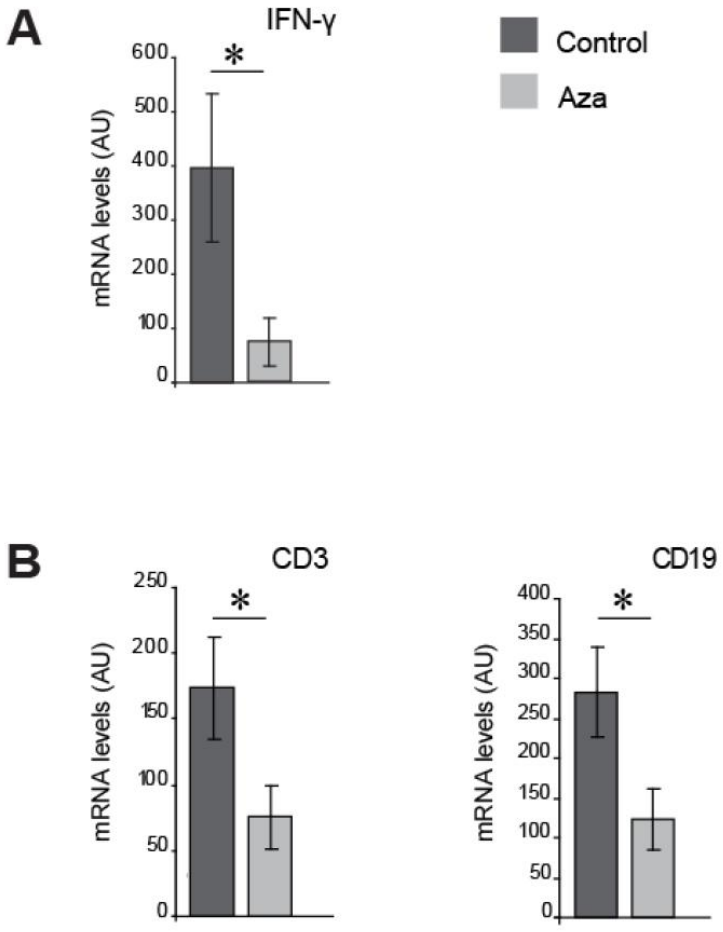
Supplemental Figure I. 6-MP does not interfere with basic cellular processes. 6-MP [10µM] does not influence proliferation (A) or migration capacity (B).



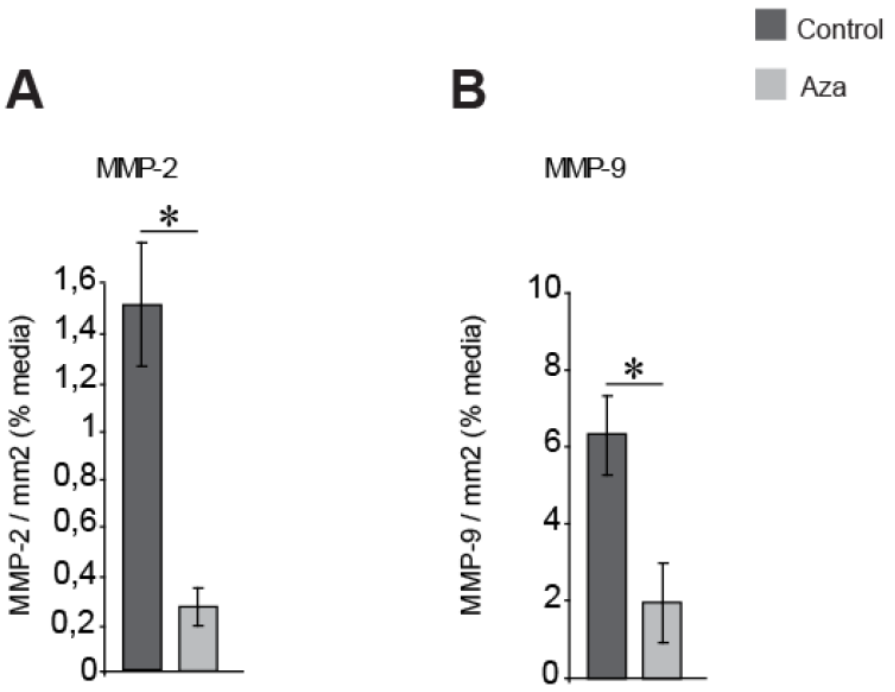
Supplemental Figure II. Aza dose finding study. Quantification of the number of red (A) and white (B) blood cells was used to determine an optimal dose of Aza in the aneurysm experiments. The first dosage which did not influence red blood cell counts was 0,1 mg/kg/day.



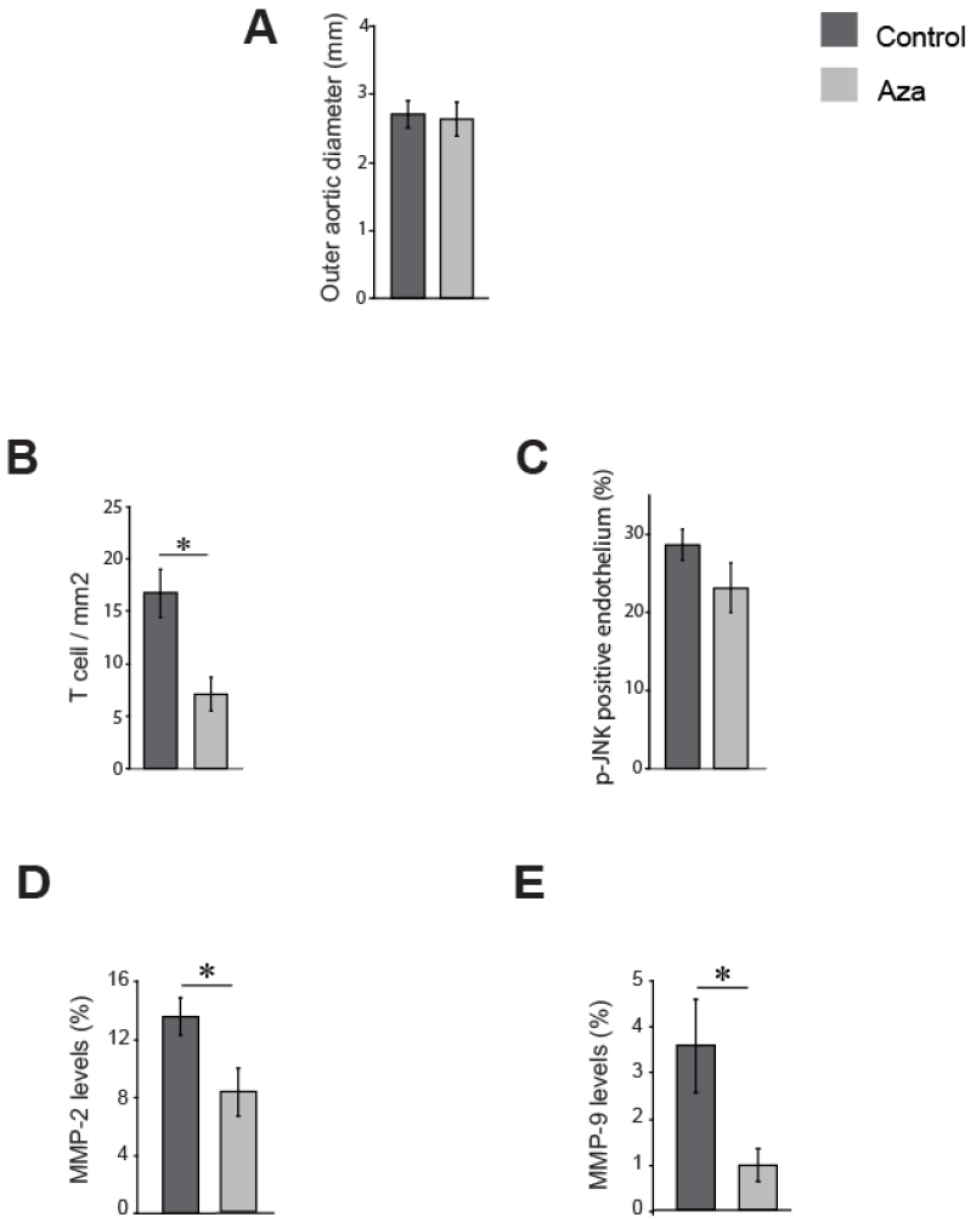
Supplemental Figure III. Histological overview of saccular aneurysms in the ascending aorta. Upper row: Common site of saccular aneurysm presence (box) after AngII treatment. Lower row: Enlargement of the box in the upper row reveals detailed histology of the saccular aneurysm, characterized by almost complete absence of the medial smooth muscle cell layer.



Supplemental Figure IV. Aza reduces systemic inflammation reflected by reduced mRNA levels of IFN-gamma (A, *P<0.03), T cell marker CD3 (B, *P<0.04) and B cell marker CD19 (C, *P<0.03) in thoracic lymph nodes.



Supplemental Figure V. Aza reduces production of Matrix-metalloproteinases (MMP). Protein expression of MMP-2 (A, *P<0.008) and MMP-9 (B, *P<0.009) was measured in the vessel wall, and is significantly reduced in Aza treated mice. Positive MMP surface area is expressed as percentage of total medial area.



Supplemental Figure VI. Application of Aza did not affect macroscopically measured outer aortic diameter of treated mice (A). Aza reduces infiltration of T cells into the aortic wall (B, *P<0.003). P-JNK activity was not alterd in endothelial cells within aneurysms upon Aza application (C). Aza did reduce production of MMP-2 and MMP-9 (D,E, respectively, *P<0.03)