Immunosuppressive Drug Azathioprine Reduces Aneurysm Progression Through Inhibition of Rac1 and c-Jun-Terminal-N-Kinase in Endothelial Cells

Goran Marinković, Stijntje Hibender, Mark Hoogenboezem, Amber van Broekhoven, Arginell F. Girigorie, Natascha Bleeker, Anouk A.J. Hamers, Jan Stap, Jaap D. van Buul, Carlie J.M. de Vries, Vivian de Waard

Objective—In aortic aneurysms the arterial vessel wall is dilated because of 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 (EC) activation with immunosuppressive drug azathioprine (Aza).

Approach and Results—Aza and its metabolite 6-mercaptopurine have anti-inflammatory effects on leukocytes. We here demonstrate that treatment of ECs with 6-mercaptopurine inhibits cell activation as illustrated by reduced expression of interleukin-12, CCL5, CCL2, and vascular cell adhesion molecule-1 and inhibition of monocyte–EC adhesion. The underlying mechanism of 6-mercaptopurine involves suppression of GTPase Rac1 activation, resulting in reduced phosphorylation of c-Jun-terminal-N-kinase and c-Jun. Subsequently, the effect of Aza was investigated in aneurysm formation in the angiotensin II 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 c-Jun-terminal-N-kinase activation in ECs and reduced leukocyte influx in the aortic wall.

Conclusions—The immunosuppressive drug Aza has an anti-inflammatory effect and in ECs inhibits Rac1 and c-Jun-terminal-N-kinase activation, which may explain the protective effect of Aza in aneurysm development and, most importantly for clinical implications, aneurysm severity. (Arterioscler Thromb Vasc Biol. 2013;33:2380-2388.)

Key Words: aneurysm ■ endothelium ■ inflammation

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. Because of decreased smoking habits in the Western society, the AAA incidence has declined recently from ≈5% to 2% of the elderly male population.1 However, as the number of elderly people in Western society increases, AAA remains a serious health problem. In addition, a larger number of elderly people will be diagnosed by coincidence with a small aneurysm because of the use of more sophisticated imaging techniques. Small aneurysms are screened regularly on discovery to monitor the growth rate. When the size exceeds 5.5 cm, open or endovascular repair surgery is the only form of treatment. To date, commonly used medication, such as angiotensin-converting enzyme 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 To date, many animal studies using anti-inflammatory drugs were successful in inhibition of initiation of aneurysm formation. For example, celecoxib, a nonsteroidal anti-inflammatory drug inhibiting cyclooxygenase-2, has been shown to prevent aneurysm formation 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.\textsuperscript{13} Furthermore, cyclosporine A has been described as a potent inhibitor of de novo aneurysm formation in mice,\textsuperscript{14} whereas hydrocortisone causes increased aortic rupture and aneurysm formation in mice.\textsuperscript{15} The inconclusive observations with immunosuppressive therapy to treat aneurysm progression in humans recently raised a relevant discussion.\textsuperscript{16} It seemed obvious that anti-inflammatory therapy would be beneficial to stop aneurysm progression; however, it does not seem that simple in clinical practice.\textsuperscript{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 patients with AAA 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,\textsuperscript{17} in contrast to sirolimus, which induces apoptosis, causing the thrombotic problems in sirolimus-coated coronary stents,\textsuperscript{18} or cyclosporine A that is toxic to ECs and provokes vasculopathy.\textsuperscript{19} We and others have previously shown that Aza maintains the contractile phenotype of smooth muscle cells\textsuperscript{20,21} and has an anti-inflammatory effect in macrophages\textsuperscript{22} and T cells.\textsuperscript{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-N-kinase (JNK) in ECs. JNK is a kinase with a pivotal role in aneurysm pathology.\textsuperscript{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**

Materials and Methods are available in the online-only Supplement.

**Results**

**6-MP Decreases EC Activation**

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

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

Because treatment of just the ECs revealed reduced monocyte adherence, we analyzed the effect of low-dose 6-MP on the expression of several characteristic cytokines and adhesion molecules in ECs. ECs were pretreated overnight with or without 6-MP and then incubated with or without TNF\(\alpha\) for 24 hours. TNF\(\alpha\) significantly induced mRNA expression of Rantes (CCL5), monocyte chemotactic protein-1 (CCL2), interleukin-12, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 (Figure 1B). 6-MP strongly inhibited expression of interleukin-12, CCL2, CCL5, and vascular cell adhesion molecule-1, whereas mRNA levels of intercellular adhesion molecule-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 Rac1 Activation and Rac1-Mediated Signaling**

In activated CD4+ T lymphocytes, it has been established that the small GTPase Rac1 is a target of Aza/6-MP, which...
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 to 4 mg Aza/kg per day. Therefore, apolipoprotein E–deficient mice were exposed to 10, 5, 1, and 0.1 mg Aza/kg per day, which was administered orally in a Western type diet. Circulating cell numbers were determined after 4 weeks of treatment, and we observed that only the low dose of 0.1 mg Aza/kg per day did not affect red blood cell counts and that these mice showed only limited reduction in white blood cell numbers (Figure II in the online-only Data Supplement). 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 1 mouse (1/13) in the Aza-treated group died because of aortic rupture within the first week of the experiment. After 4 weeks, aortas were isolated and revealed that aneurysm formation is strongly inhibited by Aza because only 17% (2/12) of the Aza-treated mice develop an aneurysm (Figure 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 with the Aza-treated mice (Figure 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 (Figure 4A, pink star; enlarged in Figure III in the online-only Data Supplement) 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 (Figure 4C; P<0.01).

Azathioprine Reduces JNK Activation in ECs 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. Application of Aza indeed results in decreased mRNA expression of interferon-γ in lymph nodes, as well as decreased T-cell (CD3) and B-cell (CD19) content, which represents the reduced inflammatory status of the mouse on Aza treatment (Figure IV in the online-only Data Supplement). To understand the protective effect of Aza on aneurysm formation, we aimed to study JNK activation and inflammation in the aortic vessel wall. Only 2 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. p-JNK is low in medial smooth muscle cells in these sections. However, p-JNK is abundantly present in ECs on AngII infusion and significantly decreased by Aza in endothelium lining the lumen of the aorta segment (Figure 4D). On average 64% of the ECs show p-JNK in AngII-infused mice, whereas only 47% are p-JNK positive in the Aza-treated AngII mice (P<0.0004).

Figure 2. 6-Mercaptopurine (MP) reduces activation of Rac1 in endothelial cells (ECs). Western blot showing pull down of active GTP-bound Rac1, revealing increased active Rac1 in response to tumor necrosis factor-α (TNFα) stimulation and a lower level of active Rac1 on 6-MP pretreatment. Levels of active Rac1 are also reduced only on 6-MP. Quantification of active Rac1 was normalized for total Rac1. Intercellular adhesion molecule-1 (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).
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 (Figure 4E and 4F). It is of importance to mention that in the AngII model, there is fibrolast hypertrophy, which causes thickening of the adventitia, which was not significantly different between control and Aza-treated aortas, with regard to the aortic ring sections that were quantified here (data not shown); therefore, the decreased inflammation on Aza was not just a reflection of adventitial area. In line with reduced leukocyte recruitment to the vessel wall, the expression of matrix metalloproteinase (MMP) 2 and MMP9 protein is also reduced after Aza treatment (Figure V in the online-only Data Supplement). We conclude that Aza reduces endothelial JNK activation, recruitment of inflammatory cells to the aortic vessel wall, and attenuates protease expression.

**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 1 week. 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 1 day before harvest (day 30) because of immobility caused by 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 types II and III, whereas Aza-treated mice develop predominantly type I and II aorta pathology (Figure 5A and 5B). Aza attenuates the progression of aneurysm formation, resulting in a significantly lower severity score of 1.8 compared with 2.7 in controls (Figure 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 with 47% in the delayed Aza-treated aortas (Figure 5D; P<0.0001). Decreased EC activation is reflected by decreased inflammation in the vessel wall. In the control mice, 1 saccular aneurysm is detected in the outer wall of the ascending aorta (Figure 5A, pink star). 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 time points; however, we find it in 9 of 10 mice after 31 days of AngII infusion at this location. Interestingly, the Aza-treated mice

**Figure 3.** 6-Mercaptopurine (MP) reduces activation of the Rac1-dependent c-Jun-terminal-N-kinase (JNK) signaling pathway. A, Tumor necrosis factor-α (TNFα)-activated endothelial cells (ECs) show reduced levels of JNK phosphorylation (p-JNK) in the presence of either 6-MP or Rac1 inhibitor. B, Quantification of active p-JNK is normalized for GAPDH (n=5). The amount of active p-JNK increases on 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 transcriprional activity in the presence of either 6-MP or Rac1 inhibitor (n=3; *P<0.01). D, A model of 6-MP interference in the JNK signaling cascade. 6-MP inhibits Rac1 activation and thus subsequent phosphorylation of JNK, which results in decreased c-Jun activation, which together with transcription factor c-Fos forms activator protein-1 (AP-1) transcription factor. Consequently transcription of downstream genes is inhibited, resulting in a decreased inflammatory response. OD indicates optical density.
show a strongly reduced macrophage influx into the media (Figure 5E), demonstrating the anti-inflammatory effect of Aza treatment, even when the onset of treatment was delayed in the disease process.

**Azathioprine Inhibits Inflammation Within AAA**

Although 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 because all mice (n=10 per group) had developed an AAA. The outer (maximal) diameter is measured macroscopically (Figure VIA in the online-only Data Supplement) and microscopically (Figure 6A) and is equal in control and Aza-treated mice, revealing that both methods are valid to determine aneurysm size. Also, the lumenal maximal diameter is measured and did not differ significantly between the groups. In addition, the matrix components collagen (Figure 6B) and elastin (Figure 6C) colocalize mostly with the smooth muscle cell area (Figure 6D) but are similar between the 2 groups. Although the macrophages show only a modest trend toward a decrease in the Aza-treated group (Figure 6E), the T-cell (Figure VIB in the online-only Data Supplement) 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 ECs is not different within the AAA (Figure VIC in the online-only Data Supplement), but a significant decrease in total AAA p-JNK is observed in the Aza group (Figure 6F). p-JNK is abundantly present in different cell types within the AAA and overlapped, in part, with macrophage positive area (compare Figure 6E and 6F). As a readout of the inflammatory state of the AAA, we analyzed proteases MMP2- and MMP9-positive area. Indeed, MMP2 and MMP9 area is significantly lower in the Aza-treated mice (Figure VID and VIE in the online-only Data Supplement).

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.

**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. In ECs, active Rac1 is essential to activate signal transduction via JNK and subsequent activation of c-Jun, a constituent of the proinflammatory transcription factor activator protein-1. We show decreased Rac1 activation on 6-MP treatment in ECs and a strong reduction of p-JNK and c-Jun activation. Because the Rac1-JNK signaling pathway is one of the main proinflammatory pathways downstream of the AngII receptor type 1 and because JNK has been shown to play an important role in aneurysm development, 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 and aneurysm severity are 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 and even correlate with increased size and rupture risk in the latter, 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. Along this line, we observe a reduction of MMP2 and MMP9 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 interleukin-12, CCL2/MCP1, CCL5/Rantes, and vascular cell adhesion molecule-1, which are involved in different steps of vascular inflammation. Cytokine interleukin-12 is secreted and can activate surrounding cells, chemokines CCL2 and CCL5 attract specific leukocytes, and vascular cell adhesion molecule-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. Interestingly, the role of CCL5 is more apparent because recently, Iida et al showed in 2 different AAA mouse models that inhibition of CCL5 prevented AAA development and progression. In addition, a polymorphism has been described for the receptor of CCL5 (CCR5 Δ32 deletion) that correlates with aneurysm formation in 2 of 3 human studies, which suggests an active role for CCL5 in the human aneurysm.
Attraction and penetration of macrophages to sites of activated aorta are key to achieve continuous aortic expansion from as early as 1 day after AngII infusion ≤56 days.10,29 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 macrophages22 and T cells.23,25 This may also explain our reduced p-JNK found in the AAA on Aza treatment. Because Rac1-mediated proinflammatory 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 patients with inflammatory bowel disease with Aza43–45 may involve Rac1 inhibition in inflammatory cells and 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 that is necessary to change cell shape when migrating or for cell adhesion,46 which we have not studied here.

We think that the lack of success to translate anti-inflammatory drug regimes that are successful in mouse models to the clinic is, in part, because of the limited progression studies that have been performed in mice. An informative example is the antibiotic doxycycline that has been demonstrated to effectively prevent aneurysm development in multiple aortic aneurysm mouse models,47–50 yet the first human clinical trial of 18-month 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. 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, suggesting that aneurysm initiation and progression involve more and different processes. The mouse JNK study by Yoshimura et al.24 does comprise a progression study in which JNK inhibitors indeed provoke aneurysm regression. 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-week Aza treatment was too short to expect a significant effect, when comparing it with the 6-week treatment in the CaCl2 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 are highly relevant to eventually define the necessary characteristics of the ideal drug to control aneurysm growth in humans.

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Disclosures

None.

References

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 large, there is no standardized pharmacological 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 on endothelial cells via inhibition of GTPase Rac1 and thereby reducing downstream Jun-N-terminal-Kinase signaling. Azathioprine inhibited de novo AAA and progression of aneurysm development in the angiotensin II mouse model. We show that azathioprine 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.

Significance
Immunosuppressive Drug Azathioprine Reduces Aneurysm Progression Through Inhibition of Rac1 and c-Jun-Terminal-N-Kinase in Endothelial Cells

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MATERIALS AND METHODS

Expanded 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 2x10³ 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 1-2x10⁵ 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 2x10⁵ 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

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**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 beads1. 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; Immobilon™-P). Following blocking 1 h 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 Active 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\(^2\). 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 determined 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

**References:**


Supplemental Figure I. 6-MP does not interfere with basic cellular processes. 6-MP [10 μM] 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 altered 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)