Thiopurines and inhibition of Rac1 in vascular disease
Marinkovi, G.

Link to publication

Citation for published version (APA):
Marinkovi, G. (2015). Thiopurines and inhibition of Rac1 in vascular disease Belgrade: Don-Vas

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Inhibition of GTPase Rac1 in Endothelium by 6-Mercaptopurine results in Immunosuppression in non-Immune Cells; New target for an old drug

Goran Marinković¹, Jeffrey Kroon², Mark Hoogenboezem², Kees A. Hoeben³, Matthijs S. Ruiter¹, Kondababu Kurakula¹, Iker Otermin Rubio¹, Mariska Vos¹, Carlie J.M. de Vries¹, Jaap D. van Buul², Vivian de Waard¹

1. Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, The Netherlands
2. Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, The Netherlands
3. Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands

J Immunol. 2014 May 1;192(9):4370-8
ABSTRACT

Azathioprine and its metabolite 6-mercaptopurine (6-MP) are well-established immunosuppressive drugs. Common understanding of their immunosuppressive properties is largely limited to immune cells. However, here we study the mechanism underlying the protective role of 6-MP in endothelial cell activation. Since 6-MP and its derivative 6-thioguanosine-5'-triphosphate (6-T-GTP) were shown to block activation of GTPase Rac1 in T-lymphocytes, we focused on Rac1-mediated processes in endothelial cells. Indeed, 6-MP and 6-T-GTP decreased Rac1 activation in endothelial cells. As a result, the compounds inhibited tumor necrosis factor–alpha (TNFα)-induced downstream signaling via c-Jun-N-terminal Kinase (JNK) and reduced activation of transcription factors c-Jun, Activating Transcription Factor-2 (ATF2), and additionally Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB), which led to decreased transcription of pro-inflammatory cytokines. Moreover, 6-MP and 6-T-GTP selectively decreased TNFα-induced vascular cell adhesion molecule-1 (VCAM-1), but not intracellular adhesion molecule-1 (ICAM-1) protein levels. Rac1-mediated generation of cell membrane protrusions, that form docking structures to capture leukocytes, were also reduced by 6-MP/6-T-GTP. Consequently, leukocyte transmigration was inhibited after 6-MP/6-T-GTP treatment. These data underscore the anti-inflammatory effect of 6-MP and 6-T-GTP on endothelial cells, by blocking Rac1 activation. Our data provide mechanistic insight that supports development of novel Rac1-specific therapeutic approaches against chronic inflammatory diseases.
INTRODUCTION

Transmigration of leukocytes through the endothelial barrier is a crucial event in tissue inflammation, which results in a local immune response that can either promote tissue repair or lead to tissue damage depending on the type of immune cells that transmigrate. In chronic inflammatory conditions where the excessive immune response harms tissue repair, patients receive immunosuppressive drugs to halt disease progression. Azathioprine is one of the oldest immunosuppressive drugs used in the clinic, with an established reputation, yet its working mechanism is not extensively studied. Azathioprine and its metabolite 6-mercaptopurine (6-MP) are used for treatment of various autoimmune and chronic inflammatory diseases\(^1\), such as rheumatoid arthritis\(^2\), inflammatory bowel disease (IBD)\(^{[Escher, 2003] [id;Louis, 2003] 33 [id]}\), as well as after kidney transplantations\(^5\) or in acute lymphoblastic leukemia\(^6\). Both azathioprine and 6-MP are biologically inactive prodrugs that demand intracellular enzymatic conversion. Azathioprine is converted to 6-MP, which is converted to 6-thioguanosine monophosphate (6-T-IMP), of which either the purine analogues 6-thioguanosine-5’-monophosphate (6-T-GMP) or 6-thioadenosine-5’-monophosphate (6-T-AMP) and downstream thio-purines can be synthesized\(^7\). Thereby, normal purine synthesis is hampered and incorporation of these alternative purines into newly synthesized DNA has long been the proposed therapeutic mechanism\(^7\). This effect is well described by the ability of 6-MP to prevent T-cell proliferation, relevant in a severe inflammatory condition with rapid T-cell expansion\(^8\). This effect is observed at a relatively high dose of 6-MP\(^8\), beyond clinically relevant dosages used chronically for IBD patients. In approximately 50% of all patients with IBD, a low dose of azathioprine or 6-MP is given and well tolerated without major complications\(^9\). This suggests that there may be a more subtle mechanism involved.

Indeed, in the previous decade a new role for 6-T-GTP has been proposed, which involves small Rho-GTPase Rac1 that cycles between an active and inactive conformation. A GTPase is activated when guanosine-5’-diphosphate (GDP) is exchanged by guanosine-5’-triphosphate (GTP). This process is catalyzed by guanine-nucleotide exchange factors (GEFs). Upon activation, the GTP-bound form of Rac1 can be hydrolyzed to Rac1-GDP, thereby becoming inactive. This reaction is regulated through GTPase-activating proteins (GAPs). It has been shown that 6-MP and its metabolite 6-T-GTP can target Rac1 in CD4\(^+\) T-cells, blocking T-cell activation\(^10\). Interestingly, 6-T-GTP can also bind other small Rho-GTPases such as Cdc42, RhoA and Rac2, yet, it can only block the activity of Rac1 and Rac2\(^11\). The GEF Vav2 is unable to exchange 6-T-GDP, preventing re-activation of Rac1. These data indicate that 6-T-GDP may irreversibly inhibit Rac1, including its downstream signaling\(^11\).

Migration of leukocytes through the vascular wall into injured tissues is a multi-step process\(^12,13\). A crucial phase is adhesion to inflamed endothelium. Adhesion initiates the formation of apical cell membrane protrusions on endothelial cells, which are called docking structures or transmigratory cups. These structures comprise cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1)\(^{[Escher, 2003] [id;Louis, 2003] 33 [id]}\). Rac1 is essential for the formation of these docking structures in a VCAM-1 and ICAM-1 dependent manner\(^17,18\).

In this study we demonstrate that 6-MP and 6-T-GTP exhibit an anti-inflammatory effect on endothelial cells via inhibition of Rac1 and thus attenuate downstream signal transduction, resulting in downregulated transcription of pro-inflammatory proteins as well as preventing actin polymerization in docking structure formation.
MATERIALS AND METHODS

Human endothelial cell culture
Primary human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to standard protocol. Briefly, HUVECs were isolated and cultured in M199 medium with 20% fetal bovine serum (FCS; Invitrogen), penicillin/streptomycin (P/S; 100 U/ml) and addition of heparin (0.05μg/ml), L-glutamin (2mM) and endothelial cell growth-supplement (ECGS; 25μg/ml). All experiments were performed in passage 1-3. One day prior to treatment of HUVECs with 6-MP, 6-T-GTP or Rac-1 inhibitor, medium was changed to M199-medium without purine-based compounds adenosine-sulphate, ATP-disodium salt, guanine-hydrochloride (Gibco). HUVECs were stimulated for 24h with tumor necrosis factor alpha (TNFa) [10ng/ml] to mimic inflammation with or without overnight pretreatment with 6-MP [10μM], 6-T-GTP [10μM] or Rac1 inhibitor (ITX-3; 100μM).

Monocyte-Endothelial cell co-culture
To study the effect of azathioprine on monocyte adhesion to endothelial cells, HUVECs were pretreated or not with 6-MP and activated with TNFa. Human monocyctic THP-1 cells were cultured in RPMI1640 medium, supplemented with 10% FCS and P/S. THP-1 cells were added to a confluent monolayer of endothelial cells at a concentration of 2x10^5 cells/ml and incubated for 4h. Prior to co-culturing, the THP-1 cells were fluorescently labeled using the Cell Trace™ CFSE cell Proliferation Kit (Invitrogen), to visualize cell adhesion. After 4h co-culturing, cells were washed and adhesion was analyzed by fluorescent microscopy (Zeiss, Axiovert 40 CFL). Fluorescent cells were counted per field of view (3 fields per well, 3 wells per condition).
For RNA isolation a similar experiment was performed. After 4h of co-culturing, all cells attached to the well (endothelium and THP1 together) were harvested after 4h of co-culturing for RNA isolation. Expression of CD11b was determined as readout of the number of adhered monocytes.

Rac1 and RhoA activation assays
Levels of Rac1-GTP and RhoA-GTP were measured using colorimetric based G-LISA Rac1 (BK128; Cytoskeleton) and RhoA (BK124; Cytoskeleton) activation assays. For these assays, HUVECs were plated in 6-well plates and cultured to 100% confluency followed by the switch to M199 medium without purine-based compounds. HUVECs were then treated with 6-MP, 6-T-GTP or ITX-3 and subsequently activated or not 24h with TNFa. Cell lysates were prepared and the assay performed following the protocols provided by the G-LISA kit manufacturer. Optical density (OD;490 nm) was measured with an EL808 Ultra Microplate Reader (Bio-Tek Instruments Inc.). Absorbance units in each sample were expressed after subtraction of the background units measured in protein-free lysis buffer.

Constitutively active Rac1Q61L mutant overexpression in HUVECs
HUVEC cells were grown in M199 medium (Gibco) supplemented with 20% FCS and P/S. Cells (8x10^5) were infected with the Adenovirus containing the constitutively active mutant Rac1Q61L construct or empty (mock) construct. This Rac1 mutant has a glutamine to leucine substitution at residue 61 (Rac1Q61L), stabilizing the protein in the active state independent of GTP. Multiplicity of infection (MOI) of 100 was used. Empty adenovirus carrying GFP was used as internal control for estimation of the success rate of the infection.
Medium was changed 24h after infection and the estimated infection rate was approximately 80-90%. After 6h, the compounds 6-MP, 6-T-GTP or ITX-3 was added to the cells. After 24h of TNFα stimulation, cells were lysed and Rac1 activity was measured.

Constitutively active Rac-1Q61L mutant overexpression in HEK293 cells
HEK-293 were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FCS and P/S. Cells were transfected with the mutant Rac1Q61L construct according to the manufacturer's protocol with Fugene HD® reagent (Promega). Two days later, both transfected and non-transfected cells were treated either with 6-MP or 6-T-GTP or Rac-1 inhibitor, left overnight and subsequently treated or not with TNFα for the next 24h. Thereafter, cells were lysed and Rac-1 activity measurement was performed using G-LISA Rac-1 activation kit according to manufacturer's instructions.

Electric cell-substrate impedance sensing (ECIS)
Permeability of endothelial monolayer was determined by measuring the electrical resistance using ECIS. Electrode arrays (8W10E; Applied BioPhysics) were pretreated with 10mM L-cysteine (Sigma-Aldrich) for 15 min at 37°C after which they were washed with 0.9% NaCl and coated with fibronectin (Sigma-Aldrich) for 1h at 37°C. Cells were seeded at 100.000 cells per well and grown to confluency. Electrical resistance was continuously measured at 37°C with ECIS Model 9600 Controller (Applied BioPhysics). Increase or decrease of the resistance is measured to monitor when the cells have a resistance above 1000 ohm, indicating that the cells have formed a confluent monolayer. At this point 6-MP, 6-T-GTP or ITX-3 are added for overnight incubation. The next day, resistance measurements were started in real time and after 4h, TNFα was added.

Transcription factor activity assays
Transcriptional activity of c-Jun, ATF-2 and NFκB was measured using the TransAM™ Transcription Factor Assay Kits (Active Motif). Nuclear extracts were prepared according to manufacturers’ instructions. DNA binding activity of the transcription factors was measured in an ELISA setup. Optical density was measured at 450nm.

Gene expression
RNA was isolated from cultured cells using the Aurum™ Total RNA Mini Kit (BioRad) and cDNA was generated by reverse transcription of 200ng RNA with the iScript cDNA synthesis kit (BioRad). Real-time PCR was performed on cDNA samples using SYBR Green Supermix (BioRad) and specific forward and reverse primers, in an iCycler thermal cycler system (BioRad). The primer sequences are as listed in Table I. After amplification, mRNA levels were normalized for the average value of two housekeeping genes; namely large ribosomal phosphoprotein P0 and hypoxanthine-guanine phosphoribosyltransferase.
Table I - List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD11b</strong> Homo Sapiens</td>
<td>5'-CAGCAGCAGCCAGACAGACAG-3'</td>
<td>5'-GAGGTTCCCGAAAAGAGCAGACATTG-3'</td>
</tr>
<tr>
<td><strong>IL-1</strong> Homo Sapiens</td>
<td>5'-TGGCAGAAAGGAACAGAAAGG-3'</td>
<td>5'-GTGAGTAGGAGAGGTGAGGAGG-3'</td>
</tr>
<tr>
<td><strong>IL-6</strong> Homo sapiens</td>
<td>5'-CGCCTTCGTCGAGTTG-3'</td>
<td>5'-TCGTTCTGAAGAGGTGAGT-3'</td>
</tr>
<tr>
<td><strong>IL-8</strong> Homo sapiens</td>
<td>5'-TGTCACATGTCCTCACACATC-3'</td>
<td>5'-TGCTTCCACATGTCCTCACACATC-3'</td>
</tr>
<tr>
<td><strong>IP-10</strong> Homo Sapiens</td>
<td>5'-AGCAGAGGAACCCAGCT-3'</td>
<td>5'-ATGCAGGTACAGCTACAGT-3'</td>
</tr>
<tr>
<td><strong>HPRT</strong> Homo Sapiens</td>
<td>5'-TGACACTGGCAAACATCGCA-3'</td>
<td>5'-GGTCTCTTCAGCAGAAGCA-3'</td>
</tr>
<tr>
<td><strong>PO</strong> Homo Sapiens</td>
<td>5'-TCGACAATGGCAGCATCTAC-3'</td>
<td>5'-ATCCGTCTCCACAGCAAAGG-3'</td>
</tr>
</tbody>
</table>

Western blot analysis
HUVECs were pretreated with 6-MP, 6-T-GTP or ITX-3. The next day the HUVECs were activated for 15min (for p-JNK/JNK) or 24h (for VCAM-1/ICAM-1) with TNFα. The cells were washed using ice-cold PBS and lysed in NP-40 lysis buffer containing Complete Protease Inhibitor Mix (EDTA-free; Roche). Lysates were made by 20 min incubation at 4 °C, after which insoluble material was removed by centrifugation. The resulting supernatant was used for SDS-PAGE. Between 40 and 60μg of total protein was loaded on gel and blotted to an Immobilon-FL Transfer Membrane (Millipore) by a TransBlot Turbo™ transfer system (BioRad). Membranes were stained overnight using antibodies specific for phosphorylated JNK (p-SAPK/JNK Rabbit mAb; Cell Signaling), JNK (JNK1 2C6 Mouse mAb; Cell Signaling), VCAM-1 (C-19, Santa Cruz), ICAM-1 (Rabbit polyclonal, Santa Cruz) and beta actin (Rabbit polyclonal, Cell Signaling). For detection, IRDye-tagged secondary antibodies (LI-COR Biotechnology) were used. Analysis and quantification was performed on an Odyssey Infrared Imaging system (LI-COR). Values were corrected for beta-actin.

Antibody-coated beads
Polystyrene beads (3μm; Polysciences) were pretreated with 8% glutaraldehyde overnight, washed with PBS, and incubated with 300μg/ml ICAM1 monoclonal antibody (R&D systems) to create ICAM-1-antibody coated beads to induce docking structure formation on endothelial cells.17

ICAM-1 crosslinking-dependent Rac1 activation
To analyze whether cross-linking of ICAM-1 can induce Rac1 activation and whether that activation can be dampened by 6-MP we cultured a confluent monolayer of HUVECs, pretreated with/without 6-MP overnight and activated the cells by TNFα for 4h. Thereafter, we added anti-ICAM-1 Ab coated beads (1:60 dilution). The beads were incubated for 30min, after which the medium with unattached beads was removed, cell were washed twice with ice-cold PBS and lysed to measure Rac1 activity as described above.
Apical docking structure quantification
Using confocal laser-scanning microscopy, Z-stacks were taken to reveal the formation of docking structures around beads. HUVECs were stained for F-actin with phalloidin (Invitrogen) and Vascular Endothelial (VE)-cadherin (BD Bioscience). The endothelial cell protrusions can reach 6μm above of the HUVEC apical surface. To quantify the protrusions, images were taken 2μm above the apical plane. When protrusions appeared as F-actin-positive rings, they were scored as docking structures. Differential interference contrast microscopy (DIC) was included to visualize the beads.

Scanning Electron Microscopy
HUVECs were grown on glass coverslips coated with fibronectin and treated with the experimental conditions. Fixation was performed in 2.5% glutaraldehyde/PBS for 20 min, and the cells were dehydrated in a graded ethanol series and hexamethyldisilazane. Samples were mounted on aluminium SEM specimen mount stubs, and sputter-coated with gold, using Balzers Union SCD040. Cells were examined in a scanning electron microscope (Phillips 525, Orion Frame Grabber), operated at 15kV with a spot size of 30nm. Scanning electron microscopy images were used to assess the maturation of the docking structures. A scoring system was acquired to quantify ICAM-1-mediated docking structures maturation, starting with: 0 = no visible interaction between the endothelial cell and the bead; 1= attachment of bead by endothelial cell "fingers”; 2= bottom of bead is covered by endothelial membrane; 3= up to 50% bead coverage by cell membrane; 4= >50% coverage of the bead (mature cup).

Neutrophil transendothelial migration across Transwell
A cell migration assay was performed using Transwell plates (Falcon, HTS Fluoroblock™ Insret) of 6.5mm diameter with 3μm pore-filters. HUVECs were seeded on fibronectin-coated Transwell filters (black) and cultured for two days, after which medium was changed to M199 without purine based compounds. The following day cells were treated or not with 6-MP, 6-T-GTP or ITX-3, and after 3h followed by activation with TNFα overnight. Neutrophils were freshly isolated from healthy volunteers using density gradient cell separation with Lymphoprep. Cells were labeled by green fluorescent Cell Trace™. Neutrophils (2x10⁵cells/well) were added to the upper compartment and were allowed to migrate to 100nM Formyl-Methionyl-Leucyl-Phenylalanine (fMLP, Sigma-Aldrich) placed in the lower chamber to create a chemotactic gradient. Immediately after neutrophil addition, the plate was placed in a pre-warmed NovaStar system and green fluorescent signal was measured in time in the lower compartment up to 45 min.

Neutrophil transendothelial migration under physiological flow conditions
HUVECS were cultured in a fibronectin-coated Ibidi μ-slide VI⁰.⁴ (Ibidi, München, Germany) for two days until confluency and subsequently changed to experimental medium, as described above. One day prior to the experiment the compounds 6-MP and 6-T-GTP (each 10μM) were added to the cells and the next day, cells were stimulated with TNFα (10 ng/ml) for 4h. Freshly isolated neutrophils were resuspended at 0.4 x10⁶ cells/ml in HEPES medium and were incubated for 20 minutes at 37 °C. Neutrophils were perfused over the HUVEC monolayers at 0.5 ml/min (correlates to shear stress of 0.9 dyn/cm²). Subsequently, HEPES medium was perfused for a minimum of 20 minutes. During this time, leukocyte-endothelial interactions were recorded in three random fields with a Zeiss Axiovert 200 microscope (10x
objective) equipped with a motorized stage. Images were recorded with Zeiss Zen 2012 software. Live imaging was performed at 37 °C and 5% CO$_2$. Upon flow, adhesion is measured 2 min after a single bolus injection of cells. Thereafter buffer was introduced and the number of transmigrated leukocytes is quantified after 15 min and calculated as percentage of adherent cells.

**Statistics**
For all experiments a Students t-test was performed. P-values ≤0.05 are considered significant. Data are represented as mean value ± SEM.
RESULTS

Inhibition of monocyte adhesion and Rac1 activation
Leukocyte recruitment and transmigration through the endothelial cell layer into the subjacent tissues is a crucial event in inflammatory diseases. We show that TNFα-treated endothelial cells, that were pre-incubated with 6-MP, bare reduced adhesion capacity of monocytes (Figure 1A,B). Azathioprine, 6-MP and the downstream metabolite 6-T-GTP have been shown to decrease Rac1 activation in T-cells\textsuperscript{11}, which could be the underlying mechanism in endothelial cells as well. Indeed, we recently revealed that TNFα-induced Rac1 activation is impaired when endothelial cells are pretreated with 6-MP, by pulldown of active Rac1\textsuperscript{19}. Here we show that pretreatment of the endothelial cells with 6-T-GTP also blocked TNFα-induced Rac1 activation to a similar extent as 6-MP and Rac1 inhibitor ITX-3, in an ELISA-like setup (Figure 1C). This Rac1 inhibitor is specific for guanine-nucleotide exchange factor (GEF) Trio, the most relevant Rac1 targeting GEF in endothelial cells\textsuperscript{20}. Clearly, Rac1 inhibition by 6-MP and 6-T-GTP is not reserved for T-cells only, and 6-T-GTP is presumably a Rac1-binding metabolite of azathioprine and 6-MP. Interestingly, pretreatment of endothelial cells with 6-MP and 6-T-GTP was not able to dampen the TNFα-induced activity of another small GTPase, namely RhoA (Figure 1C), pointing out specificity of these compounds for Rac1. We now further investigate the consequence of Rac1 inhibition in endothelial cells by 6-MP and 6-T-GTP.

GTP-dependent inhibition of Rac1 by 6-MP/6-T-GTP
To show that 6-MP and 6-T-GTP affect Rac1 via a GTP-dependent mechanism, adenoviral overexpression experiments were performed in endothelial cells with a Rac1-Q61L mutant construct. This mutant Rac1 is constitutively active and thus lacks the GTP-dependency, which is key to activate endogenous Rac1. In mock-infected endothelial cells, endogenous Rac1 can be induced by TNFα and inhibited again by 6-MP, 6-T-GTP and the Rac1 inhibitor (Figure 1D). Overexpression of constitutive active Rac1 in endothelial cells already shows high Rac1 activity without TNFα stimulation. Yet, Rac1 activity was equal in all conditions, indicating that 6-MP, 6-T-GTP and the Rac1 inhibitor were unable to block Rac1-Q61L activity (Figure1D). A similar experiment in HEK293 cells, transfected with the Rac1-Q61L mutant construct, gave equal results, showing that the Rac1 inhibitory function of 6-MP and 6-T-GTP are not unique to endothelial cells (Supplemental Figure I). The lack of Rac1-Q61L inhibition by the compounds demonstrates that 6-MP and 6-T-GTP inhibit endogenous Rac-1 via a GTP-dependent mechanism, which is absent in Rac1-Q61L protein.
**Figure 1. 6-MP inhibits monocyte adhesion to activated endothelium and reduces Rac-1 activity.**

(A) Fluorescently labeled THP1 monocytes show increased adherence to TNFα-activated HUVEC monolayers. Pre-incubation of HUVECs with 6-MP reduced the number of adherent THP1 cells (*P<0.0002). (B) 6-MP also reduces monocyte adhesion as determined by levels of mRNA for monocyte marker CD11b (*P<0.005). (C) 6-MP as well as its metabolite 6-T-GTP reduce Rac-1 activity in TNFα-activated HUVECs to a similar extent as Rac1 inhibitor ITX-3, but there is no effect on the activity of GTPase RhoA. (D) Upper panel; In HUVEC cells endogenous Rac-1 activity was increased after TNFα stimulation and markedly reduced by 6-MP, 6-T-GTP and Rac1-inhibitor (*P<0.03). Lower panel; Upon overexpression of constitutively active Rac-1 mutant (Q61L) in HUVECs, overall Rac1 activity was increased and could not be inhibited by the compounds.
The effect of 6-MP and 6-T-GTP on endothelial barrier function

Rac1 is known to influence endothelial barrier function and therefore we determined the effect of 6-MP and 6-T-GTP on endothelial cell monolayer integrity, using electric cell-substrate impedance sensing (ECIS). The electrical resistance of the endothelial monolayer is monitored in real time in the absence or presence of TNFα. The experiment started when the endothelial cells had formed a confluent monolayer, which is marked by a resistance above 1000Ω. The resistance was continuously increasing, even in the presence of the compounds (Supplemental Figure II). TNFα gave a rapid, but transient increase in resistance in all conditions. Where 6-T-GTP slightly decreased the resistance compared to control and 6-MP treated cells, they were all significantly higher than the Rac1 inhibitor-treated cells. Thus, 6-MP and 6-T-GTP behave differently than the Rac1 inhibitor, suggesting that these compounds also have other functions beyond Rac1 inhibition. Interestingly, all conditions remained above 1000Ω, suggesting that the reduction of Rac1 activity did not harm the endothelial monolayer integrity. Apparently, the concentrations that we use are not strong enough to induce a detrimental effect on endothelial cell barrier function.

Inhibition of signaling cascades by 6-MP/6-T-GTP

TNFα-induced Rac1 activation initiates downstream signaling, leading to phosphorylation of specific transcription factors, resulting in altered gene expression. Rac1 is best known for its involvement in the c-Jun N-terminal kinase (JNK)-mediated pathway \(^{21,22}\) (via activation of mitogen-activated protein kinase kinase 4/7 (MEKK 4/7) \(^{22,23}\)). Here, we study if suppression of Rac1 activity by 6-MP or 6-T-GTP will influence TNFα-induced downstream signaling. Both, 6-MP and 6-T-GTP efficiently perturb TNFα-induced JNK phosphorylation (p-JNK), like the Rac1 inhibitor (Figure 2A).

Downstream of activated JNK are the transcription factors c-Jun \(^{24-27}\) and activating transcription factor 2 (ATF2; also known as cAMP-dependent transcription factor) \(^{24,27,28}\). c-Jun usually forms heterodimers with transcription factor c-Fos and as such is a constituent of early response transcription factor complex AP-1 \(^{29,30}\). In addition, c-Jun forms heterodimers with ATF2 and then binds to the cAMP-response-element binding (CREB) site (CRE). Both 6-MP and 6-T-GTP reduce TNFα-induced transcriptional activation of c-Jun and ATF2, similar as the Rac1 inhibitor (Figure 2B,C).

Yet, another pro-inflammatory transcription factor that can be activated by TNFα, and regulated by Rac1 activation, is NFκB \(^{31}\). In addition to c-Jun and ATF2, TNFα-induced NF-κB activation is also significantly reduced by 6-MP, 6-T-GTP or the Rac1 inhibitor (Figure 2D). Interestingly, under baseline culture conditions (without TNFα), c-Jun and ATF2 are somewhat active, whereas NFκB is not. 6-MP can down-regulate c-Jun and ATF2 activity below the activity in baseline conditions (P<0.03 and P<0.0009, respectively). Together, these data indicate that both 6-MP and 6-T-GTP effectively suppress TNFα-induced gene transcription. We explore this further by measuring expression of hallmark pro-inflammatory endothelial cell genes.
Figure 2. 6-MP reduces activation of signaling pathways downstream of Rac-1.
(A) In HUVECs, increased (active) phosphorylated (p)-JNK is observed in response to TNFα stimulation, which is lowered upon 6-MP, 6-T-GTP and Rac-1 inhibitor pre-treatment. Quantification of p-JNK is shown in the right panel (n=3; *P<0.05). (B-D) The activity of the transcription factors was significantly reduced by 6-MP, 6-T-GTP and Rac1-inhibitor in TNFα treated HUVECs; c-Jun (*P<0.008), ATF2 (*P<0.002) and NFκB (*P<0.05).

A suppressed pro-inflammatory gene expression profile by 6-MP
Knowing that 6-MP inhibits TNFα-induced activation of the transcription factors c-Jun, ATF2 and NFκB, we analyzed gene expression of a subset of inflammatory markers to determine the effect of 6-MP on their expression profile. Previously, we have shown that 6-MP can potently reduce the mRNA expression level of pro-inflammatory cytokine interleukin (IL)-12 and chemokines C-C motif ligand (CCL)-2 and CCL5, also known as monocyte chemoattractant protein-1 (MCP1) and Rantes, respectively. Here, more cytokines and chemokines are studied, namely IL-1β, IL-6, IL-8, as well as C-X-C motif chemokine 10 (CXCL10) also known as interferon gamma-induced protein 10 (IP-10). In TNFα-stimulated endothelial cells that were pre-treated with 6-MP, IL-6, IL-8 and IP-10 are significantly down-regulated (Figure 3) in addition to IL12, CCL2 and CCL5 that we reported earlier. The fact that IL-1β mRNA expression is not suppressed, shows that 6-MP inhibits specific pro-
inflammatory pathways. In conclusion, a decreased inflammatory gene expression profile is observed upon 6-MP treatment, probably as a result of 6-MP-mediated transcriptional inactivation of c-Jun, ATF2 and NF-κB.

Figure 3. 6-MP decreases pro-inflammatory gene expression response.
6-MP modulates the pro-inflammatory gene expression response of HUVECs by suppressing mRNA production of cytokines IL-6, and chemokines IL-8 and IP-10 (*P<0.04). Expression of IL-1β is not affected.

Reduced VCAM-1 protein by 6-MP/6-T-GTP
After activation and attraction of leukocytes by cytokines and chemokines, these immune cells need to adhere to the endothelium to transmigrate into the injured tissue. To accomplish leukocyte adhesion, cell surface adhesion molecules are essential. Previously, we have demonstrated in TNFα-stimulated endothelial cells that 6-MP reduces the mRNA expression level of VCAM-1, but not of ICAM-119. Here, we show that 6-MP also suppresses VCAM-1 protein expression. Not only 6-MP, but also 6-T-GTP and the Rac1 inhibitor effectively prevent VCAM-1 protein upregulation by TNFα (Figure 4A), suggesting that 6-T-GTP is a potent purine analogue that is responsible for Rac1 blockade. Interestingly, while VCAM-1 protein levels are markedly abrogated, ICAM-1 protein levels are unaffected by 6-MP, 6-T-GTP or the Rac1 inhibitor (Figure 4B), again showing that 6-MP and 6-T-GTP inhibit specific inflammatory processes.
Figure 4. 6-MP/6-T-GTP decreases VCAM-1 protein.
(A) An increase in the amount of VCAM-1 protein in response to TNFα stimulation is observed, which is significantly reduced upon 6-MP, 6-T-GTP and Rac1-inhibitor pretreatment (*P<0.04). (B) ICAM-1 levels are significantly increased in the presence of TNFα, but are not changed in response to pretreatment with 6-MP, 6-T-GTP or Rac1-inhibitor.

6-MP/6-T-GTP inhibit ICAM-1 mediated docking structure formation
It has been established that cross linking of ICAM-1 or VCAM-1 results in activation of Rac1.17,32 Moreover, ICAM-1 or VCAM-1 clustering is essential in the formation of docking structures, required for proper leukocyte transendothelial migration, for which Rac1 is the molecular engine.14,16 Since 6-MP does not affect ICAM-1 protein levels, this allows us to study the effect of 6-MP on ICAM-1-mediated docking structure formation, also known as transmigratory cup formation. ICAM-1-induced docking structure formation was achieved with beads that were coated with anti-ICAM-1 antibodies, mimicking leukocytes. Endothelial cells were incubated with these beads to provoke Rac1 activation. F-actin and VE-cadherin staining visualizes the actin cytoskeleton and cell membrane, respectively. Using confocal laser-scanning microscopy, Z-stacks were taken to reveal the docking structures around the beads. Differential interference contrast microscopy (DIC) is included to visualize the beads. The endothelial cell protrusions around the beads reach approximately 6μm above the endothelial cell apical surface (Supplemental Figure III). To quantify these protrusions, images were taken at 2μm above the apical plane, where protrusions appear as F-actin-positive rings around a bead and were scored as docking structures (Supplemental Figure III). The number of attached beads, after washing, reveal that 6-MP significantly reduces adhesion of beads to the endothelial cells (Figure 5A,B).
This is in line with our observations that 6-MP reduces the binding of THP-1 monocytes to endothelial cells (Figure 1). In addition, our results show that 6-MP significantly prevents the
formation of ICAM-1-induced docking structures because a reduced number of captured beads (beads surrounded by an F-actin ring) is observed (Figure 5A,C).

In conclusion, even though 6-MP does not affect TNFα-induced ICAM-1 expression, ICAM-1 function is strongly inhibited by 6-MP, most likely through Rac1 inhibition resulting in decreased formation of docking structures that can capture anti-ICAM-1 antibody-coated beads.

ICAM-1-clustering mediated activation of Rac1
As it was previously reported that clustering of ICAM-1 results in Rac1 activation\textsuperscript{17,32}, and based on our findings that 6-MP impairs docking structure formation, it is important to verify whether 6-MP can actually reduce Rac1 activity induced by cross-linking of ICAM-1. In the absence of TNFα, addition of anti-ICAM-1 antibody-coated beads does not induce Rac1 activity (Supplemental Figure IV A). On the other hand, in the presence of TNFα, when also ICAM-1 protein production is induced (Figure 4B), there is induced Rac1 activation, which is significantly further increased by the anti-ICAM-1 antibody-coated beads (Supplemental Figure IV A). Activation of Rac1, could again be inhibited by 6-MP. From these data, it can be concluded that the reduction in docking structure formation surrounding the beads is the direct consequence of reduced Rac1 activity by 6-MP.
Figure 5. 6-MP inhibits ICAM-1 mediated docking structure formation.
(A) Anti-ICAM-1 antibody-coated beads were incubated with TNFα-activated HUVECs. F-actin and VE-cadherin staining was performed and confocal microscopy was performed at the basolateral (0μm) and apical (2μm) plane. Cell membranes at the basolateral plane are detected with VE-cadherin. Membrane protrusions that form the docking structures are visible as F-actin rings surrounding anti-ICAM-1-antibody-coated beads in the apical plane (see Supplemental Figure III for detailed scheme). To assess bead localization on the membrane surface Differential Interference Contrast (DIC) microscopy was performed. (B) Treatment of HUVECs with 6-MP prior to TNFα activation leads to a reduction in overall number of adherent beads (*P<0.02). (C) 6-MP treatment results also in a decrease in beads captured by a mature docking structure (*P<0.0001).
6-MP/6-T-GTP reduces docking structure maturation and neutrophil migration

In the experiments with anti-ICAM-1 antibody-coated beads mainly mature F-actin-rich docking structures can be detected. To visualize maturation of these transmigratory cups we performed scanning electron microscopy. Furthermore, we included 6-T-GTP and the Rac1 inhibitor in these experiments to confirm that the observed docking structure formation is Rac1-dependent. We introduce a scoring system to quantify docking structure maturation (induced by anti-ICAM-1 antibody-coated beads) by determining the amount of bead coverage by the endothelial cell membrane protrusions (Figure 6A). Similarly as observed for adhesion of beads to 6-MP-treated endothelium, the application of either 6-T-GTP or Rac1 inhibitor leads to a significant reduction in docking structure maturation (Figure 6B). Thus, 6-MP and 6-T-GTP prevent maturation of ICAM-1-induced transmigratory cups.

Transendothelial migration of monocytes is mediated through both VCAM-1 and ICAM-1, whereas neutrophils show exclusive ICAM-1 dependent transmigration across endothelial cells. Therefore, we examined whether reduced docking structure maturation by 6-MP or 6-T-GTP has functional implications on neutrophil adhesion and transmigration. Transwell migration assays were performed in which endothelial cells were pretreated with the 6-MP, 6-T-GTP or Rac1 inhibitor, and activated with TNFα as a pro-inflammatory stimulus to induce ICAM-1 expression. Analogous to the reduction in numbers of captured anti-ICAM-1 antibody coated beads, application of either 6-MP or 6-T-GTP or Rac1 inhibitor leads to a reduction in neutrophil migration through the confluent endothelial cell monolayer, confirming that indeed ICAM-1 mediated adhesion and transmigration is functionally diminished by inhibition of Rac1 (Supplemental Figure IV B). Still, it could be argued that due to decreased adhesion, there is decreased transmigration. In an attempt to discriminate between these two processes, we performed neutrophil transendothelial cell migration studies under flow conditions using in vitro imaging (Figure 6C). In this perfusion-based flow model, we quantified neutrophil adhesion and transmigration under physiological flow conditions. We previously described that Rac1 inhibition by blocking GEF Trio in endothelial cells decreased both adhesion and transmigration in this setup18. When using 6-MP or 6-T-GTP, we again observed a large reduction in neutrophil adhesion to endothelial cells, under flow. In addition, when studying the attached cells only, and then calculate the percentage of cells that actually transmigrate through the endothelial layer, the compounds also reduce the diapedesis capacity of the neutrophils. Thus both adhesion and transmigration is inhibited by 6-MP and 6-T-GTP.

Taken together, these results show that 6-MP disables Rac1-mediated signaling downstream from TNFα via 6-T-GTP, resulting in an impaired transcriptional inflammatory response and impaired cytoskeletal rearrangement in endothelial cells affecting leukocyte adhesion and transmigration (Fig 6D).
Figure 6. 6-MP/6-T-GTP inhibit transmigratory cup maturation and neutrophil transmigration.

(A) Scanning electron microscopy images illustrate the scoring system (explained in Methods section) used to determine the extent of transmigratory cup maturation. (B) 6-MP and 6-T-GTP reduce transmigratory cup maturation back to the baseline level of unstimulated endothelial cells, similar as the Rac1 inhibitor (*P<0.04). (C) 6-MP and 6-T-GTP functionally disable ICAM-1-dependent transmigration of neutrophils in a flow chamber; Upper panel: Number of adherent neutrophils per mm² after 2 min of flow (*P<0.05), Lower panel: The percentage of transmigrated neutrophils per mm² after 15 min of flow (*P<0.03). (D) A schematic overview of the mechanism by which azathioprine (Aza) and its metabolites inhibit Rac1 activation and the subsequent consequences are shown. Gene expression is influenced by inhibition of the NFκB and JNK signaling cascades. Decreased phosphorylation (P-) of JNK, c-Jun and ATF-2 results in reduced AP-1 and CRE dependent transcription. Rac1 inhibition also prevents cytoskeletal rearrangement to form transmigratory cups, to capture leukocytes.
DISCUSSION

We have recently shown that low dose azathioprine effectively prevents the chronic inflammatory aortic disease of abdominal aortic aneurysm formation in mice, by reducing endothelial JNK activation and macrophage infiltration of the vessel wall. To date, there is limited information on the exact cellular mechanism underlying the immunosuppressive properties of azathioprine in T cells and monocytes/macrophages. Poppe and colleagues demonstrated that azathioprine, and its metabolites 6-MP and 6-T-GTP have the ability to potently block the activity of Rac1 and Rac-2 in T cells. These observations are in line with our results, showing that treatment of endothelial cells with 6-MP or 6-T-GTP strongly reduces Rac1 activity, comparable with the inhibition by Rac1 inhibitor ITX-3, which is an inhibitor of GEF Trio, the most relevant Rac1 targeting GEF in endothelial cells. Moreover, Rac1 activity of constitutively active Rac1 mutant Q61L could not be inhibited by 6-MP, 6-T-GTP or Rac1 inhibitor, revealing that 6-MP and 6-T-GTP inhibit Rac1 in a GTP-dependent fashion.

In the present study, we demonstrate in depth that azathioprine-derived metabolites 6-MP and 6-T-GTP have the ability to diminish monocyte and neutrophil adhesion and transmigration, by inhibiting TNFα-induced Rac1-dependent pro-inflammatory signaling pathways, as well as ICAM-1-induced Rac1-mediated transmigratory cup formation. Both activation pathways, i.e. TNFα- and ICAM-1-mediated Rac1 activation, are believed to occur in parallel but with distinct kinetics during inflammation. ICAM-1-induced Rac1 activation occurs when leukocytes adhere to the endothelium and engage ICAM-1 through integrin-binding. This process also involves recruitment of actin-adapter proteins such as filamin. Actin remodeling, resulting in docking structure formation, is a prominent downstream effect from ICAM-1-induced Rac1 activation. We show here that 6-MP as well as its metabolite 6-T-GTP blocks the maturation of ICAM-1-induced docking structures, which are necessary to capture leukocytes.

In the TNFα signaling pathway, filamin is not involved although actin remodeling takes place. Rather, TNFα induces stress fiber formation and upregulation of essential adhesion molecules like ICAM-1 and VCAM-1. Clustering of these molecules has been shown to induce phosphorylation of VE-cadherin and as a consequence loss of VE-cadherin-mediated cell-cell contact. This in turn facilitates leukocyte migration. Pro-inflammatory stimuli, such as TNFα, lead to strong activation of Rac1, which in turn is a potent activator of the JNK signaling cascade. The Rac1-dependency of JNK activation was demonstrated by downregulation of Rac1 expression by an siRNA approach that led to decreased phosphorylation (activation) of JNK, which we also observe when using 6-MP or 6-T-GTP. A less well described Rac1-mediated signaling pathway is induing NFκB activation, and the Rac1-dependency is demonstrated in different cell types. We observe that 6-MP and 6-T-GTP effectively reduce transcriptional activation of JNK-dependent transcription factors c-Jun /ATF2, and of (JNK-independent) NFκB and their downstream inflammatory gene expression profiles. It should however be noted that some of the 6-MP effects may also be caused by “off target” effects on other GMP/GDP/GTP- or AMP/ADP/ATP-dependent pathways, whereas 6-T-GTP may influence alternative GTPases or GTP-dependent processes, which deserves more extensive investigation.

Previous studies have shown that azathioprine treatment of T cells leads to downregulation of inflammatory gene expression and here, where application of 6-MP reduces the expression level of most cytokines, chemokines and specifically VCAM-1 upon TNFα stimulation, which may be
attributed to reduced activity of the above mentioned transcription factors. Interestingly, although TNFα-induced VCAM-1 expression was effectively inhibited by 6-MP, ICAM-1 expression was not changed neither at mRNA\textsuperscript{19} nor at protein level. Since both adhesion molecules have NFκB and AP-1 sites in their promoter\textsuperscript{44-46}, there must be additional transcription factors involved that determine ICAM-1 transcription, since the 6-MP- and 6-T-GTP-mediated decrease in NFκB and AP-1 does not reduce ICAM-1, but is sufficient to reduce VCAM-1. Indeed, in TNFα-induced activation of the ICAM-1 promoter the AP-1 sites are not essential\textsuperscript{46}.

The increase in expression of ICAM-1 in response to TNFα is stable in the presence of 6-MP or 6-T-GTP, and thus provided the opportunity to study ICAM-1 functionality on the cell membrane. ICAM-1 ligation, induced by anti-ICAM-1 antibody-coated beads, resulted in Rac1-dependent transmigratory cup formation, which was inhibited by 6-MP and 6-T-GTP. Since neutrophils are solely dependent on ICAM-1 for transmigration, we assessed the biological relevance of diminished ICAM-1 functionality, by showing that neutrophils transmigrate less over an endothelial cell monolayer in the presence of 6-MP or 6-T-GTP.

Taken together, our data suggest that azathioprine inhibits adhesion and transmigration of leukocytes through the endothelial barrier in three ways. First, endothelial cells are less activated and therefore do not contribute to leukocyte attraction and activation. Second, VCAM-1 mRNA and protein expression is inhibited, leading to reduced VCAM-1-mediated adhesion of predominantly monocytes, which are more dependent on VCAM-1. Third, ICAM-1-mediated adhesion is reduced due to inhibition of docking structure maturation, which predominantly affects neutrophil adhesion and migration.

Our results demonstrate that low dose 6-MP and 6-T-GTP have an intricate mechanism of action, which goes beyond its described effect on incorporation of purine antagonists that block DNA synthesis as observed in immune cells in response to high dose azathioprine or 6-MP. Part of the anti-inflammatory effect of azathioprine can now be attributed to inhibition of endothelial cell Rac1 activity. In conclusion, this implicates that Rac1 inhibition has potential to block excessive leukocyte invasion in inflammatory diseases. Interestingly, one of the pleiotropic anti-inflammatory effects of statins to protect against cardiovascular disease is also via inhibition of endothelial cell Rac1 activity\textsuperscript{47}. Since the effect of 6-MP and 6-T-GTP is presumably not limited to T cells, monocytes/macrophages and endothelial cells, the Rac1 pathways in other cell types are probably also affected and in part responsible for reduced tissue inflammation when azathioprine is used in a clinical setting. This study provides insight into the crucial role of Rac1 in inflammatory responses of endothelial cells, revealing this signaling molecule as a valid target for future pharmaceutical approaches to treat tissue inflammation.

**Sources of Funding:**
This research forms part of the Project P1.02 NEXTREAM of the research program of the BioMedical Materials Institute, co-funded by the Dutch Ministry of Economic Affairs. The financial contribution of the Netherlands Heart Foundation is gratefully acknowledged.
CHAPTER 4

Reference List


Supplemental figure I. Upper panel; In HEK293 cells endogenous Rac-1 activity was increased after TNFα stimulation and markedly reduced by 6-MP, 6-T-GTP and Rac1-inhibitor (P<0.02). Lower panel; Upon overexpression of constitutively active Rac-1 mutant (Q61L) in HEK293, overall Rac1 activity was increased and could not be inhibited by the compounds.
Supplemental figure II. Electric Cell-substrate Impedance Sensing (ECIS) shows that 6-T-GTP slightly decreases the resistance compared to control and 6-MP treated cells, on the other hand, they were all significantly higher than the Rac1 inhibitor-treated cells. Importantly, all conditions remained above 1000Ω, suggesting that the reduction of Rac1 activity did not harm the endothelial monolayer integrity.
Supplemental Figure III. Schematic overview of a mature docking structure on the cell membrane surface. On the left: apical view of the endothelial cell where the red ring represents the cell membrane protrusion with F-actin as the main structural component of the docking structure. On the right: view from the side, showing ICAM-1 positive cell membrane forming the transmigratory cup surrounding a differentiated HL60 cell.
Supplemental figure IV. (A) Cross-linking of ICAM-1 does not induce Rac1 activation without presence of TNFα. It is noticable that 6-MP potently reduce activity of Rac1 in the presence or absence of TNFα. Addition of anti-ICAM-1 antibody coated beads further increases Rac1 activity which could be efficiently dampened by 6-MP (B) 6-MP and its derivative 6-T-GTP are reducing ICAM-1 dependent transmigration of neutrophils in a similar extent as ITX-3.