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Inhibition of GTPase Rac1 in Endothelium by 6-Mercaptopurine Results in Immunosuppression in Nonimmune Cells: New Target for an Old Drug

Goran Marinković,* Jeffrey Kroon, † Mark Hoogenboezem, † Kees A. Hoeben, ‡ Matthijs S. Ruiter,* Kondababu Kurakula,* Iker Otermin Rubio,* Mariska Vos,* Carlie J. M. de Vries,* Jaap D. van Buul, † and Vivian de Waard*

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, in this study, the mechanism underlying the protective role of 6-MP in endothelial cell activation is investigated. Because 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 TNF-α-induced downstream signaling via JNK and reduced activation of transcription factors c-Jun, activating transcription factor-2 and, in addition, NF-κ-light-chain-enhancer of activated B cells (NF-κB), which led to decreased transcription of proinflammatory cytokines. Moreover, 6-MP and 6-T-GTP selectively decreased TNF-α-induced VCAM-1 but not ICAM-1 protein levels. Rac1-mediated generation of cell membrane protrusions, which form docking structures to capture leukocytes, was 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. The Journal of Immunology, 2014, 192: 4370–4378.

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) (3, 4), 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, of which either the purine analogs 6-thioguanosine-5’-monophosphate or 6-thiadenosine-5’-monophosphate 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 ~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-thioguanosine-5’-triphosphate (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 and downstream thio-purines can be synthesized (7).
GTPase-activating proteins. 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 also can bind other small Rho-GTPases such as Cdc42, RhoA, and Rac2, yet, it can only block the activity of Rac1 and Rac2 (11). The GFP Vav2 is unable to exchange 6-T-GDP, preventing reactivation 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 multistep 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 VCAM-1 and ICAM-1 (14–16). 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 proinflammatory proteins as well as preventing actin polymerization in docking structure formation.

Materials and Methods

Human endothelial cell culture

Primary HUVECs were isolated and cultured, according to standard protocol. Briefly, HUVECs were isolated and cultured in M199 medium with 20% FCS (Invitrogen), penicillin/streptomycin (P/S; 100 U/ml) and addition of heparin (0.05 μg/ml), t-glucone (2 mM), and endothelial cell growth supplement (25 μg/ml). All experiments were performed in passage 1–3. One day prior to treatment of HUVECs with 6-MP, 6-T-GTP, or Rac1 inhibitor, medium was changed to M199 medium without purine-based compounds adenosine-sulfate, ATP-disodium salt, and guanine-hydrochloride (Life Technologies). HUVECs were stimulated for 24 h with TNF-α (10 ng/ml) to mimic inflammation with or without overnight pretreatment with 6-MP (10 μM), 6-T-GTP (10 μM), or Rac1 inhibitor (ITX-3, 100 μM).

Monocyte–endothelial cell coculture

To study the effect of azathioprine on monocyte adhesion to endothelial cells, HUVECs were pretreated or not with 6-MP and activated with TNF-α. Human mononuclear THP-1 cells were cultured in RPMI 1640 medium, supplemented with 10% FCS and P/S. THP-1 cells were added to a confluent monolayer of endothelial cells at a concentration of 2 × 10^5 cells/ml and incubated for 4 h. Prior to coculturing, the THP-1 cells were fluorescently labeled using the Cell Trace CFSE cell Proliferation Kit (Invitrogen) to visualize cell adhesion. After 4 h of coculturing, cells were washed, and adhesion was analyzed by fluorescent microscopy (Axiovert 200M; Zeiss). Fluorescent cells were counted per field of view (three fields per well, three wells per condition).

For RNA isolation, a similar experiment was performed. After 4 h of coculturing, all cells attached to the well (endothelium and THP1 together) were harvested 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 24 h with TNF-α. Cell lysates were prepared, and the assay was performed following the protocols provided by the G-LISA kit manufacturer. OD (490 nm) was measured with an EL808 Ultra Microplate Reader (Bio-Tek Instruments). 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 (Life Technologies) supplemented with 20% FCS and P/S. Cells (8 × 10^5) were infected with the adeno virus 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 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 24 h postinfection, and the estimated infection rate was ∼80–90%. After 6 h, the compounds 6-MP, 6-T-GTP, or ITX-3 were added to the cells. After 24 h of TNF-α stimulation, cells were lysed, and Rac1 activity was measured.

Constitutively active Rac1Q61L mutant overexpression in HEK293 cells

HEK-293 were grown in DMEM (Life Technologies) 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 nontransfected cells were treated either with 6-MP or 6-T-GTP or Rac1 inhibitor, left overnight, and subsequently treated or not with TNF-α for the next 24 h. Thereafter, cells were lysed, and Rac1 activity measurement was performed using G-LISA Rac1 activation kit, according to the manufacturer’s instructions.

Electric cell–substrate impedance sensing

Permeability of endothelial monolayer was determined by measuring the electrical resistance using electric cell–substrate impedance sensing (ECIS). Electrode arrays (8W10E; Applied BioPhysics) were pretreated with 10 mM 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 1 h at 37°C. Cells were seeded at 100,000 cells/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 Ω, 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 4 h, TNF-α was added.

Transcription factor activity assays

Transcriptional activity of c-Jun, activating transcription factor 2 (ATF2), and NF-κB 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. OD was measured at 450 nm.

Gene expression

RNA was isolated from cultured cells using the Aurum Total RNA Mini Kit (Bio-Rad), and cDNA was generated by reverse transcription of 200 ng RNA with the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed on cDNA samples using SYBR Green Supersmix (Bio-Rad) and specific forward and reverse primers in an iCycler thermal cycler system (Bio-Rad). 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.

Western blot analysis

HUVECs were pretreated with 6-MP, 6-T-GTP, or ITX-3. The next day, the HUVECs were activated for 15 min (for p-JNK/JNK) or 24 h (for VCAM-1/ICAM-1) with TNF-α. The cells were washed using ice-cold PBS and lysed in Nonidet P-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 60 μg total protein was loaded on gel and blotted to an Immobilon-FL Transfer Membrane (Millipore) by a TransBlot Turbo transfer system (Bio-Rad). Membranes were stained overnight with Abs specific for p-JNK (p-SAPKJNK Rabbit mAb; Cell Signaling Technology), JNK (JNK1/2 C6 Mouse mAb; Cell Signaling Technology), VCAM-1 (C-19, Santa Cruz Biotechnology), ICAM-1 (rabbit polyclonal; Santa Cruz Biotechnology), and β-actin (rabbit polyclonal; Cell Signaling Technology). For detection, IRDye-tagged secondary Abs (LI-COR Biotechnology) were used. Analysis and quantification were performed on an Odyssey Infrared Imaging system (LI-COR). Values were corrected for β-actin.

Ab-coated beads

Polystyrene beads (3 μm; Polysciences) were pretreated with 8% glutaraldehyde overnight, washed with PBS, and incubated with 300 μg/ml

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Table I. Sequences of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>CD11b</td>
<td>5′-AGCCACACAGACGACGACACAG-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>5′-GAGGTCTCCGAAAAGAGCAGACAAATGG-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5′-TGGCAGAAAGGGAAACAGAAAAGG-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>5′-GTGAATAGAGGAGGAGAGGAGGAG-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-CGCATTCCATGCTACCTGTTCTC-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>5′-TCGTTCTGAAGAGTGTAGT-3′</td>
</tr>
<tr>
<td>IL-8</td>
<td>5′-TGGCAATGGCAGAGAAGCAGACGACAG-3′</td>
</tr>
<tr>
<td>Hom sapiens</td>
<td>5′-ATGCCATTCCAGACGCAAGAAG-3′</td>
</tr>
<tr>
<td>IP-10</td>
<td>5′-AGCAGAGAAGAAGTTCCAGTCTC-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>5′-ATGCAGTACAGCCTGTAAGT-3′</td>
</tr>
<tr>
<td>HPRT</td>
<td>5′-TGCAGCTGGCAAAGAATGCA-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
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<tr>
<td>PO</td>
<td>5′-TGCAGATGGACAGATCTAC-3′</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>5′-ATCGCTTTCCACAGACGAGG-3′</td>
</tr>
</tbody>
</table>

ICAM-1 mAb (R&D Systems) to create ICAM-1–Ab–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 TNF-α for 4 h. Therefore, we added anti-ICAM-1 Ab–coated beads (1:60 dilution). The beads were incubated for 30 min, after which, the medium with unattached beads was removed, and cells 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 Biosciences). The endothelial cell protrusions can reach 6 μm above the HUVEC apical surface. To quantify the protrusions, images were taken 2 μm above the apical plane. When protrusions appeared as F-actin–positive rings, they were scored as docking structures. Differential interference contrast (DIC) microscopy 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 aluminum 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 15 kV with a spot size of 30 nm. 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; and 4 = >50% coverage of the bead (mature cup).

**Neutrophil transendothelial migration across Transwell**

A cell migration assay was performed using Transwell plates (HTS Fluoroblock Insert; BD Falcon) of 6.5-mm diameter with 3-μm pore filters. HUVECs were seeded on fibronectin-coated Transwell filters (black) and cultured for 2 d, 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 3 h, followed by activation with TNF-α overnight. Neutrophils were freshly isolated from healthy volunteers using density gradient cell separation with Lymphoprep. Cells were labeled by green fluorescent Cell Trace. Neutrophils (2 × 10⁶ cells/well) were added to the upper compartment and were allowed to migrate to 100 nM fMLP (Sigma-Aldrich) placed in the lower chamber to create a chemotactic gradient. Immediately after neutrophil addition, the plate was placed in a prewarmed 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 VI²⁻⁴ (Ibidi, Munich, Germany) for 2 d 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 10 μM) were added to the cells, and the next day, cells were stimulated with TNF-α (10 ng/ml) for 4 h. Freshly isolated neutrophils were resuspended at 0.4 × 10⁶ cells/ml in HEPES medium and were incubated for 20 min 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 min. During this time, leukocyte–endothelial interactions were recorded in three random fields with a Zeiss Axiosvert 200 microscope (×10 objective) equipped with a motorized stage. Images were recorded with Zeiss Zen 2012 software. Live imaging was performed at 37°C and 5% CO₂. 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 Student t test was performed. A p value ≤ 0.05 is 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 TNF-α–treated endothelial cells, that were preincubated with 6-MP, bare reduced adhesion capacity of monocytes (Fig. 1A, 1B). Azathioprine, 6-MP, and the downstream metabolite 6-T-GTP have been shown to decrease Rac1 activation in T cells (11), which could be the underlying mechanism in endothelial cells as well. Indeed, we recently revealed that TNF-α–induced Rac1 activation is impaired when endothelial cells are pretreated with 6-MP, by pulldown of active Rac1 (19). In this study, we show that pretreatment of the endothelial cells with 6-T-GTP also blocked TNF-α–induced Rac1 activation to a similar extent as 6-MP and Rac1 inhibitor ITX-3, in an ELISA-like setup (Fig. 1C). This Rac1 inhibitor is specific for GEF Trio, the most relevant Rac1 targeting GEF in endothelial cells (20).

Clearly, Rac1 inhibition by 6-MP and 6-T-GTP is not reserved for inflammatory diseases. We show that TNF-α–treated endothelial cells, that were preincubated with 6-MP and 6-T-GTP was not able to dampen the TNF-α–induced activity of another small GTPase, namely RhoA (Fig. 1C), pointing out specificity of these compounds for.
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 ECIS. The electrical resistance of the endothelial monolayer is monitored in real time in the absence or presence of TNF-α. 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 Fig. 2). TNF-α gave a rapid but transient increase in resistance in all conditions. Where 6-T-GTP slightly decreased the resistance compared with 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

TNF-α–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 JNK-mediated pathway (21, 22) (via activation of MAPK kinase 4/7 (22, 23). In this study, we investigated whether suppression of Rac1 activity by 6-MP or 6-T-GTP will influence TNF-α–induced downstream signaling. Both, 6-MP and 6-T-GTP efficiently perturb TNF-α–induced p-JNK, like the Rac1 inhibitor (Fig. 2A).

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**FIGURE 1.** 6-MP inhibits monocyte adhesion to activated endothelium and reduces Rac1 activity. (A) Fluorescently labeled THP1 monocytes show increased adherence to TNF-α–activated HUVEC monolayers. Preincubation 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 monocye marker CD11b ($^{*} p < 0.005$). (C) 6-MP as well as its metabolite 6-T-GTP reduce Rac-1 activity in TNF-α–activated HUVECS to a similar extent as Rac1 inhibitor ITX-3, but there is no effect on the activity of GTPase RhoA. ($^{*} p < 0.03$). (D) 6-MP also reduces monocyte adhesion to TNF-α–activated HUVEC monolayers. Preincubation 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 monocye marker CD11b ($^{*} p < 0.005$). (C) 6-MP as well as its metabolite 6-T-GTP reduce Rac-1 activity in TNF-α–activated HUVECS to a similar extent as Rac1 inhibitor ITX-3, but there is no effect on the activity of GTPase RhoA. ($^{*} p < 0.03$). Lower panel, Upon overexpression of constitutively active Rac1–Q61L mutant construct, gave equal results, showing that the Rac1 inhibitor (Fig. 1D).

**FIGURE 2.** 6-MP reduces activation of signaling pathways downstream of Rac-1. (A) In HUVECS, increased (active) p-JNK is observed in response to TNF-α stimulation, which is lowered upon 6-MP, 6-T-GTP, and Rac1 inhibitor pretreatment. 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 TNF-α–treated HUVECS: c-Jun ($^{*} p < 0.008$), ATF2 ($^{*} p < 0.002$), and NF-κB ($^{*} p < 0.05$).
Downstream of activated JNK are the transcription factors c-Jun (24–27) and 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 CREB site (cAMP response element). Both 6-MP and 6-T-GTP reduce TNF-α–induced transcriptional activation of c-Jun and ATF2, similar as the Rac1 inhibitor (Fig. 2B, 2C).

Yet, another proinflammatory transcription factor that can be activated by TNF-α, and regulated by Rac1 activation, is NF-κB (31). In addition to c-Jun and ATF2, TNF-α–induced NF-κB activation is also significantly reduced by 6-MP, 6-T-GTP, or the Rac1 inhibitor (Fig. 2D). Interestingly, under baseline culture conditions (without TNF-α), c-Jun and ATF2 are somewhat active whereas NF-κB is not. 6-MP can downregulate c-Jun and ATF2 activity below the activity in baseline conditions ($p < 0.03$ and $p < 0.0009$, respectively). Taken together, these data indicate that both 6-MP and 6-T-GTP effectively suppress TNF-α–induced gene transcription. We explore this further by measuring expression of hallmark proinflammatory endothelial cell genes.

A suppressed proinflammatory gene expression profile by 6-MP

Knowing that 6-MP inhibits TNF-α–induced activation of the transcription factors c-Jun, ATF2, and NF-κB, 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 proinflammatory cytokine IL-12 and chemokines CCL2 and CCL5, also known as MCP-1 and RANTES, respectively (19). In this study, more cytokines and chemokines are studied including IL-1β, IL-6, IL-8, as well as CXCL10, also known as IFN-γ–induced protein 10 (IP-10). In TNF-α–stimulated endothelial cells that were pretreated with 6-MP, IL-6, IL-8, and IP-10 are significantly downregulated (Fig. 3) in addition to IL-12, CCL2, and CCL5 that we reported earlier (19). The fact that IL-1β mRNA expression is not suppressed shows that 6-MP inhibits specific proinflammatory 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.

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 TNF-α–stimulated endothelial cells that 6-MP reduces the mRNA expression level of VCAM-1 but not of ICAM-1 (19). In this study, 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 TNF-α (Fig. 4A), suggesting that 6-T-GTP is a potent purine analog that is responsible for Rac1 blockade. Interestingly, although VCAM-1 protein levels are markedly abrogated, ICAM-1 protein levels are unaffected by 6-MP, 6-T-GTP, or the Rac1 inhibitor (Fig. 4B), again showing that 6-MP and 6-T-GTP inhibit specific inflammatory processes.

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). Because 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 Abs, 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. With confocal laser-scanning microscopy, Z-stacks were taken to reveal the docking structures around the beads. DIC microscopy is included to visualize the beads. The endothelial cell protrusions around the beads reach ~6 μm above the endothelial cell apical surface (Supplemental Fig. 3). To quantify these protrusions, images were taken at 2 μm above the apical plane, where protrusions appear as F-actin–positive rings around a bead and were scored as docking structures (Supplemental Fig. 3). The number of attached beads, after washing, reveal that 6-MP significantly reduces adhesion of beads to the endothelial cells (Fig. 5A, 5B). This is in line with our observations that 6-MP reduces the binding of THP-1 monocytes to endothelial cells (Fig. 1). In conclusion, 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 (Fig. 5A, 5C).

In conclusion, even though 6-MP does not affect TNF-α–induced ICAM-1 expression, ICAM-1 function is strongly inhibited by 6-MP, most likely through Rac1 inhibition resulting in decreased VCAM-1 mediated docking structure formation.

![Figure 3](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 3.** 6-MP decreases proinflammatory gene expression response. 6-MP modulates the proinflammatory 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.

![Figure 4](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 4.** 6-MP/6-T-GTP decreases VCAM-1 protein. (A) An increase in the amount of VCAM-1 protein in response to TNF-α 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 TNF-α but are not changed in response to pretreatment with 6-MP, 6-T-GTP, or Rac1-inhibitor.
treatment results also in a decrease in beads captured by a mature docking structure formation, leading to a reduction in overall number of adherent beads (*p < 0.0001).

FIGURE 5. 6-MP inhibits ICAM-1–mediated docking structure formation. (A) Anti–ICAM-1 Ab–coated beads were incubated with TNF-α–activated HUVECs. F-actin and VE-cadherin staining was performed, and confocal microscopy was performed at the basolateral (0 μm) and apical (2 μm) 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 Ab–coated beads in the apical plane (see Supplemental Fig. 3 for detailed scheme). To assess bead localization on the membrane surface, DIC microscopy was performed. (B) Treatment of HUVECs with 6-MP prior to TNF-α 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).

increased formation of docking structures that can capture anti–ICAM-1 Ab–coated beads.

ICAM-1–clustering mediated activation of Rac1
As it was previously reported that clustering of ICAM-1 results in Rac1 activation (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 TNF-α, addition of anti–ICAM-1 Ab–coated beads does not induce Rac1 activity (Supplemental Fig. 4A). In contrast, in the presence of TNF-α, when also ICAM-1 protein production is induced (Fig. 4B), there is induced Rac1 activation, which is significantly further increased by the anti–ICAM-1 Ab–coated beads (Supplemental Fig. 4A). 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.

6-MP/6-T-GTP reduces docking structure maturation and neutrophil migration
In the experiments with anti–ICAM-1 Ab–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 Ab–coated beads) by determining the amount of bead coverage by the endothelial cell membrane protrusions (Fig. 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 (Fig. 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 6-MP, 6-T-GTP, or Rac1 inhibitor and activated with TNF-α as a proinflammatory stimulus to induce ICAM-1 expression. Analogous to the reduction in numbers of captured anti–ICAM-1 Ab–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 Fig. 4B). Still, it could be argued that because of 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 (Fig. 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 setup (18). 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 calculating 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 TNF-α 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).

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 (19). To date, there is limited information on the exact cellular mechanism underlying the immunosuppressive properties of azathioprine in T cells (10, 11) and monocytes/macrophages (33). Poppe et al. (11) 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 current 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 TNF-α–induced Rac1-dependent proinflammatory signaling pathways as well as ICAM-1–induced Rac1-mediated transmigratory cup formation.

Both activation pathways, that is, TNF-α– 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–adaptor proteins such as filamin (20). Actin remodeling, resulting in docking structure formation, is a prominent downstream effect from ICAM-1–induced Rac1 activation. We show in this study that 6-MP as well as its metabolite 6-T-GTP block the maturation of ICAM-1–induced docking structures, which are necessary to capture leukocytes.

In the TNF-α signaling pathway, filamin is not involved, although actin remodeling takes place (20, 34). Rather, TNF-α 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 (35) and, as a consequence, loss of VE-cadherin-mediated cell–cell contact. This in turn facilitates leukocyte migration (35, 36). Proinflammatory stimuli, such as TNF-α, lead to strong activation of Rac1 (37, 38), which in turn is a potent activator of the JNK signaling cascade (21, 22). The Rac1 dependency of JNK activation was demonstrated by downregulation of Rac1 expression by a small interfering RNA approach that led to decreased phosphorylation (activation) of JNK (39), which we also observe when using 6-MP or 6-T-GTP. A less well described Rac1-mediated signaling pathway is inducing NF-κB activation, and the Rac1 dependency is demonstrated in different cell types (40–42). 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) NF-κB 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 (11, 43). We show a similar effect in endothelial cells, previously (19) and in this study, where application of 6-MP reduces the...
expression level of most cytokines, chemokines, and specifically VCAM-1 upon TNF-α stimulation, which may be attributed to reduced activity of the above mentioned transcription factors. Interestingly, although TNF-α–induced VCAM-1 expression was effectively inhibited by 6-MP, ICAM-1 expression was not changed neither at the mRNA (19) nor at the protein level. Because both adhesion molecules have NF-κB and AP-1 sites in their promoter (44–46), there must be additional transcription factors involved that determine ICAM-1 transcription, because the 6-MP– and 6-T-GTP–mediated decrease in NF-κB and AP-1 does not reduce ICAM-1 but is sufficient to reduce VCAM-1. Indeed, in TNF-α–induced activation of the ICAM-1 promoter, the AP-1 sites are not essential (46).

The increase in expression of ICAM-1 in response to TNF-α 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 Ab–coated beads, resulted in Rac1–dependent transmigratory cup formation, which was inhibited by 6-MP and 6-T-GTP. Because 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 because of 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 by azathioprine or 6-MP can now be attributed to inhibition of endothelial cell Rac1 pathways via inhibition of endothelial cell Rac1 activity (47). Because the effects of statins to protect against cardiovascular disease is also partly 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.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental figure I

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 an anti-ICAM-1 antibody-coated bead.
Supplemental figure IV. (A) Cross-linking of ICAM-1 does not induce Rac1 activation without presence of TNFα. It is noticeable 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.