NR4A nuclear receptors in atherosclerosis
Pols, T.W.H.

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6-mercaptopurine induces anti-atherogenic effects in monocytes/macrophages and inhibits atherosclerosis in ApoE*3-Leiden transgenic mice

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Submitted for publication
Abstract

6-Mercaptopurine (6-MP) is derived from the immunosuppressive pro-drug Azathioprine and is commonly used in autoimmune diseases and transplant recipients. In the current study, we aimed to gain knowledge on the effect of 6-MP in atherosclerosis with a focus on effects of 6-MP on monocytes and macrophages. We demonstrate in THP-1 monocytes that 6-MP decreases intracellular purine levels and enhances caspase activity, resulting in monocyte apoptosis and decreased monocyte numbers. The induction of apoptosis by 6-MP is preceded by a strong suppression of the intrinsic anti-apoptotic factors Bcl-xL and Bcl-2. 6-MP also decreases expression of the adhesion integrins VLA-4, and platelet endothelial cell adhesion molecule-1 (PECAM-1) in THP-1 monocytes, and reduces monocyte adhesion. In THP-1-derived macrophages, screening of a panel of atherosclerosis-related cytokines, in response to lipopolysaccharide (LPS) stimulation, revealed that 6-MP robustly inhibits expression and secretion of monocyte chemoattractant protein-1 (MCP-1), while viability of these cells was only mildly affected by 6-MP. Finally, local delivery of 6-MP to the vessel wall, using a drug-eluting cuff, inhibits atherosclerosis in ApoE*3-Leiden transgenic mice fed a Western diet. This inhibition of lesion formation was accompanied by decreased lesion macrophage content, which is consistent with our data obtained in cultured cells. Taken together, we here report that 6-MP induces anti-atherogenic effects in monocytes/macrophages and inhibits atherosclerosis development, providing novel insight on the role of the immunosuppressive drug Azathioprine in atherosclerosis.
Introduction

Atherosclerosis is the major cause of cardiovascular disease (CVD) and has been recognized as an inflammation-driven process. Inflammatory cells present in an atherosclerotic lesion are predominantly monocyte-derived macrophages that are crucial both in initiation and as well as in progression of atherosclerosis (Lusis, 2000). Atherosclerosis initiates and progresses usually over decades in silence until the vessel lumen is severely narrowed and compromises the required blood flow to limbs or organs. Often lethal complications of atherosclerosis are sudden myocardial infarctions and strokes, resulting from ruptured lesions.

Transplant recipients or patients suffering from chronic systemic inflammatory conditions are at high risk to develop CVD (Lusis, 2000; Cara et al., 2004). Azathioprine is widely used as an immunosuppressive agent to prevent transplant rejection and to treat several chronic inflammatory conditions, such as inflammatory bowel diseases (IBD), multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Miller, 2002; Dubinsky, 2004; Casetta et al., 2007; Dooley and Ginzler, 2006). It has been proposed that Azathioprine is among the more beneficial immunosuppressive drugs with regard to CVD (Miller, 2002).

The pro-drug Azathioprine is rapidly converted to 6-mercaptopurine (6-MP), whereafter it is further metabolized (Cara et al., 2004). The immunosuppressive mechanism of 6-MP is believed to be mainly derived from anti-proliferative and pro-apoptotic effects on T- and B-cells (Cara et al., 2004). Well-described mechanisms of action of 6-MP and its metabolites include inhibition of purine synthesis, incorporation into DNA and RNA, and apoptosis of CD4+ T cells through inhibition of Rac1 activation (Cara et al., 2004; Tiede et al., 2003). Recently, enhancement of the transcriptional activity of the Nur77-family of nuclear receptors (Nur77, Nurr1 and NOR-1) has been proposed to contribute to biological effects of 6-MP (Ordentlich et al., 2003; Wansa et al., 2003). In agreement with the latter, 6-MP inhibits smooth muscle cell (SMC) proliferation, and increases hypoxia inducible factor-1α (HIF-1α) in endothelial cells with involvement of Nur77 (de Waard et al., 2006; Yoo et al., 2007).

Next to the suppression of T cells and the inhibition of SMC proliferation, only very limited knowledge is available on 6-MP in atherosclerosis, or on monocytes/macrophages, cells that are crucial and abundant in this process. Here, we report that 6-MP modulates key processes in monocytes and macrophages that are important in atherosclerosis development, notably adhesion, apoptosis, and cytokine secretion. We subsequently demonstrate that local delivery of 6-MP inhibits atherosclerosis development in ApoE*3-Leiden transgenic mice. We
propose that our data provide novel insight into actions of the immunosuppressive
drug Azathioprine in the atherosclerotic vessel wall.

**Materials and methods**

**Cell culture and lentiviral transductions**

Human monocytic THP-1 cells were cultured at a density of 1-2×10^5 cells/ml in RPMI 1640 medium (GIBCO-Invitrogen, Breda, The Netherlands), supplemented with 10% (vol/vol) fetal bovine serum (GIBCO-Invitrogen) and 100 U/ml penicillin/streptomycin (GIBCO-Invitrogen). THP-1 monocyctic cells were differentiated into macrophages by culturing cells for 24 hrs in the presence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). THP-1-derived macrophages were activated by stimulation with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, Zwijndrecht, The Netherlands). THP-1 monocytes were counted using a Bürker chamber; dead cells were excluded by trypan blue staining. Cell viability of macrophages was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) as described before (Pires et al., 2007). Adenine (Sigma-Aldrich) and 6-MP (Sigma-Aldrich) were dissolved in dimethylsulfoxide (DMSO) and added to cell cultures at a final DMSO concentration of 0.1% (v/v). For control conditions, similar amounts of DMSO were added to the cells. To silence Nur77 expression, we transduced THP-1 cells with lentivirus encoding short hairpin (sh) RNA against Nur77 and used a non-specific sequence as control, as described before (Bonta et al., 2006). Alternatively, we transduced THP-1 cells with lentivirus encoding a dominant-negative variant of Nur77 (ΔTA), which was generated by cloning human Nur77 cDNA (GenBank D49728, bp 8-1920) lacking the N-terminal transcriptional activation (TA) domain into the XbaI-NdeI sites of the pRRL-cPPt-PGK-PreSIN vector. Lentiviral particle production and transduction of THP-1 cells was performed as described before (Bonta et al., 2006).

**Nucleotide measurements**

Ribonucleotides were extracted by adding 150 µl of ice-cold 0.4 M perchloric acid to a cell pellet of 5 × 10^6 cells. After 10 min. on ice, the suspension was centrifuged at 11,000 × g at 4 °C for 15 min. The supernatant was removed and neutralized with approximately 7.5 µl 5.0 M K₂CO₃. After centrifugation (11,000 x g at 4 °C for 5 min) the supernatant was used for HPLC analysis. The pellet containing total protein was dissolved in 500 µl 0.2 M NaOH and the protein content was measured with a copper-reduction method using bicinechonic acid, essentially as described by Smith et al. (Smith et al., 1985). Nucleotide profiles were determined by ion exchange HPLC
using a Whatman Partisphere SAX column (4.6 x 125 mm, 5 µm particle size) and a Whatman AX (10 x 25 mm) guard column. Buffer A consisted of 9 mM NH₄H₂PO₄ (pH 3.5) and buffer B consisted of 325 mM NH₄H₂PO₄ (pH 4.4) and 500 mM KCL. Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in 60 min at a flow-rate of 1 ml/min.

**Real-time RT-PCR analysis**
RNA was extracted from cells using the Total mRNA extraction Kit (Biorad, Veenendaal, The Netherlands) after which cDNA was synthesized from 500 ng total RNA (iScript; Biorad). Semi-quantitative real-time reverse transcriptase (RT)-PCR was performed using iQ SYBR-Green Super-Mix in the MyiQ RT-PCR system (Biorad), using gene-specific primers (see Supplemental Table S1). All expression levels were corrected for expression of the housekeeping gene β-2-microglobulin (B2M).

**Western blotting, ELISA, and FACS analysis**
Poly(ADP-ribose) polymerase 1 (PARP1)-cleavage was detected by Western blotting with antibodies recognizing both cleaved and uncleaved PARP1 (BD Pharmingen, Breda, The Netherlands). Protein levels of monocyte chemotactic protein-1 (MCP-1/CCL2) were determined by an ELISA (DuoSet, R&D Systems, Minnesota), according to manufacturers’ instructions. To measure apoptosis by FACS, approximately 5×10⁵ cells were suspended in 50 μl Annexin-V binding buffer (Molecular Probes), and stained with propidium iodine (PI; 100 µg/ml; Molecular Probes-Invitrogen, Breda, The Netherlands), and with APC or FITC-labeled Annexin-V (Molecular Probes-Invitrogen), at dilutions of 1:50 and 1:100, respectively. Analysis was performed with the FACSCalibur, using cellquest software (BD Biosciences, Breda, The Netherlands).

**Monocyte adhesion assay**
Flatbottom 96-well plates (Nunc, Roskilde, Denmark) were coated with 1 mg/ml fibronectin, or with gelatin (0.1% w/v; Merck, Darmstadt, Germany) for 30 minutes. THP-1 monocytes, pre-treated with 10µM 6-MP for 24 hours, or control-treated cells, were transferred to the coated wells in 100µl 10% FCS RPMI 1640 medium at a density of 2×10⁴ cells/well. Cells were subsequently stimulated with 100ng/ml PMA to induce adherence. At indicated times after PMA addition, the wells were extensively washed with PBS, and the amount of adherent THP-1 monocytes was quantified using the MTT assay.
Mouse femoral artery cuff model

Animal care and experimental procedures were approved by the animal experimental committee at our institute. Male ApoE*3-Leiden transgenic mice in a C57Bl/6 background aged 12 weeks (n=6/group) were fed a high fat diet containing 15% cacao butter and 1% cholesterol (Arie Blok, Woerden, The Netherlands), which was initiated one week prior to cuff placement and continued until the end of the experiment. At time of cuff placement, mice were anaesthetized with an intraperitoneal injection of 5 mg/

### Supplemental Table S1. Gene specific primers.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Genbank no.</th>
<th>Forward (Fw) and reverse (Rv) primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
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</tbody>
</table>
| IL-1β | NM_000576.2 | Fw: 5’-TGGCAGAAAGGAAACAGAAGG-3’
Rv: 5’-GTGAGTGGAGAGGTGAGAGG-3’ |
| IL-6 | NM_000600.2 | Fw: 5’-GTGAGGCGCCCCACACAG-3’
Rv: 5’-GCTGCTTTCAACATGTACTCTTG-3’ |
| IL-8 | NM_000584.2 | Fw: 5’-CTGCGCCAACAGAAATT-3’
Rv: 5’-ATTCGACTCTGGCAACCTAC-3’ |
| TF | NM_001993.2 | Fw: 5’-ACCGACAGATTTGAGGATG-3’
Rv: 5’-TGTTGCTGTCGAGGAGTTG-3’ |
| TNF-α | NM_000594.2 | Fw: 5’-AGGAGAAGAGGCCTGAGAAACAG-3’
Rv: 5’-AGGAGAGAGGCTGAGAAACAG-3’ |
| CCL2 | NM_002982.3 | Fw: 5’-CTTAGTTCCCAGAAGACC-3’
Rv: 5’-CCAGGAAGGAGGCAACAG-3’ |
| CCL3 | NM_002983.2 | Fw: 5’-ACGAGGACAGAGCACAGG-3’
Rv: 5’-GCGGCTGGACGGACAGAG-3’ |
| CCL4 | NM_002984.2 | Fw: 5’-GGCTGACTCTCTGCTCTCC-3’
Rv: 5’-ACACAAAGTGGCGAGAACG-3’ |
| CCL5 | NM_002985.2 | Fw: 5’-CGCTGTCATCTCTATTGC-3’
Rv: 5’-CCACTGGTGATAGAATCTCC-3’ |
| CCL18 | NM_002988.2 | Fw: 5’-GTCCTGTCGTCAGCAGATTG-3’
Rv: 5’-AACCTTTTGTTGAAACTCTGCCAGG-3’ |
| **Miscellaneous** | | |
| B2M | NM_000404.8 | Fw: 5’-CTGGCGCTCTCTTTCTTCT-3’
Rv: 5’-TCGCTCCAATTTGTTCTC-3’ |
| CD11b | NM_000632.3 | Fw: 5’-CAGCACACGCAGACACAGACAG-3’
Rv: 5’-GGGCTGGGAAGGAGAAGACGC-3’ |
| SR-A | NM_138715.2 | Fw: 5’-CTGGTCCCCACTCTGAGAG-3’
Rv: 5’-GAGAAGCTTGAATGGGCCAT-3’ |
| CD36 | NM_00072.2 | Fw: 5’-CTGAACCTGGCAGAAAGGAG-3’
Rv: 5’-TTCAACTTGGAAGGCAAAAA-3’ |
| **Bcl members** | | |
| Bcl-2 and Bcl-x | NM_000567.2 | Fw: 5’-GGGGAGGATTGTGGCTTC-3’
Rv: 5’-GAAGGAGGGAAGGAGCACC-3’ |
| Bcl-x | NM_001191.2 and NM_138578.1 | Fw: 5’-GCAGGCTGGCAGGGAGGAGCACC-3’
Rv: 5’-GGGAACCTGGCAGAAAGGAG-3’ |
| **Adhesion** | | |
| PECAM-1 | NM_000442.3 | Fw: 5’-TGCACTCTCTTTCTCCTACCTG-3’
Rv: 5’-TCCCACCTCGCGACATGTCTGAG-3’ |
| Integrin-β2 | NM_000211.3 | Fw: 5’-ATGGAGTGCTGGAGGCTCTCTG-3’
Rv: 5’-TGCCACTTTCTCCTCTTGGGTTG-3’ |
| VLA-4 | NM_000854.2 | Fw: 5’-CTGTAGCAGAAAGCAGACTACAGG-3’
Rv: 5’-AAGTGGAACTGGAAGGACCAGG-3’ |

Primer sequences were obtained from literature, designed (Beacon designer 3, Premier Biosoft International, Palo Alto, CA) or derived from the Harvard primer bank (Xiaowei Wang and Brian Seed (2003); A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Research 31(24): e154; pp.1-8.).
kg midazolam (Roche, Basel, Switzerland), 0.5 mg/kg medetomidine (Orion, Helsinki, Finland) and 0.05 mg/kg fentanyl (Janssen, Geel, Belgium). Control or 6-MP eluting cuffs (1% or 2.5% w/w) were placed loosely around the femoral artery as previously described (Pires et al., 2007). The cuffs were manufactured, as described before (Pires et al., 2007). Release of 6-MP from the cuff was determined as described before (Pires et al., 2007). Briefly, 6-MP showed a sustained and dose-dependent release from the 6-MP eluting cuffs, which was 6.3 ± 0.7 μg total release in 14 days for 1% 6-MP eluting cuffs and 12.0 ± 1.3 μg total release in 14 days for 2.5% 6-MP eluting cuffs. Plasma cholesterol levels were determined with a colorimetric assay (Biomerieux, France).

**Tissue preparation and morphometry**

Two weeks after cuff placement, mice were euthanized and cuffed vessel segments were perfused with Shandon Formal fixx (Thermo Scientific, Breda, The Netherlands) and embedded in paraffin. Approximately 6-8 cross-sections of 5 μm, each separated by 200 μm, throughout the cuffed femoral artery were used for quantification. The vessels’ lamina elastica were visualized by Lawson stain (Klinipath, Duiven, The Netherlands). Morphometric data was obtained using image analysis software (Leica Qwin, Wetzlar, Germany). For visualization, sections were stained with Lawson stain and subsequently with hematoxylin-eosin (HE) stain.

**Immunohistochemistry**

Macrophages were detected by immunohistochemistry using antibodies against Mac3 (M3/84, BD Pharmingen), followed by horse radish peroxidase (HRP)-conjugated donkey-anti-rat antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) and DAP (Immunologic, Duiven, The Netherlands) substrate color development. Mac3 positive area was determined using image analysis software (Leica Qwin).

**Statistical analysis**

All data are presented as mean ± SEM and analyzed using the Student t-test. P values < 0.05 were regarded as statistically significant.

**Results**

6-MP decreases THP-1 monocyte numbers by interfering with purine synthesis

To our best knowledge, the effect of 6-MP on monocytes has not been reported before. Inhibition of purine synthesis is one of the well-described mechanisms by which 6-MP is proposed to affect cellular behavior (Cara et al., 2004). We therefore first examined a potential inhibition of purine synthesis in THP-1 monocytes by
analyzing intracellular nucleotide levels in response to 6-MP. HPLC analysis revealed that 10 μM 6-MP decreased both ATP (1.5 fold reduction at 8 hrs, $P<0.05$; Figure 1A) as well as GTP levels (4.1 fold reduction at 8 hrs, $P<0.05$; Figure 1B). Addition of 10 μM of the purine adenine restored ATP and GTP levels (Figure 1C), indicating that 6-MP reduces intracellular nucleotide levels in monocytes through interfering with purine synthesis.

Recruitment and proliferation of monocytes plays a major role in the growth of atherosclerotic lesions (Lusis, 2000; Lessner et al., 2002). To evaluate the effect of 6-MP on monocyte numbers, we treated proliferating THP-1 monocytes with 10 μM 6-MP, and observed that 6-MP decreased the number of monocytes in time as compared to control conditions (1.9 fold reduction at 48 hrs, $P<0.05$; Figure 1D). Exogenous

**Figure 1.** 6-MP decreases THP-1 monocyte numbers by interfering with purine synthesis. Timecourse of intracellular (A) ATP levels and (B) GTP levels in THP-1 monocytes treated with 10 μM 6-MP (diamonds) and treated under control conditions (squares). (C) Intracellular ATP and GTP levels in monocytes treated for 8 hrs with 10 μM 6-MP (white bars) or treated under control conditions (black bars) with and without simultaneous addition of 10 μM adenine (light grey bars and dark grey bars). (D) Timecourse of monocyte numbers in response to 10 μM 6-MP (diamonds) as compared to the number of monocytes cultured under control conditions (squares). (E) Monocyte numbers after 48 hrs treatment with 6-MP (10 μM 6-MP, grey bars; 50 μM 6-MP, white bars) as compared to control conditions (black bars), with and without simultaneous addition of 10 μM adenine. Results represent means±SEM, n=3; *statistically significant, $P<0.05$. NS, not significant.
addition of 10 μM adenine rescued the effect of 6-MP on monocyte numbers (Figure 1E), indicating that this effect 6-MP involved inhibition of purine synthesis.

6-MP induces apoptosis of THP-1 monocytes

We next assessed whether the decrease in the number of THP-1 monocytes by 6-MP involves apoptosis, for which we analyzed the number of early apoptotic (PI-/Annexin-V-FITC+) cells by FACS. 10 μM 6-MP increased the number of early apoptotic cells 5.3 fold after 48 hrs \( (P<0.05; \text{Figure 2A and 2B}). \) Next, we examined cleavage of

**Figure 2. 6-MP induces apoptosis of THP-1 monocytes.** (A) FACS-derived dot plot of monocytes stained with Annexin-V-FITC and PI treated for 48 hrs with 10 μM 6-MP (lower panel) and of monocytes treated under control conditions (upper panel). Early apoptotic cells were identified by Annexin-V-FITC+/PI staining (lower right quadrants). (B) Timecourse of the percentage apoptosis (Annexin-V-FITC+/PI-) in monocyte cultures in response to 10 μM 6-MP (diamonds) and in control-treated monocyte cultures (squares) as analyzed by FACS. (C) Western blot of intact PARP1 (113kD band) and the larger fragment of cleaved PARP1 (83kD band) in cell lysates of monocytes treated for 48 hrs with 6-MP. (D) Percentage of apoptosis (Annexin-V-FITC+/PI-) in monocyte cultures treated for 48 hrs with 10 μM 6-MP (white bars) and treated under control conditions (black bars) with and without simultaneous addition of 10 μM adenine. (E-F) mRNA expression of the intrinsic anti-apoptotic genes (E) Bcl-xL (F) Bcl-2 and (G) Bcl-xL in monocytes in response to a 24 hrs treatment with 10 μM 6-MP (white bars) and in control treated monocytes (black bars). Results represent means±SEM, n=3; *statistically significant, \( P<0.05 \). NS, not significant.
poly ADP-ribose polymerase-1 (PARP-1), which is a substrate of caspases and may be considered as a read-out for caspase activity in the cell. Clear PARP-1 cleavage in response to 6-MP was observed (Figure 2C), reflecting increased caspase activity which further confirms that 6-MP induces apoptosis of THP-1 monocytes. Preventing depletion of purine levels by adding 10 μM adenine blocked the apoptosis induction by 6-MP (Figure 2D), indicating that the enhanced apoptosis in response to 6-MP in THP-1 monocytes involved inhibition of purine synthesis. 6-MP has been described to enhance the transcriptional activity of Nur77-family members (Ordentlich et al., 2003; Wansa et al., 2003), which are implicated in apoptosis of myeloid progenitor cells (Mullican et al., 2007). We therefore assayed involvement of Nur77 in the induction of apoptosis of THP-1 monocytes by 6-MP. For this, we silenced Nur77 by lentiviral shRNA delivery or overexpressed a dominant negative variant of Nur77 (ΔTA), which blocks the transcriptional activity of all Nur77-family members, and cultured these cells in the presence of 6-MP or in control conditions. We observed no change in the number of apoptotic cells in response to 6-MP in either ΔTA-transduced cells or shNur77-transduced cells as compared to control cells, demonstrating that 6-MP induces apoptosis independent of Nur77 (See supplemental data; Figure S1A).

To gain further insight into the mechanism underlying the enhanced apoptosis in response to 6-MP, we analyzed gene expression of monocytes treated with 10 μM 6-MP. We chose to analyze gene expression of monocytes after 24 hours 6-MP treatment, a timepoint prior to excessive apoptosis (Figure 1D and Figure 2B), thereby minimizing interference of ongoing apoptotic processes on gene expression. We analyzed expression of Bcl-2, Bcl-x, and Bcl-xL, which are key regulatory proteins in the intrinsic apoptotic signaling pathway. Although expression of Bcl-x was not modulated by 6-MP (Fig 2E), we observed that the anti-apoptotic factors Bcl-xL (1.7 fold reduction, P<0.05; Fig 2F) and Bcl-2 (3.2 fold reduction, P<0.05; Fig 2G) were potently inhibited by 6-MP, which is entirely consistent with the observed apoptosis induction.

6-MP inhibits adhesion of THP-1 monocytes
The entry of monocytes into the vessel wall is highly relevant in the initiation and progression of atherosclerosis (Lusis, 2000; Lessner et al., 2002). To investigate potential effects of 6-MP in this process, we analyzed expression levels of the integrins VLA-4, integrin-β2, and PECAM-1, which are critical in adhesion of monocytes, and influence monocyte infiltration (Lusis, 2000). Expression of integrin-β2 was not modulated in THP-1 monocytes treated with 10 μM 6-MP for 24 hrs (Figure 3A), but we did observe that 6-MP reduced expression of the
6-Mercaptopurine in monocytes and macrophages

The downregulation of PECAM-1 and VLA-4 suggested that 6-MP might inhibit monocyte adhesion. We therefore assessed adhesion of THP-1 monocytes treated for 24 hrs with 10 μM 6-MP. 6-MP did not modulate adhesion of THP-1 monocytes to gelatin-coated surfaces in response to PMA treatment (Figure 3D). However, most interestingly, 6-MP decreased adhesion of THP-1 monocytes to fibronectin-coated surfaces as compared to control-treated THP-1 monocytes (1.3 fold decrease at 30 min., \( P < 0.05 \); Figure 3E). This is in full agreement with the knowledge that VLA-4, most strongly suppressed by 6-MP, interacts next to vascular cell adhesion molecule (VCAM)-1 with fibronectin (Chan et al., 1992). These findings indicate that 6-MP may reduce monocyte recruitment to atherosclerotic lesions by decreasing adhesion capabilities of monocytes.

6-MP inhibits expression and secretion of MCP-1 in macrophages

Lesion macrophages are major players in atherosclerosis and locally secrete abundant amounts of cytokines (Lusis, 2000). To investigate the influence of 6-MP on the expression and secretion of monocyte chemoattractant protein-1 (MCP-1), cells were treated with 10 μM 6-MP for 24 hrs. MCP-1 mRNA expression was measured by quantitative real-time PCR (Figure 3). 6-MP significantly decreased MCP-1 mRNA expression in control-treated THP-1 monocytes as compared to control-treated monocytes (1.2 fold decrease, \( P < 0.05 \); Figure 3A). Similarly, 6-MP significantly reduced MCP-1 protein secretion in control-treated THP-1 monocytes as compared to control-treated monocytes (1.5 fold decrease, \( P < 0.05 \); Figure 3B). These findings indicate that 6-MP may reduce monocyte recruitment to atherosclerotic lesions by decreasing adhesion capabilities of monocytes.
Supplemental Table S2. Lipopolysaccharide (LPS)-stimulated cytokine expression of THP-1-derived macrophages pre-treated for 48 hrs with 10 μM 6-MP or control conditions.

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>4 hrs LPS</th>
<th>8 hrs LPS</th>
<th>24 hrs LPS</th>
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<tr>
<td></td>
<td>Ctrl</td>
<td>6-MP</td>
<td>Ctrl</td>
<td>6-MP</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.0 ± 0.4</td>
<td>1.1 ± 0.5</td>
<td>9.5 ± 2.7</td>
<td>11.8 ± 6.6</td>
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<tr>
<td>IL-6</td>
<td>1.0 ± 1.1</td>
<td>1.9 ± 1.1</td>
<td>2853.8 ± 936.4</td>
<td>3677.3 ± 1242.3</td>
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<tr>
<td>IL-8</td>
<td>1.0 ± 0.1</td>
<td>3.7 ± 3.5</td>
<td>5.1 ± 1.9</td>
<td>5.9 ± 2.9</td>
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<tr>
<td>TF</td>
<td>1.0 ± 0.2</td>
<td>0.6 ± 0.5</td>
<td>6.5 ± 0.4</td>
<td>7.0 ± 1.1</td>
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<td>TNF-α</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>115.3 ± 20.6</td>
<td>148.4 ± 20.6</td>
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<td>CCL2 (MCP-1)</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.1*</td>
<td>3.4 ± 0.2</td>
<td>1.6 ± 0.6*</td>
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<tr>
<td>CCL3 (MIP-1α)</td>
<td>1.0 ± 0.5</td>
<td>1.5 ± 0.3</td>
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<td>CCL4 (MIP-1β)</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>367.9 ± 141.0</td>
<td>357.3 ± 175.7</td>
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<td>CCL5 (RANTES)</td>
<td>1.0 ± 0.5</td>
<td>1.6 ± 0.2</td>
<td>9.8 ± 4.0</td>
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<td>CCL18 (PARC)</td>
<td>1.0 ± 0.5</td>
<td>1.6 ± 1.0</td>
<td>22.6 ± 8.4</td>
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All mRNA expression data are corrected for expression of the housekeeping gene B2M, and are expressed as fold change of basal expression in control-treated macrophages. Results represent Means ± SEM, n=4. *Statistically significant to control, P<0.05.
MP on inflammatory gene expression in macrophages, we differentiated THP-1 monocytes into macrophages by treatment with PMA. In contrast to our observations in THP-1 monocytes, a high concentration of 50 μM 6-MP only modestly reduced macrophage cell viability (1.2 fold reduction at 72 hrs, $P<0.05$; Figure 4A). 6-MP did not change the cellular morphology of macrophages (Figure S2A-S2C), nor modulate macrophage differentiation, as is shown by unchanged mRNA expression levels of CD11b (Figure S2D), and of the scavenger receptors CD36 and scavenger receptor-A (SR-A; Figure S2E and Figure S2F).

To examine a potential modulation of inflammatory gene expression by 6-MP, we pre-treated macrophages for 48 hrs with 10 μM 6-MP and subsequently induced inflammatory gene expression by LPS stimulation. Using real-time RT-PCR analysis, we analyzed mRNA expression levels of tissue factor (TF) and of a panel of cytokines involved in atherosclerosis, which consisted of interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor-α (TNF-α), MCP-1 (CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β (CCL4), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), and pulmonary and activation-regulated chemokine (PARC/CCL18). From all examined cytokines, only MCP-1 was robustly suppressed by 6-MP at 4, 8 and 24 hrs after LPS stimulation (1.8 fold reduction at 8 hrs, $P<0.05$; Table S2). Inhibition of MCP-1 expression was also observed in

![Fig. 4](image-url)

**Fig. 4. 6-MP modulates cytokine secretion of THP-1 derived macrophages.** (A) Cell viability of macrophages treated for 72 hrs with 10 μM 6-MP (grey bars) and 50 μM 6-MP (white bars) as compared to the cell viability of macrophages treated under control conditions (black bars). (B) MCP-1 mRNA expression of 8 hrs LPS-stimulated macrophages pre-incubated for 48 hrs with increasing 6-MP concentrations (white bars) or without 6-MP treatment (black bar), expressed as fold induction over non-LPS treated macrophages. (C) MCP-1 protein in the supernatant of 24 hrs LPS-stimulated macrophages pre-incubated for 48 hrs with 6-MP (2 μM 6-MP, dark grey bars; 10 μM 6-MP, light grey bars; 50 μM 6-MP, white bars) or without 6-MP treatment (black bars) with and without simultaneous addition of 10 μM adenine. Results represent means±SEM, n=3; *statistically significant, $P<0.05$. NS, not significant.
response to 2, 25 and 50 μM 6-MP (1.9 fold reduction, \( P<0.05 \); 2.6 fold reduction, \( P<0.05 \) and 4.3 fold reduction, \( P<0.05 \), respectively; Figure 4B). Of note, even the higher concentrations of 6-MP did not modulate mRNA expression of the other tested cytokines in response to LPS stimulation (data not shown).

We quantified MCP-1 protein levels in the supernatant of macrophages by ELISA after 24 hrs of LPS stimulation. In line with our results that 6-MP inhibits mRNA levels of MCP-1, we observed reduced MCP-1 protein in response to 6-MP treatment (2.4 fold reduction at 10 μM 6-MP, \( P<0.05 \); Figure 4C), indicating that the reduction in MCP-1 mRNA resulted in reduced MCP-1 protein secretion. The inhibitory action of 6-MP on MCP-1 synthesis was reversed by restoring purine concentrations through addition of adenine, indicating involvement of purine synthesis in this action of 6-MP (Figure 4C). 6-MP reduced MCP-1 expression to a similar extent in macrophages in which Nur77 was silenced by shRNA as compared to control-treated cells, indicating that downregulation of Nur77 expression does not affect this action of 6-MP (Figure S1B). Overexpression of the dominant negative variant of Nur77 (ΔTA) strongly decreased MCP-1 levels in response to LPS stimulation. The latter observation is in agreement with the data presented by Hong et al. who showed that the C-terminal domain of Nur77, which is unaltered in the ΔTA-variant, is involved in the inhibition of NF-κB through direct protein-protein interaction (Hong et al.,

Figure 5. 6-MP inhibits atherosclerotic lesion formation in ApoE*3-Leiden transgenic mice fed a Western diet. (A-F) Photomicrographs of representative cross-sections of cuffed femoral arteries stained with HE-Lawson (A-C) or with immunohistochemistry to detect Mac3 (D-F) of vessels around which control cuffs were placed (A and D) or from vessels around which 1% (B and E) and 2.5% 6-MP (C and F) eluting cuffs were placed (right panels); Arrows indicate the internal elastic lamina; Original magnification 40×, scalebar is 100 μm. (G) Intimal lesion formation after 14 days in control cuffed vessels (black bars) and in vessels around which 1% and 2.5% 6-MP eluting cuffs were placed (white bars), as determined by morphometric analyses. Results represent means±SEM; *statistically significant, \( P<0.05 \). NS, not significant.
6-MP inhibits atherosclerosis development in ApoE*3-Leiden transgenic mice and decreases lesion macrophage content.

The effect of 6-MP on apoptosis, adhesion, and MCP-1 expression in monocytes/macrophages suggested that 6-MP modulates atherosclerosis development. We therefore examined the effect of locally applied 6-MP in atherosclerotic lesion formation by placing 6-MP eluting cuffs (or control cuffs) loosely around the femoral arteries of ApoE*3-Leiden transgenic mice fed a Western diet (Lardenoye et al., 2000). Two weeks after cuff placement, equal high plasma cholesterol levels of approximately 9 mmol/L were observed in all mice (data not shown). Cross-sections stained with HE-Lawson (Figure 5A-5C), and with immunohistochemistry for Mac3 to detect macrophages (Figure 5D-5F), revealed the induction of atherosclerotic lesion formation. Quantification of the lesion area demonstrated that the total lesion surface area was reduced, respectively 4.1 fold reduction and 7.1 fold reduction, in cuffs containing 1% (2.423 ± 541 μm², P<0.05) and 2.5% 6-MP (1.409 ± 466 μm², P<0.05) as compared to lesions induced by control cuffs (9962 ± 1.644 μm²; Figure 5G and Table 1). Furthermore, the intima/media ratio and the percentage of lumen stenosis were greatly reduced by 6-MP, while media and total vessel surface areas were not significantly affected (Table 1).

Since the in vitro data revealed that 6-MP increases monocyte apoptosis, decreases monocyte adhesion, and inhibits secretion of the chemotactic molecule MCP-1, we quantified the macrophage-positive area. The Mac3 staining unmasked decreased macrophage content in the intima of arteries treated with 1% or 2.5% 6-

### Table 1. Morphometric data.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>6-MP-Eluting Cuff 1%</th>
<th>6-MP-Eluting Cuff 2.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vessel area², ×10¹ μm²</td>
<td>28.7 ± 2.6</td>
<td>25.6 ± 1.6</td>
<td>26.0 ± 3.6</td>
</tr>
<tr>
<td>Media, ×10¹ μm²</td>
<td>10.2 ± 0.7</td>
<td>9.1 ± 0.6</td>
<td>7.5 ± 0.9</td>
</tr>
<tr>
<td>Neointima, ×10¹ μm²</td>
<td>10.0 ± 1.6</td>
<td>2.4 ± 0.5 *</td>
<td>1.4 ± 0.5 *</td>
</tr>
<tr>
<td>I/M ratio</td>
<td>0.97 ± 0.17</td>
<td>0.28 ± 0.07 *</td>
<td>0.20 ± 0.08 *</td>
</tr>
<tr>
<td>Lumenstenosis, %</td>
<td>54.3 ± 9.4</td>
<td>13.5 ± 2.2 *</td>
<td>7.9 ± 1.9 *</td>
</tr>
<tr>
<td>Total Mac3 positive area, ×10¹ μm²</td>
<td>2.5 ± 0.9</td>
<td>0.6 ± 0.3 *</td>
<td>0.4 ± 0.1 *</td>
</tr>
<tr>
<td>Intima</td>
<td>3.4 ± 1.0</td>
<td>1.3 ± 0.4 *</td>
<td>0.7 ± 0.3 *</td>
</tr>
<tr>
<td>Media</td>
<td>20.2 ± 7.2</td>
<td>29.8 ± 13.3</td>
<td>25.9 ± 5.4</td>
</tr>
<tr>
<td>Relative Mac3, %</td>
<td>35.6 ± 10.1</td>
<td>15.6 ± 4.8 *</td>
<td>8.4 ± 3.2 *</td>
</tr>
</tbody>
</table>

# Total vessel area comprises the surface within the external elastic lamina, including the lumen. Result represent means ± SEM, *statistical significant as compared to control group, P<0.05.
MP cuffs as compared to lesions induced by control cuffs (565 ± 259 μm², \(P<0.05\) and 434 ± 139 μm², \(P<0.05\), respectively vs 2448 ± 901 μm²; Table 1) as well as in the media of arteries (1263 ± 397 μm², \(P<0.05\) and 672 ± 297 μm², \(P<0.05\) respectively vs 3439 ± 958 μm²; Table 1). Next, the relative macrophage content was calculated, and revealed a reduction in the macrophage ratio of the media of 1% and 2.5% 6-MP-treated vessels, as compared to control-cuff treated vessels (15.6 ± 4.8%, \(P<0.05\) and 8.4 ± 3.2%, \(P<0.05\), respectively vs 35.6 ± 10.1%; Table 1). Although the total macrophage content in the intima was reduced, the relative macrophage content in the intima was not modulated by 6-MP treatment (Table 1). This is consistent with our previous observations, showing that 6-MP also reduces lesion size by inhibiting the contribution of SMCs to lesion formation (de Waard et al., 2006; Pires et al., 2007).

**Discussion**

6-MP is derived from the pro-drug Azathioprine, which is a commonly used immunosuppressive drug in clinical practice to chronically treat transplant recipients and autoimmune diseases. The cardiovascular risk profiles of the

Supplemental Figure S1. Effect of Nur77 knockdown and overexpression of ΔTA, the dominant-negative variant of Nur77 family members, on 6-MP-induced apoptosis and MCP-1 expression. (A) Percentage of early apoptotic cells (Annexin-V-APC+/PI-) in monocyte cultures treated with 10 μM 6-MP for 48 hrs (black bars) or in control-treated cultures (white bars) in which Nur77 was silenced with shRNA (shNur77) or in which control shRNA (shCON) was used, and monocytes overexpressing a dominant negative variant of Nur77 (ΔTA) as compared to control-treated monocytes (Mock). (B) MCP-1 mRNA expression of 24 hrs LPS-stimulated macrophages pre-incubated for 48 hrs with 6-MP (white bars) and control conditions (black bars) of cells in which Nur77 was silenced by shRNA (shNur77) or in which control shRNA (shCON) was used. (C) MCP-1 mRNA expression in 24 hrs LPS-stimulated macrophages pretreated with 10 μM 6-MP for 48 hrs (black bars) or in control-treated cells (white bars) overexpressing the dominant negative variant of Nur77 (ΔTA) or control (Mock) transduced cells. Results represent means±SEM, n=3; *statistically significant, \(P<0.05\). NS, not significant.
immunosuppressive drugs used in these patients are extremely relevant, particularly since these individuals are due to their specific condition already at increased risk for CVD. In the current study, we investigated 6-MP in monocytes and macrophages, and demonstrate that vascular application of 6-MP inhibits intimal lesion formation in a mouse model of atherosclerosis.

To investigate 6-MP in atherosclerosis development, we used ApoE*3-Leiden transgenic mice, which develop accelerated atherosclerosis in cuffed arteries when fed a Western diet (Lardenoye et al., 2000). We demonstrated that 6-MP-eluting cuffs placed around the femoral artery inhibit atherosclerotic lesion formation, which was accompanied with decreased macrophage content in both the intima as well as in the media. Our data obtained in cultured cells provide insight into the mechanisms by which 6-MP decreases lesion macrophage content and inhibits atherosclerosis, which is discussed in the paragraph below.

First, we propose that effects of 6-MP on viability of THP-1 monocytes and THP-1-derived macrophages contributes to the observed atheroprotective effects of 6-MP. This hypothesis is in line with Stoneman and colleagues, who reported that monocyte/macrophage apoptosis is beneficial in early atherosclerosis (Stoneman et al., 2007). The decreased expression of the anti-apoptotic factors Bcl-2 and Bcl-x_L provides insight

Supplemental Figure S2. 6-MP does not modulate morphology or reverse differentiation of THP-1-derived macrophages. (A-C) Monocytes (A) were differentiated into macrophages (B and C), and treated with control-medium (B) or with 50 μM 6-MP for 48 hrs (C); Original magnification 20×, scalebar is 100 μm. (D-F) mRNA expression of the macrophage differentiation genes (D) CD11b, (E) SR-A and (F) CD36 in monocytes (white bars) and of macrophages treated for 48 hrs with and without 50 μM 6-MP (black bars). Results represent means±SEM, n=3; *=statistically significant, P<0.05. NS, not significant.
into the mechanism by which 6-MP induces apoptosis (Adams and Cory, 2007), although the precise action by which 6-MP modulates expression of the latter genes remains to be elucidated. Second, we demonstrated in cultured THP-1 monocytes that 6-MP potently inhibits mRNA expression of the adhesion molecules PECAM-1 and VLA-4, and inhibits monocyte adhesion, a key event in atherosclerosis (Lusis, 2000; Lessner et al., 2002). The relevance of VLA-4, most strongly suppressed by 6-MP, in atherosclerosis is further underlined by a report demonstrating that inhibition of VLA-4 inhibits atherosclerosis in LDL-receptor-deficient mice (Lusis, 2000; Shih et al., 1999). Finally, we demonstrated that 6-MP inhibits MCP-1, a key component of the chemotactic system, in THP-1 derived macrophages. MCP-1 is relevant to monocyte infiltration, and has a well-described, pivotal function in atherosclerosis (Kitamoto and Egashira, 2002). We propose that decreased expression of MCP-1 by atherosclerosis-resident macrophages, and perhaps also in other vascular cells, in response to 6-MP contributes to the decreased atherosclerosis development observed in the 6-MP-treated ApoE*3-Leiden transgenic mice.

6-MP is known to inhibit purine synthesis, amongst others via inhibition of glutamine-5-phosphoribosylpyrophosphate amidotransferase (Cara et al., 2004). We demonstrate that the effects of 6-MP observed in monocytes/macrophages were attributed to decreased intracellular nucleotide levels, most probably by inhibition of purine synthesis. At this point, the causal link between nucleotide levels and the biological actions of 6-MP is unclear. It is possible that the induction of apoptosis and the inhibition of MCP-1 by 6-MP involves inhibition of the transcription factor NF-κB, which is upstream of MCP-1, involved in apoptosis and inhibited by 6-MP in CD4+ T cells (Tiede et al., 2003).

The clinical indications for which 6-MP is currently applied as an immunosuppressive pro-drug in the form of Azathioprine include MS, SLE and IBD, such as Crohn’s disease. In these diseases, macrophages contribute to disease progression (Barnett et al., 2006; Pringe et al., 2007; Herfarth et al., 2003). Furthermore, MCP-1 is elevated in Crohn’s disease and is associated with the degree of intestinal inflammation (Herfarth et al., 2003). Although beyond the scope of this study, we speculate that effects of 6-MP on monocytes/macrophages, including effects on MCP-1 expression, may contribute to its beneficial action in these diseases.

In conclusion, we provide evidence that local, vascular application of 6-MP inhibits atherosclerotic lesion formation and decreases lesion macrophage content, which is explained by yet unrecognized effects of 6-MP on monocyte apoptosis, monocyte adhesion, and MCP-1 secretion by macrophages. Our results thereby provide further insights into actions of the immunosuppressive drug Azathioprine in atherosclerosis.
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