Chapter 6

6-Thioguanine Increases Human Monocyte Innate Immunity via Inhibition of the Rac1 Signaling Pathway

Submitted
6-Thioguanine Increases Human Monocyte Innate Immunity via Inhibition of the Rac1 Signaling Pathway

Lu Zhou\textsuperscript{1,2}, Gerard Dijkstra\textsuperscript{2}, Kaushal Parikh\textsuperscript{1}, Lydia Visser\textsuperscript{3}, Anouk Regeling\textsuperscript{2}, Gwenny M. Fuhler\textsuperscript{1}, Maikel P. Peppelenbosch\textsuperscript{1}, Klaas Nico Faber\textsuperscript{2}

1. Department of Cell Biology, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands.
2. Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands.
3. Department of Pathology, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands.
ABSTRACT

**Background:** Accumulating evidence suggests that the primary immune defect of Crohn’s disease patients lies in impaired innate immunity. Azathioprine is an effective immunomodulator for treating Crohn’s disease. Its actions in autoimmunity were assumed to its cytostatic properties on lymphocytes and thus far its effect on innate immunity has not been established. Here, we studied the effects of Azathioprine on monocyte, the main innate mediators of immunity.

**Methods:** Human primary monocytes from healthy individuals and Crohn’s disease patients were exposed to 6-thioguanine, the active metabolite of Azathioprine, and its effect on bacterial phagocytosis, cytokine production and Rac1 signaling was analyzed.

**Results:** 6-thioguanine (10 μM) stimulated bacterial phagocytosis in monocytes from healthy individuals (n=18, P<0.001) and Crohn’s disease patients (n=7, P<0.01). 6-thioguanine strongly inhibited the activity of Rac1-GTPase and its downstream kinase PAK2 in monocytes, while no Rac1 inhibition was observed in lymphocytes under these conditions. Monocyte phagocytosis was also stimulated by the unrelated Rac1 inhibitor NSC23766, supporting that 6-thioguanine acts via Rac1 to enhance monocyte phagocytosis. The Rac1 pathway is hyperactivated in Crohn’s disease patients, both in peripheral monocytes and in the intestinal mucosa, and monocytes from Crohn’s diseases patients show reduced phagocytosis compared to control monocytes. Finally, 6-thioguanine increased interleukin-8 production by monocytes while slightly reducing IL-10 production.

**Conclusions:** 6-thioguanine enhances human monocyte innate immunity by increasing monocyte phagocytosis and IL-8 production. We propose that the defect in innate immunity in Crohn’s disease patients originates from Rac1 hyperactivation and that Azathioprine acts by counteracting Rac1.

**Keywords:** 6-Thioguanine; Azathioprine; Crohn’s disease; innate immunity
INTRODUCTION
Azathioprine (AZA) is an immunosuppressant used to treat autoimmune diseases, including inflammatory bowel diseases (Crohn’s disease and ulcerative colitis), rheumatoid arthritis, pemphigus, and multiple sclerosis. After oral administration and absorption, the prodrug AZA undergoes approximately 90% conversion to 6-mercaptopurine (6-MP) by nonenzymatic attack of sulphhydryl-containing compounds, such as glutathione or cysteine (1, 2). In subsequent steps, 6-MP is enzymatically converted to 6-thiouric acid by xanthine oxidase, to 6-methyl-MP by thiopurine S-methyltransferase (TPMT) and to 6-thioguanine (6-TG) by hypoxanthine phosphoribosyl transferase (1) 6-TG mediates the immunomodulatory properties of AZA, both in \textit{in vitro} experiments and in patients (3, 4) Despite four decades of widespread clinical use, our knowledge of the molecular and cellular mechanisms underlying the action of AZA in human immune system is far from complete (5)
Recent studies have shown that AZA and its metabolites impair lymphocyte proliferation and induce apoptosis of human CD4+ T lymphocytes through inhibition of the Rac1 signaling pathway (4,6). Hence, AZA may temper disease activity via inhibition of the T lymphocyte compartment, which shows exaggerated activity in patients with Crohn’s disease (CD). Accordingly, induction of apoptosis of T-lymphocytes is linked with clinical remission of CD (7) However, in contrast to the anti-TNFα-antibody infliximab that induces both T cell apoptosis and rapid remission in CD (8). AZA is a slow-acting compound in CD, suggesting that its activity lies more in remission maintenance than in remission induction (9). Thus, further investigations as to its mode of action are called for.
Evidence that defects in innate immunity contribute to the pathogenesis of autoimmune disease is rapidly accumulating, especially for CD. Recent genome-wide association studies (GWAS) have identified more than 30 genes or loci associated to CD and most of them confer reduced activity in the innate immunity (10-12). Moreover, CD patients exhibit a phagocyte immunodeficiency that combines a primary macrophage defect and a secondary granulocytic defect (13,14). In CD patients, monocytes show lower phagocytic activity towards \textit{Candida albicans} as compared to monocytes obtained from healthy individuals (15). Furthermore, neutrophil recruitment and its bacterial clearance to \textit{E. coli} loading after acute trauma in the gut mucosa and skin are attenuated. Macrophages from these patients secreted reduced amounts of IL-8 and when IL-8 was exogenously applied to the infected sites, normal neutrophil recruitment was restored (16,17). These findings prompt investigations to the possible consequences of drug treatment on human innate immunity.
In the present study, we show that AZA is a highly effective inhibitor of Rac1 signaling in the monocyte compartment and that this inhibition results in enhanced innate functionality of these cells. Our data provide a molecular explanation as to the remarkable effects of AZA on human immunity and support the concept that deficiencies in innate immunity underlie the pathogenesis of CD.
MATERIALS AND METHODS

Isolation of human primary monocyte and 6-TG treatment

Heparinized blood was obtained from healthy volunteers and patients with Crohn’s disease after informed consent. Patients were identified through the gastroenterology outpatient clinics at University Medical Center Groningen. Patients were not receiving immunomodulatory medication and all had quiescent disease according to the Harvey-Bradshaw score. Human peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque gradients. Monocytes were further purified using CD14 monoclonal antibodies conjugated to microbeads according to the manufacturer’s protocol (Miltenyi Biotec, Germany). Cell cultures of primary monocytes were performed in RPMI-1640 medium (PPA laboratories, Austria) supplemented with 10% heat-inactivated FCS and 10 μg/ml Gentamicine at 37°C in 5% CO₂ humidified air. The in vitro 6-TG pretreatment for monocytes is 10 μM 30 minutes or other time points and concentrations where indicated. It has been shown that 10 μM 6-TG incubation results in a clinical relevant intracellular 6-TG level in human primary lymphocytes (2-4).

Phagocytosis assays

For phagocytosis assays, monocytes were plated at 1×10⁴ cells per ml on coverslips in 12-well plates and pretreated with/without 6-TG (Sigma-Aldrich) or NSC23766 (Calbiochem) for 30 minutes. Subsequently, cells were challenged with FITC-labeled Escherichia coli (E. coli) at a 1:5 cell-bacteria ratio for 5 minutes or other time points where indicated. Cells were washed with phosphate-buffered saline (PBS), and fixed in 4% formaldehyde before mounting in 50% PBS / 50% Glycerol. Phagocytosis was evaluated microscopically by counting both the number of monocytes exhibiting phagocytosis and the number of bacteria phagocytosed per monocyte. At least 300 cells were counted for each slide. The engulfment of E. coli was confirmed by confocal laser scanning microscopy (Fig. S1).

Cell stimulation and signaling transduction assays

Monocytes (1×10⁶/ml) were stimulated in the presence or absence of 6-TG for a given time with peptidoglycan (PGN, 30 μg/ml, Fluka) from Staphylococcus aureus, lipopolysaccharide (LPS, 2 Cg/ml, Sigma) purified by gel-filtration chromatography, or muramyl dipeptide (MDP, 10 μg/ml, Sigma). The levels of GTP-bound Rac1 were measured by G-LISA™ Rac1 activation assay biochem kit (Cytoskeleton, USA) according to the manufacturer’s protocol. The accuracy of G-LISA™ measurement was confirmed by a PAK domain pull-down based method previously established in our lab (Fig. S2) (18). The levels of PK2 phosphorylation were determined by western blots using anti-human PK2, phosphorylated (pSer20) antibody (1:1000 dilution; USBiological) and HRP-conjugated anti-rabbit Ig (1:2000 dilution; Cell signaling technology).

Immunofluorescence for monocyte spreading

For spreading assays, monocytes in the presence or absence of 6-TG 10 μM pre-incubation for 30 minutes were cultured with or without E. coli loading for 5 minutes and then fixed in 4% formaldehyde in PBS before permeabilized with 0.1% Triton X-100 in PBS. Cells were
stained with 25 \( \mu \text{g/ml} \) TRITC-conjugated phalloidin solution in PBS for 40 minutes and mounted in 50% PBS/50% glycerol. Digitized images of cells (at least 30 cells from each individual condition) acquired by confocal microscopy were analyzed with Image J software (19).

**Cytokine measurement**

Monocytes (2\( \times \)10\(^5\)/ml) were plated in 96-well plates and pretreated with 6-TG (10 \( \mu \text{M} \)) for 30 minutes. Subsequently, cells were stimulated with PGN (30 \( \mu \text{g/ml} \)) or vehicle. Culture supernatants were collected at 48 hours and analyzed by enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) for IL-8 or IL-10 production. The absolute concentrations of cytokine were normalized to the numbers of viable cells in each well, as determined by the MTT assay (20). In addition, IL-8 production was analyzed at mRNA levels by real-time PCR. Total RNA of monocytes was extracted at 48 hours using NucleoSpin® RNAII kit (Bioke, The Netherlands). IL-8 mRNA expression was determined using the assay-on-demand kit on the ABI PRISM 7700 (Applied Biosystems, Foster City, CA). 18S levels were used as endogenous control.

**PAK kinase activity in colonic biopsies**

Kinase activity in the colonic mucosa of CD patients (n=3) and control patients (colon polyps, n=3) towards p65PAK sensitive undecapeptides was determined by Pepscan presto array. The protocol is described in detail on the website (http://www.pepscanpresto.com/files/PepChip%20Kinase%20Lysate%20Protocol_v5.pdf) (21). Biopsies were lysed as described by Diks et al.. The experiments were performed using 3 CD patients and 3 control patients (polyps). Results were shown as the average values of spot intensities obtained from 9 kinase reactions (3 technical replicas from 3 individual patients).

**Statistical analysis**

Tests for statistical significance of differences were made by 2-tailed student t test using the program SPSS 16.0. Differences were considered significant when \( P < 0.05 \).

**RESULTS**

**6-TG enhances monocyte phagocytosis in healthy individuals and CD patients**

We first studied the effect of 6-TG on bacterial phagocytosis by monocytes, which is the key innate immune function of these cells. In order to optimize our experimental conditions, monocytes from healthy volunteers were incubated with various concentrations 6-TG (0 \( \mu \text{M} \), 10 \( \mu \text{M} \), 30 \( \mu \text{M} \), 60 \( \mu \text{M} \) and 100 \( \mu \text{M} \)) for 30 minutes and subsequently challenged with FITC-labeled *E. coli*. Phagocytosis was evaluated after a 5-minute and a 15-minute *E. coli* challenge. 6-TG dose-dependently enhanced monocyte phagocytotic activity at concentrations between 10 and 60 \( \mu \text{M} \) (Fig. S3). The stimulation of monocyte phagocyte function by 10 \( \mu \text{M} \) 6-TG was confirmed by analyzing monocytes from 18 healthy controls and 7 CD patients. Compared to vehicle condition, 6-TG significantly stimulated both the number of monocytes exhibiting phagocytosis, as well as the number of bacteria phagocytozed per monocyte (Fig.
6-TG efficiently inhibits Rac1 GTP-loading in monocytes

Rac1 plays a key role in monocyte biology, especially in the phagocytotic process, by modulating actin rearrangements. We analyzed the link between monocyte phagocytosis and 6-TG-modulated Rac1 activity, as it has been shown that 6-TG inhibits Rac1 in T-lymphocytes. In otherwise unchallenged monocytes, a 30 minute incubation with 6-TG strongly reduced both Rac1-GTP levels (Fig. 2 A) and autophosphorylation of its crucial downstream target p21 protein-activated kinase 2 (PAK2) (Fig. 2 B; n=5, P<0.05). In contrast,
exposure of T-lymphocytes to the same 6-TG treatment left the levels of PAK2 autophosphorylation unchanged (Fig. 2 B). Monocyte spreading, in which Rac1-mediated actin rearrangement plays a key role, was strongly reduced following 6-TG treatment (Fig. 2 C), again showing that such treatment interferes with Rac1 activation in monocytes. Upon stimulation of monocytes with bacterial cell wall constituents, Rac1-GTP levels and PAK2 autophosphorylation were consistently induced by the TLR2 ligand peptidoglycan (PGN) (Fig. 2 D; n=7, P<0.01) and to a lesser extent by the TLR4 ligand lipopolysaccharide (LPS) or the NOD2 ligand muramyl dipeptide (MDP). In the presence of 6-TG, PGN stimulation is no longer capable of inducing PAK2 autophosphorylation in monocytes (Fig. 2 E), while such an effect was not observed in T-lymphocytes. Thus, 6-TG is a potent Rac1 inhibitor in the monocyte compartment.

**Figure 2.** 6-TG reduces GTP-loaded Rac1 in monocytes from healthy individuals. (A) The levels of GTP-loaded Rac1 of monocytes were reduced by 6-TG (10 μM, 30 minutes) incubation compared to the untreated condition (n=3). (B) At basal level, 6-TG incubation (10 μM, 30 minutes) reduced autophosphorylation of PAK2 in monocytes (n=5, P<0.05), but not in lymphocytes (n=2). Results are shown as representative blots. (C) Monocyte spreading was reduced by 6-TG incubation. Data are presented as the mean ± SEM of individual monocytes from two independent experiments; * P<0.05. (D) Rac1-GTP levels and PAK2 autophosphorylation were consistently induced by the TLR2 ligand peptidoglycan (PGN, 30 μg/ml, 7 minutes) (n=7, P<0.01) and to a lesser extent by the TLR4 ligand lipopolysaccharide (LPS, 2 μg/ml, 5 minutes) or NOD2 ligand muramyl dipeptide (MDP, 10 μg/ml, 10 minutes). Results are shown as representative blots. (E) In the presence of 6-TG (10 μM ~ 100 μM), PGN stimulation is no longer capable of inducing PAK2 autophosphorylation in monocytes, while in lymphocyte compartment, PAK2 autophosphorylation remains unchanged. Results are
shown as representative blots of 4 independent experiments.

**Rac1 inhibition leads to enhanced monocyte phagocytosis**

An inverse relationship between cell spreading and the phagocytic activity of individual monocytes was observed in the 6-TG treatment experiments (Fig S4). Monocyte spreading requires active Rac1. This suggests that 6-TG-mediated Rac1 inhibition may lead to increased monocyte phagocytosis. To obtain independent evidence for this surprising finding, we exposed monocytes to the unrelated Rac1 activation inhibitor NSC23766 and studied its effect on phagocytosis. Consistent with the effect of 6-TG, NSC23766 enhanced monocyte phagocytosis of FITC-*E. coli* to a comparable extent as the 6-TG treatment, both for the number of phagocytosing monocytes and the total number of *E. coli* phagocytosed per 100 monocytes (Fig. 3). Thus, inhibition of Rac1 directly results in increased phagocytic activity of human monocytes.

![Figure 3](image)

**Figure 3.** The Rac1-specific inhibitor NSC23766 enhances monocyte phagocytosis. Monocytes isolated from 3 healthy individuals were preincubated with 5 μM NSC23766 for 30 minutes before exposure to FITC-labeled *E. coli*. Monocyte phagocytosis was enhanced by NSC23766, evaluated by both the number of monocytes exhibiting phagocytosis (A) and the number of bacteria phagocytosed per monocyte (B). Data are presented as the mean ± SEM where * P<0.05; *** P<0.001.

**Rac1/PAK2 overactivation in monocytes from Crohn’s disease patients**

The therapeutic effect of AZA in CD is routinely linked to repression of an overactivated adaptive immune compartment. Our data suggest that, in addition, AZA may stimulate the activity of innate immunity through repression of Rac1 signaling. To study the clinical relevance of the latter process, we analyzed the monocyte phagocytosis and the Rac1 activation status in CD patients. Monocytes from patients with CD and from age- and gender-matched healthy controls were analyzed in parallel. When monocytes were challenged with *E. coli* for 5-minute, the basal level of phagocytosis of monocytes from CD patients was lower than that from healthy controls (Fig. 4 A). In contrast, under unstimulated conditions, the
levels of PAK2 autophosphorylation in monocytes of CD patients were higher than those from healthy controls (Fig. 4 B). Moreover, colonic biopsies from CD patients and control patients were lysed and analyzed by in vitro kinase array. Colonic biopsies from CD patients displayed substantially higher PAK kinase phosphorylation compared to biopsies from control patients (Table 1). Taken together, our data suggest that increased Rac1 activity is relevant for the diminished monocyte phagocytic activity in CD. Importantly, 6-TG treatment inhibits phospho-PAK2 levels at both basal (Fig. 4 C) and PGN-stimulated (Fig. 4 D) conditions in monocytes from CD patients. Thus, Rac1 signaling is overactivated both in circulating monocytes and the intestinal mucosa of patients with CD, which is effectively suppressed by 6-TG.

Table 1. PAK kinase activities in the colonic mucosa of CD and control patients (polyps).

<table>
<thead>
<tr>
<th>PAK sensitive peptide</th>
<th>Control patients (n=3)</th>
<th>CD patients (n=3)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASRPSSRSRYV&lt;sup&gt;1&lt;/sup&gt;</td>
<td>61</td>
<td>81</td>
<td>0.0358</td>
</tr>
<tr>
<td>SRRPKSSLPPV&lt;sup&gt;2&lt;/sup&gt;</td>
<td>77</td>
<td>108</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Note: <sup>1</sup>p65PAK target peptide derived from vimetin, <sup>2</sup>p65PAK target peptide derived from myosin light chain kinase. Result numbers represent the average values of spot intensities obtained from 9 kinase reactions (3 technical replicas from 3 individual patients).

Figure 4. Diminished phagocytosis and overactivated Rac1/PAK2 signaling in monocytes from CD patients. (A) The basal levels of E. coli phagocytosis of monocytes from CD patients were significantly lower than those from age- and gender-matched healthy controls (pair-wise analysis, n=5, \( P < 0.05 \)). (B) The levels of PAK2 autophosphorylation in monocytes of CD patients (n=3) were higher than those from age- and gender-matched healthy controls (n=3).
Values are reported as the mean ± SEM of the intensities of phosphorylated PAK2 normalized by the intensities of GAPDH. (C,D) 6-TG treatment (10 µM, 30 minutes) inhibits phospho-PAK2 levels at both basal (n=3) and PGN-stimulated (n=2) conditions in monocytes from CD patients. Results are shown as representative western blots.

**6-TG increases IL-8 production by monocytes**

Monocytes of CD patients show impaired IL-8 production (16), a cytokine particularly relevant for granulocyte recruitment and thus innate immunity. We analyzed whether 6-TG also affects IL-8 production by monocytes. Figure 5A shows that even at unstimulated conditions, 6-TG incubation increased IL-8 production in monocytes (+45%, n=4, Fig. 5A). Upon PGN stimulation, a strong increase in production of both IL-8 and IL-10 by monocytes was observed (Fig. S5). Notably, 6-TG also provoked a superstimulation of PGN-dependent IL-8 production at both the protein level (+50%, n=7, P<0.05; Fig. 5A) and the RNA level (n=3, Fig. 5B). In contrast, PGN-stimulated production of IL-10 was reduced by 6-TG treatment (-6%, n=7, P<0.05; Fig 5C). Thus, also the cytokine profile induced by 6-TG in monocyte cultures is consistent with a stimulation of innate immunity.

**DISCUSSION**

Azathioprine (AZA) is a highly effective drug maintaining remission in Crohn’s disease (CD) and other autoimmune disorders, but its mechanism of action remains unclear. This study

**Figure 5.** 6-TG increases IL-8 production by monocytes. (A) At unstimulated conditions, 6-TG incubation (10 µM, 48 hours) increased IL-8 production in monocytes (+45%, n=4). Upon PGN (30ng/ml, 48 hours) stimulation, 6-TG incubation significantly increased IL-8 production in monocytes (+50%, n=7, P<0.05). (B) 6-TG incubation also stimulated PGN-dependent IL-8 production at RNA level (n=3). (D) In contrast, PGN-stimulated production of IL-10 was reduced by 6-TG treatment (-6%, n=7, P<0.05). Data are presented as the mean ± SEM.
shows that the active metabolite of AZA, 6-thioguanine (6-TG), stimulates monocyte phagocytosis and IL-8 production via inhibition of Rac1 activity. This implies that stimulation of monocyte-associated innate immunity is a crucial therapeutic effect of AZA in CD patients, which may be also its therapeutic action in other autoimmune diseases.

We observed that at clinical relevant concentrations 6-TG enhances monocyte phagocytosis and IL-8 production. This finding fits well with the emerging notion that diminished innate immunity is the primary defect in autoimmune diseases. In patients with CD, abnormally low neutrophil accumulation was observed after endoscopically-induced acute trauma in the intestinal mucosa as well as following the induction of epidermal shear trauma in the skin (16). Hence, patients with CD seem to suffer from generalized defects in neutrophil recruitment. This phenomenon is probably due to a defect of macrophage function, because macrophages from patients with CD secreted reduced amounts of IL-8 and when IL-8 was exogenously applied to the infected sites, normal neutrophil recruitment was restored (16,17).

Furthermore, in vitro experiments showed that monocytes isolated from CD patients had lower phagocytic activity towards *C. albicans* than those obtained from healthy individuals (15). The exact reason for the phagocyte dysfunction remains unclear, but has been proposed to derive from genetic defects, such as polymorphisms in autophagy-related genes that are involved in TLR receptor-mediated phagocytosis (23,24). Alternatively, some bacterial pathogens may directly cause phagocyte dysfunction. For example, the CD-associated fungal *Saccharomyces cerevisiae* mannan increased survival of *E. coli* within monocytes (25). Taken together, monocyte/macrophage dysfunction seems associated with the pathogenesis of CD-like autoimmunity. Thus, it is plausible to propose that AZA-derived 6-TG achieves its therapeutic effect, at least partially, by targeting this primary defect of innate immunity in these patients.

AZA and its metabolite 6-TG have been reported to induce apoptosis of primary human CD4+ T lymphocytes and to abrogate lymphocyte proliferation and thereby explaining the clinical effects (4,6). It is interesting to note, however, that the effects were limited to CD28-activated lymphocytes and not observed in resting lymphocytes. CD28 acts as the receptor of CD80/CD86 on monocytes. When activated by TLR ligands, CD80/CD86 expression is upregulated in monocytes and consecutively, through CD28 stimulation, activation of T lymphocytes ensues (26). Our finding of 6-TG-enhanced monocyte phagocytosis and IL-8 production opens the possibility that the 6-TG-dependent increased pathogen clearance results in CD80/CD86 downregulation, and consequently diminished lymphocyte activation. Furthermore, the induction of T lymphocyte apoptosis through 6-TG-mediated Rac1 inhibition was only observed after 5 days of culture (4). We observed that Rac1/PAK2 signaling in monocytes was more sensitive to 6-TG treatment than that in lymphocytes. A possible interpretation of our results is that for autoimmune disease, immunosuppression of the lymphocyte compartment provides only short-term clinical benefit, and prevention of new lesions depends on stimulation of the innate immune system rather than blank immunosuppression.
The most remarkable finding of this study is that inhibition of the Rac1/PAK2 signaling pathway, either by 6-TG or NCS23766, leads to enhanced monocyte phagocytosis. There are many reports that show that Rac1 is actually required for phagocytosis (27-29). However, recent data show that especially the cycling of Rac1 between its active (GTP-bound) and inactive (GDP-bound) state is crucial for efficient phagocytosis. It was shown that macrophages engulf apoptotic cells at their lamellipodia where active Rac1 is required to form phagocytic cups that consist of actin patches. After closure of the phagocytic cup, Rac1 is down-regulated leading to abrupt disappearance of the actin patches and closure of the phagocytic cup. Expression of a constitutive-active form of Rac1 (Q61L) leads to a delayed disassembly of the actin patch and closure of phagocytic cups, which strongly reduces the ability of macrophages to engulf apoptotic cells.30 In line with this observation, constitutive-active Rac1-V12 has been shown earlier to disrupt bacterial phagocytosis and suppress receptor endocytosis (31,32). The formation of lamellipodia requires the cycling of the Rac1-GTPase between the active and inactive forms, which is catalyzed by Dbl family guanine nucleotide exchange factors (GEFs). The Rac1 inhibitor NSC23766 significantly inhibits the binding of Rac1-specific GEFs including Trio and Tiam1 to Rac1, which in turn dose-dependently reduces Rac1-GTP level and the formation of lamellipodia under stimulation (33). Taken together, these data imply that the Rac1 activity needs to be balanced within a proper dynamic range and needs to cycle between an active and inactive state for optimal phagocytic activity. Our data show that Rac1 is overactivated in monocytes and the intestinal mucosa of patients with Crohn’s disease and thus may limit bacterial clearance leading to a chronic inflammatory reaction. It is plausible to predict that 6-TG sustains Rac1 activity of monocytes within a proper dynamic range, which facilitates phagocytosis. The dose-response effect of 6-TG on monocyte phagocytosis showing a gradual increase between 0 and 60 uM 6-TG and a subsequent decreased phagocytosis at 100 uM supports this proposed mechanism.

In conclusion, our findings provide a novel understanding on the therapeutic mechanism of 6-TG, which may hold for other immunosuppressive agents as well. By inhibiting the Rac1/PAK2 signaling pathway, 6-TG enhances monocyte-mediated innate immunity, which is considered the primary defect in CD.

REFERENCE


Figure S1. Analysis of monocyte phagocytosis of FITC-labeled *E. coli*. In the experiments of monocyte phagocytosis, the engulfment of *E. coli* by monocytes was analyzed by confocal microscope in randomly selected samples. Picture is shown as a representative cross section image obtained from confocal microscope.

Figure S2. Comparision of methods to determine Rac1 activation. The accuracy of G-LISA™ measurement for Rac1 activation assay is confirmed by another Rac1 activation assay based on PAK pull-down. (A) Rac1 GTP loading was measured by G-LISA™. (B) Rac1 GTP loading was measured by the PAK pull-down based Rac1 activation assay. The two measurements were performed using monocytes from 1 isolation. 6-TG reduced Rac1 GTP loading in a comparable extent when measured with the two methods.
Figure S3. 6-TG enhances monocyte phagocytosis in healthy individuals. Monocytes from healthy volunteers were incubated with various 6-TG concentrations (0 μM, 10 μM, 30 μM, 60 μM and 100 μM) for 30 minutes and subsequently challenged with FITC-labeled *Escherichia coli*. Monocyte phagocytosis was evaluated after 5-minute and 15-minute of *E. coli* loading. Phagocytosis was evaluated microscopically by counting the number of monocytes exhibiting phagocytosis (A) and the number of bacteria phagocytosed per monocyte (B). Compared to untreated conditions, 6-TG concentrations of 10 μM - 60 μM enhanced monocyte phagocytosis, while the non-physiologically high concentration of 100 μM 6-TG reduced monocyte phagocytosis.

Figure S4. Inverse correlation between monocyte spreading and the number of *E. coli* phagocytosed under 6-TG treatment. In the presence of 6-TG (30 μM, 30 minutes) preincubation, the number of engulfed *E. coli* and the spreading area of individual monocytes are inversely correlated (*P*<0.05). Data are from one representative experiment.
Figure S5. PGN significantly stimulates both IL-8 and IL-10 production by monocytes from healthy individuals. Monocytes from 3 healthy individuals were stimulated with PGN (30 µg/ml, 48 hours) in the presence or absence of 6-TG (10 µM, 48 hours). Absolute IL-8 concentrations in culture supernatants are shown in the upper panel. Absolute IL-10 concentrations in culture supernatants are shown in the lower panel.