Azathioprine Suppresses Ezrin-Radixin-Moesin-Dependent T Cell-APC Conjugation through Inhibition of Vav Guanosine Exchange Activity on Rac Proteins

Daniela Poppe, Imke Tiede, Gerhard Fritz, Christoph Becker, Brigitte Bartsch, Stefan Wirtz, Dennis Strand, Shinya Tanaka, Peter R. Galle, Xosé R. Bustelo and Markus F. Neurath

*J Immunol* 2006; 176:640-651; ;
doi: 10.4049/jimmunol.176.1.640
http://www.jimmunol.org/content/176/1/640

**References**
This article cites 65 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/176/1/640.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Azathioprine Suppresses Ezrin-Radixin-Moesin-Dependent T Cell-APC Conjugation through Inhibition of Vav Guanosine Exchange Activity on Rac Proteins

Daniela Poppe,1* Imke Tiede,1* Gerhard Fritz,† Christoph Becker,* Brigitte Bartsch,* Stefan Wirtz,* Dennis Strand,* Shinya Tanaka,‡ Peter R. Galle,* Xosé R. Bustelo,§ and Markus F. Neurath2*1

We have shown recently that the azathioprine metabolite 6-Thio-GTP causes immunosuppression by blockade of GTPase activation in T lymphocytes. In the present study, we describe a new molecular mechanism by which 6-Thio-GTP blocks GTPase activation. Although 6-Thio-GTP could bind to various small GTPases, it specifically blocked activation of Rac1 and Rac2 but not of closely related Rho family members such as Cdc42 and RhoA in primary T cells upon stimulation with oCD28 or fibronectin. Binding of 6-Thio-GTP to Rac1 did not suppress Rac effector coupling directly but blocked Vav1 exchange activity upon 6-Thio-GTP hydrolysis, suggesting that 6-Thio-GTP loading leads to accumulation of 6-Thio-GDP-loaded, inactive Rac proteins over time by inhibiting Vav activity. In the absence of apoptosis, blockade of Vav-mediated Rac1 activation led to a blockade of ezrin-radixin-moesin dephosphorylation in primary T cells and suppression of T cell-APC conjugation. Azathioprine-generated 6-Thio-GTP thus prevents the development of an effective immune response via blockade of Vav activity on Rac proteins. These findings provide novel insights into the immunosuppressive effects of azathioprine and suggest that antagonists of the Vav-Rac signaling pathway may be useful for suppression of T cell-dependent pathogenic immune responses. The Journal of Immunology, 2006, 176: 640–651.

Azathioprine, a mercaptopurine analog of adenine and hypoxanthine, was developed by Elion and Hitchings as a long-lived agent to improve the rate of inactivation of its parent drug, 6-mercaptopurine (6-MP)3 (1). Both azathioprine and 6-MP are immunosuppressive agents with potent anti-inflammatory functions that have been used in childhood leukemia, organ transplantation, and the treatment of autoimmune and chronic inflammatory diseases (2–5). In particular, azathioprine has been used therapeutically in kidney transplantation (4) and inflammatory bowel diseases (IBD), such as Crohn’s disease and ulcerative colitis (2, 6–8), and is considered the gold standard of organ transplantation, and the treatment of autoimmune and chronic inflammatory diseases (2–5). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Laboratory of Immunology, I. Department of Medicine, University of Mainz, Mainz, Germany; 2Laboratory of Molecular and Cellular Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and 3Centro de Investigación del Cáncer, University of Salamanca-Spanish Council for Scientific Research, Salamanca, Spain

Received for publication November 2, 2004. Accepted for publication October 10, 2005.


Markus F. Neurath2*

Copyright © 2005 by The American Association of Immunologists, Inc. 0022-1767/05/$02.00

*Laboratory of Immunology, I. Department of Medicine, and 1Department of Toxicology, University of Mainz, Mainz, Germany; 2Laboratory of Molecular and Cellular Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and 3Centro de Investigación del Cáncer, University of Salamanca-Spanish Council for Scientific Research, Salamanca, Spain

Received for publication November 2, 2004. Accepted for publication October 10, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 D.P. and I.T. contributed equally to this manuscript.
2 Address correspondence and reprint requests to Dr. Markus F. Neurath, Laboratory of Immunology, I. Department of Medicine, University of Mainz, Langenbeckstrasse 1, 55101 Mainz, Germany. E-mail address: neurath@1-med.klinik.uni-mainz.de
3 Abbreviations used in this paper: 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; ERK, extracellular-signal-regulated kinase; PAK, p21-activated kinase; ERM, ezrin-radixin-moesin; PEBM, phospho-ERM; IBM, inflammatory bowel disease; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; GEF, guanosine exchange factor; PAK, p21-activated kinase.

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/$02.00

Downloaded from http://www.jimmunol.org/ by guest on May 1, 2017
major role in T cell development, differentiation, cytokine production, apoptosis, and proliferation (18–20). Whereas RhoA is critical for pre-T cell differentiation and proliferation (17, 21), Cdc42 regulates T cell polarization toward APCs via filopodia and controls chemokine-induced lymphocyte migration (22). Furthermore, Rac1 and Rac2 proteins have been shown to modulate T cell differentiation and cytokine production (18, 19). In fact, dominant-positive Rac mutations have been associated with increased cell proliferation and tumors, whereas functionally inactive Rac mutations are associated with immunodeficiencies and reduced T cell cytokine production (17, 18).

Although 6-Thio-GTP has been shown to suppress Rac1 activation in T lymphocytes (15), the molecular mechanism by which 6-Thio-GTP causes Rac1 blockade remained unknown. In the present study, we have identified the molecular mechanism by which 6-Thio-GTP blocks GTPase activation. Binding of 6-Thio-GTP to Rac1 did not suppress effector activation directly but blocked Vav exchange activity upon hydrolysis, suggesting that 6-Thio-GTP loading leads to accumulation of 6-Thio-GDP-loaded, inactive Rac proteins over time by inhibiting Vav guanosine exchange activity. At weak TCR signal intensity, blockade of Vav-mediated Rac1 activation led to a blockade of ezrin-radixin-moesin (ERM) dephosphorylation in primary T cells and suppression of T cell-APC conjugation. Azathioprine-generated 6-Thio-GTP thus prevents T cell-APC conjugation and the subsequent development of an effective immune response via blockade of Vav activity on Rac proteins.

Materials and Methods

Isolation of primary CD4+ T lymphocytes

Human PBMC from healthy volunteers (age, 25–42 years) were isolated using Ficoll-Hypaque gradients. PBMC were further purified using CD4 mAbs attached to immunomagnetic beads according to the protocol provided by the manufacturer followed by treatment with Detachbead (obtained from Dynal Biotech). Reanalysis of sorted populations revealed a purity of >96%.

T cell culture

Because primary T cells will die when left unstimulated for several days, we performed T cell stimulation as follows: T lymphocytes were stimulated in complete RPMI 1640 medium (15, 23) for 2–5 days with coated Abs to CD3 (BD Pharmingen; HIT3a) and soluble CD28 Abs (BD Pharmingen; 1 μg/ml) plus IL-2 (R&D Systems; 40 U/ml). Anti-CD3 concentrations varied between 0.04 μg/ml (high TCR signal intensity) and 0.001 μg/ml (low TCR signal intensity). Azathioprine, 6-MP, and 6-TG (Sigma-Aldrich) for 40 min. T cells were isolated in complete RPMI 1640 medium (15, 23) for 2–5 days with coated Abs to CD3 (BD Pharmingen; HIT3a) and soluble CD28 Abs (BD Pharmingen; 1 μg/ml) plus IL-2 (R&D Systems; 40 U/ml). Anti-CD3 concentrations varied between 0.04 μg/ml (high TCR signal intensity) and 0.001 μg/ml (low TCR signal intensity). Azathioprine, 6-MP, and 6-TG (Sigma-Aldrich) or 6-Thio-GTP (JenaBioscience) were added to the T cell cultures at indicated time points at a final concentration of 5 μM. This concentration of azathioprine has been previously shown to result in intracellular 6-TG levels that are comparable to those reported in leukocytes of patients with Crohn’s disease receiving long-term azathioprine or 6-MP treatment (14, 15). To determine the influence of IFN-γ, IL-2, and IL-4 on Rac1 activation in our experimental system, neutralizing Abs against IFN-γ (Acris; 2.5 μg/ml), IL-2 (Acris; 1 μg/ml), or IL-4 (Acris; 0.1 μg/ml) were added to the cell culture.

For fibronectin-mediated stimulation, human CD4+ T cells were isolated and treated with 6-Thio-GTP (JenaBioscience) and IL-2 (40 U/ml) for 3 days, as described in the previous paragraph. On day 3, T cells were stimulated with coated fibronectin (10 μg/ml; Sigma-Aldrich) for 40 min.

T cell proliferation

Proliferation was determined in 200 μl of culture medium containing primary human CD4+ T cells. Cells were stimulated with IL-2, anti-CD3, and anti-CD28 Abs and treated with azathioprine (5 μM) or left untreated. Cells were pulsed with [3H]thymidine (0.25 μCi/well) before analysis of thymidine incorporation by scintillation counting.

FACS analysis

FACS analysis (FACScan; BD Biosciences) was performed using FITC-labeled murine Abs to human CD4 (BD Pharmingen) and anti-mouse IgG1 isotype control Abs (BD Pharmingen). Apoptotic cells were detected by staining with annexin V and propidium iodide using the Annexin V<sup>FITC</sup> Apoptosis Detection Kit I (BD Pharmingen) (15, 23).

ELISA analysis

To measure cytokine production, human CD4+ T lymphocytes were isolated from peripheral blood and stimulated with Abs against CD3 and CD28 plus IL-2 in the presence or absence of 6-MP. After 3 days, culture supernatants were removed and assayed for cytokine concentration. Cytokine concentrations were determined by ELISA. ELISA was performed using the PeliKine-compact ELISA kit for IL-4 and the BD OptEIA kit for IFN-γ according to the manufacturer’s protocols.

Western blots

Cellular proteins were isolated as previously described (15). Western blots were made using specific anti-Rac1, anti-Rac2, anti-Cdc42, anti-Ras, and anti-RhoA Abs (Santa Cruz Biotechnology; 1/1000 dilution), anti-actin Ab (Biocarta), anti-Vav Ab (BD Transduction Laboratories), HRP-linked anti-mouse Ig (DakoCytomation; 1/1000 dilution), HRP-linked anti-rabbit Ig (DakoCytomation; 1/1000 dilution), and the ECL Western blotting analysis system (PerkinElmer).

Densitometric analysis of blots

Western blots were scanned and densitometry was performed using the ChemiImager 5500 software (Alpha Innotech) as previously described (24).

Analysis of 6-Thio-GTP binding to GTPases

To analyze the capacity of 6-Thio-GTP to bind to GTPases, chemically synthesized 6-Thio-GTP (obtained from JenaBioscience) was used. Recombinant GST-proteins were incubated with 5 μM radiolabeled GTP (0.5 μCi [3H]GTP, obtained from Amersham Biosciences) and increasing amounts of 6-Thio-GTP (20, 100, or 500 μM). Incubation was performed for 10 min at 30°C in 100 μl of buffer C containing 50 mM Tris (pH 7.5), 12.5 mM MgCl₂, and 750 mM NaCl (Sigma-Aldrich) in the presence of 10 nM EDTA (Sigma-Aldrich). After incubation, 10 mM MgCl₂ was added and the probes were put on ice. After addition of 900 μl of ice cold buffer C, the probes were pushed through filter-membranes (glass microfiber filters GF/C circles 25 mm; obtained from Whatman) and washed with 40 ml of buffer C. Finally, scintillation counting was performed.

Rac, Ras, Cdc42, Rap, and RhoA activation assays

Levels of Rac1-, Rac2-, Cdc42-, RhoA-, Rap-, and Ras-bound GTP were determined by established immunoblot techniques (Upstate Biotechnology) (15, 25). In brief, T cells were stimulated for indicated periods of time in the presence or absence of azathioprine, 6-MP, or 6-TG. According to the Upstate Biotechnology kit protocols, cell lysates were incubated with 10 μg/ml p21-activated kinase (PAK)-1 agarose (Rac/Cdc42 assays), 10 μg/ml Raf RBD agarose (Ras assay), 40 μg/ml Raf GDS-RBD (Rap assay), or 50 μg/ml Rhoelkin RBD-agarose (Rho assay) for 60 min (Rac/Cdc42/Efta assays), 45 min (Ras assay), or 50 min (Ras assays) at 4°C. Agarose beads were collected by centrifugation following by denaturation, boiling of the samples, and SDS-PAGE analysis. Proteins were transferred to nitrocellulose membranes and incubated with 1 μg/ml murine anti-human Rac1, Rac2, RhoA, Cdc42, Rap, or anti-human Ras (clone Ras10) Abs overnight at 4°C, followed by detection with goat anti-mouse HRP-conjugated IgG (1/1000 dilution) and the ECL detection system.

Immunofluorescence staining

Freshly isolated CD4+ T cells were plated on poly-L-lysine (0.1 mg/ml; obtained from Sigma-Aldrich) and CD3-coated chamber-slides (Nunc), and stimulation was performed as described above (23, 24). The medium was removed after 5 days of incubation, and the cells were fixed with 4% PFA for 15 min at room temperature. Upon washing in PBS/0.1% Tween 20 samples were treated with 3% BSA in 1× PBS/0.1% Tween 20 for 30 min at room temperature. Upon removal of the BSA solution, cells were incubated with Abs against Rac1, RhoA, Cdc42, Ras (Santa Cruz Biotechnology; 1/200 dilution), and Vav (BD Transduction Laboratories; 1/200 dilution) for 2 h at room temperature. Secondary Abs used were Cy3 (cy3-conjugated affinity pure goat anti-mouse or Cy3 (cy3-conjugated affinity pure goat anti-rabbit (1/200 dilution for 1 h, obtained from Jackson Immunoresearch Laboratories). Before examination, the nuclei were counterstained with Hoechst 3342 (Molecular Probes).

Analysis of filopodia and lamellipodia

Chamber slides were coated with fibronectin (10 μg/ml) and incubated for 30 min at 37°C. CD4+ T cells cultured for 4 days in the presence or
absence of azathioprine were harvested and centrifuged, and the pellet was resuspended in RPMI 1640 (Cambrex) stimulation-medium containing CD28 (Acris; 1 μg/ml) and CD3 (BD Pharmingen; HTTsA 0.04 μg/ml) Abs, PMA (50 ng/ml) and PHA (50 ng/ml). The cell solution was plated on the fibronectin-coated chamber slides and then incubated for 45 min at 37°C. Cells were fixed in 4% PFA (15 min, room temperature), washed in PBS, and treated with 3% BSA in PBS/0.1% Tween 20 for 15 min at room temperature. After washing in PBS and 0.1% Triton X-100 (Sigma-Aldrich) for 20 min, Texas Red-X-phalloidin (20 min, room temperature) was used for F-actin staining, while Hoechst 33342 (1/10,000 dilution for 5 min) was used for nuclear counterstaining. Quantification of the number of lamellipodia was performed on 10–15 high-power fields per conditions at ×400.

**Transient transfection assays**

Transformation of the Vav expression plasmid (obtained from X. Bustelo, Centro de Investigacion del Cancer, University of Salamanca-Spanish Council for Scientific Research, Salamanca, Spain) was performed with Solo-Pack Gold Competent cells (Stratagene) and plasmid preparation was performed with the Qiagen Plasmid Maxi Prep kit (Qiagen) according to the manufacturer’s protocol. The transient transfection of the Vav plasmid was performed with the human T cell Nucleofector kit (Amaxa Biosystems) according to the manufacturer’s protocol for unstimulated human T cells. A total of 2–7 × 10⁶ CD4⁺ T cells isolated with Dynal beads (Dynal Biotech) frombuffy coats was transfected with the Vav vector or an empty control vector (pDNAx). Four hours after transfection, the cells were stimulated with IL-2 (R&D Systems; 40 U/ml) and anti-CD3/28 Abs in the presence of 6-MP (5, 10, or 50 μM) or 6-Thio-GTP (5, 10, or 50 μM). Cells were lysed after 3 days of incubation followed by a pull-down of Rac using PAK-1 PBD agaro (Rac activation assay kit; Upstate Biotechnology).

**Immunoprecipitation**

CD4⁺ T cells were lysed on ice in RIPA buffer (10 mM Tris (pH 8.0), 140 mM NaCl; obtained from Carl Roth), 1% Triton X-100, 1% deoxycholate and 0.1% SDS (Sigma-Aldrich) for 10 min. Cleared substrates were normalized for protein content using the Bio-Rad protein assay (Bio-Rad). Proteins were immunoprecipitated with protein A/G plus agarose (Santa Cruz Biotechnology) coupled with Abs against Rac1, Cdc42, Rho A, or Vav (Santa Cruz Biotechnology) at 4°C overnight. Immunoprecipitates were washed twice in RIPA buffer, boiled for 5 min, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked 1 h at room temperature in PBS-Tween 20 containing 5% nonfat dry milk (ApplChem). Membranes were incubated overnight at 4°C with a rabbit polyclonal Vab Ab (BD Transduction Laboratories; dilution 1/200) or with a rabbit phosphoVav Ab (Ref. 26; dilution 1/5000). An anti-rabbit HRP-labeled Ab was used as secondary Ab (dilution 1/1000). Proteins were visualized by ECL (PerkinElmer).

**Analysis of phospho-ERM (pERM) levels**

Isolated CD4⁺ T cells were stimulated with anti-CD3/CD28 Abs (BD Biosciences) plus IL-2 (Strathamnn Biotech) and treated with 6-MP for 3 days. In contrast to long term culture of T cells in the presence of 6-MP, such short time stimulation in the presence of 6-MP did not result in T cell apoptosis (15). At day 3, cells were restimulated for 1 min with 10 μg of anti-CD3 per milliliter and 1 μg of anti-CD28 per milliliter and were then fixed for 15 min at 4°C with 4% paraformaldehyde (Sigma-Aldrich). Fixed cells were washed twice in PBS before permeabilization with 0.1% Triton X-100 in PBS. One microliter of rabbit anti-phosphoAb (Cell Signaling Technology) was added per 1 × 10⁶ cells in 100 μl of 0.1% Triton X-100 in PBS, and cells were incubated for 30 min on ice. After incubation, cells were washed twice in 0.1% Triton X-100 in PBS followed by addition of 4 μl of FITC-conjugated anti-rabbit Ig (DukOcytation) for 30 min on ice. Finally, cells were washed three times in PBS and analyzed by flow cytometry.

**Analysis of T cell-APC conjugates**

Isolated CD4⁺ T cells were stimulated in the absence of APCs with anti-CD3/CD28 Abs plus IL-2 and treated with azathioprine or 6-MP for 3 days. At day 3, the primary T lymphocytes were stained with CellTracker green (Molecular Probes). Accordingly, cells were resuspended in 1 μM CellTracker green in serum-free RPMI 1640 medium and incubation was performed for 30 min at 37°C. After a second incubation of the stained cells in 30 min serum-free RPMI 1640 medium for 30 min at 37°C, cells were resuspended in RPMI 1640 medium plus 10% FCS. Furthermore, Raji B cells were stained with CellTracker green (Molecular Probes) in the same way and incubated with 5 pg/ml superantigens (staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB)). Stained CD4⁺ T cells and Raji cells were mixed at a 1:1 ratio and stimulated with 5 pg/ml superantigens (SEA, SEB) plus 0.04 μg/ml anti-CD3 Abs and 1 μg/ml anti-CD28 Abs or with 5 pg/ml superantigens (SEA, SEB) alone. Formation of conjugates was analyzed by flow cytometry. The percentage of conjugates was determined as follows: (the number of CellTracker green “CellTracker orange” double-positive cells)/(total cell number) × 100.

**Analysis of cell apoptosis by annexin and propidium iodide staining**

To determine induction of apoptosis in primary T lymphocytes, cells were stimulated and coincubated with 6-TG or 6-Thio-GTP (5 μM) followed by FACS analysis. In these experiments, apoptotic T cells were detected by staining with annexin V and propidium iodide using the Annexin V FITC Apoptosis Detection Kit I (BD Pharmingen) (15). In brief, T cells were washed twice in PBS, and the pellet was resuspended in annexin V binding buffer (BD Pharmingen) at a concentration of 10⁶ cells per milliliter. Annexin V⁷/⁷ and propidium iodide were added (5 μl of each per 10⁶ cells). Samples were gently mixed and incubated for 15 min at room temperature in the dark before FACS analysis.

**Protein purification and GDP/GTP exchange assays using Vav**

The hexahistidine-tagged Vav3 (Δ1–144) protein or Vav1 was purified from Spodoptera frugiperda SF9 cells upon infection with baculoviruses, as previously described (27). GST-Rac1 was purified from E. coli using affinity chromatography onto glutathione-coated beads (Amersham Biosciences; as indicated in Schuebel et al. (28)). Exchange reactions in the presence of [γ-35S]GTP were performed as previously described (26). Loading of GST-Rac1 with GTP (Sigma-Aldrich) or 6-Thio-GTP was done as described by Movilla and Bustelo (27).

**Statistical analysis**

Tests for significance of differences were made by Student’s t test using the program StatWorks.

**Results**

Azathioprine-generated 6-Thio-GTP binds to a broad spectrum of small GTPases but specifically blocks Rac1 and Rac2 activation only

We have shown previously that azathioprine-generated 6-Thio-GTP binds to the small GTPase Rac1 and that such binding is followed by blockade of Rac1 activity (15). In contrast, 6-Thio-GTP showed little or no binding to Ras and failed to suppress Ras activation, suggesting the possibility that the selective binding of 6-Thio-GTP to GTPases is sufficient to explain its specificity for Rac1 suppression (15). To further explore this hypothesis, we tested the capacity of 6-Thio-GTP to bind to a broad spectrum of GTPases. Surprisingly, 6-Thio-GTP was able to bind to recombinant Rac1 under in vitro conditions as well as to several other Ras GTPases or GTPases from different families including Ran, Rab, RhoA, Cdc42, Rac2, and Rac3 (Fig. 1A). Furthermore, we observed that 6-Thio-GTP-bound Rac1 was able to bind to PAK (Fig. 1B), suggesting that the effector coupling of Rac1 is not directly inhibited by its binding to 6-Thio-GTP.

We next tested the capacity of azathioprine and 6-Thio-GTP to specifically suppress the activation of small GTPases of the Rho family in primary human CD4⁺ T lymphocytes. Accordingly, purified CD4⁺ T lymphocytes were stimulated with anti-CD3 and anti-CD28 Abs for 3 days in the presence or absence of azathioprine and its metabolites (5 μM) followed by analysis of GTP-bound GTPTases. As shown in Fig. 2, A and B, treatment with azathioprine and its metabolites 6-MP and 6-TG significantly suppressed the activity of Rac1 and the closely related GTPase Rac2. However, such treatment had little or no effects on basal activation of other Rho family members such as Cdc42 and RhoA, suggesting a marked specificity of azathioprine-generated 6-Thio-GTP to modulate activity of Rac proteins. These findings were not associated with changes in the expression levels of GTPases, as shown by immunofluorescence and Western blotting studies on primary CD4⁺ T lymphocytes (Fig. 2, C and D). Finally, we observed that...
Azathioprine and its metabolites block inactivation of ERM proteins and subsequent T cell-APC conjugation

In further studies on the functional consequences of 6-Thio-GTP-mediated blockade of Rac activation in T cells, we focused on ERM as well as Rap1 proteins that are involved in integrin-mediated adhesion, cell spreading, and T cell activation (31–41). As shown in Fig. 4A, no Rap1 activation was noted in our experimental system. Therefore, in subsequent studies, we focused on the effects of azathioprine metabolites on ERM proteins and subsequent T cell-APC conjugation. As Rac1 has been recently shown to control rapid inactivation of ERM proteins in T cells after Ag recognition (42), we determined in an initial series of studies the capacity of azathioprine and its metabolites to modulate ERM phosphorylation. Accordingly, primary CD4+ T lymphocytes were stimulated with anti-CD3/CD28 Abs in the presence or absence of 6-MP for 3 days followed by analysis of pERM levels. Interestingly, CD28 costimulation suppressed pERM levels, whereas 6-MP treatment blocked down-regulation of pERM levels upon CD28 costimulation in primary T lymphocytes (Fig. 4B), suggesting that 6-MP blocks Rac1-dependent pERM dephosphorylation.

Azathioprine treatment blocks formation of lamellipodia in primary CD4+ T lymphocytes

Next, we wanted to determine the functional consequences of Rac blockade in primary T cells upon azathioprine treatment. In these studies, purified CD4+ T lymphocytes were stimulated with anti-CD3 plus anti-CD28 Abs for 3 days in the presence or absence of azathioprine and its metabolites 6-MP or 6-TG. Interestingly, analysis of filopodia formation using F-actin staining showed no changes upon azathioprine treatment (Fig. 3A), suggesting that CD28-induced filopodia in primary T cells are controlled by other GTPases such as Cdc42 (29) rather than Rac proteins. As previous studies suggested that Rac proteins regulate cytoskeletal organization and lamellae in lymphocytes (30), we subsequently determined lamellipodia formation upon azathioprine treatment. It was found that CD28-dependent lamellipodia formation was suppressed by azathioprine treatment (Fig. 3B). Similarly, treatment with 6-MP, 6-TG, or 6-Thio-GTP significantly inhibited such lamellipodia formation, suggesting that azathioprine-generated 6-Thio-GTP blocks Rac1 activation and, subsequently, Rac1-dependent lamellipodia formation in primary T cells.

As azathioprine suppressed Rac2 activation and Rac2 is known to control IFN-γ cytokine production by T cells (18), we next determined cytokine production by primary T cells upon 6-MP treatment. As shown in Fig. 3C, 6-MP treatment significantly suppressed production of the Th1 cytokine IFN-γ by primary CD4+ T cells, whereas production of the Th2 cytokine IL-4 was increased. Taken together, these data suggested that 6-Thio-GTP-induced blockade of Rac GTPases leads to suppression of CD28-dependent lamellipodia formation and Th1 cytokine production.

Azathioprine-generated 6-Thio-GTP binds to various small GTPases and fails to inhibit binding of Rac1 to its effector PAK A. Competition of GT binding to various small GTPases by 6-Thio-GTP. Recombinant GTPases were incubated with radiolabeled GTP ([3H]GTP) and increasing amounts of 6-Thio-GTP followed by analysis of [3H]GTP-bound GTPases. In contrast to Ras, 6-Thio-GTP led to a concentration-dependent suppression of [3H]GTP-bound RhoA, Cdc42, Rab, Ran, Rac1, Rac-2 and Rac-3, indicating binding of 6-Thio-GTP to these small GTPases. B. 6-Thio-GTP-bound Rac1 binds to PAK. Extracts from unstimulated rat hepatoma cells were loaded with GDP (2.5 mM), GTP (250 μM), or 6-Thio-GTP (5 mM) for 15 min followed by immunoprecipitation with GST-PAK and Western blot analysis with Rac1-specific Abs. One representative experiment is shown. Similar data were obtained with Jurkat T cells (data not shown). 6-Thio-GTP preloading had no effects on binding of Rac1 to its effector PAK.

In our experimental system with primary T cells, azathioprine and 6-MP required 3 days to suppress Rac activation upon CD28 costimulation in the presence of IL-2 (15). To determine whether the suppression of Rac1 activation induced by 6-MP depends on IL-2 or other proinflammatory cytokines secreted by T cells, we performed Rac1 activation assays in the presence of anti-cytokine Abs (Fig. 2H). Interestingly, anti-IL-4, anti-IL-2, and anti-IFN-γ Abs had no effects on 6-MP-mediated suppression of Rac1 activation excluding the possibility that these cytokines control 6-MP-induced modulation of Rac1 activation in our experimental system. Finally, when using alternative stimulation pathways with fibronectin in our experimental system with primary T cells, we found that integrin engagement activated RhoA and Cdc42 (Fig. 2I). 6-Thio-GTP had little effects on fibronectin-induced Rho- and Cdc42 activation (Fig. 2I). However, azathioprine led to reduction of Rac1 activation upon integrin engagement (Fig. 2J). Thus, azathioprine and its metabolites suppress Rac rather than RhoA or Cdc42 activation in primary T cells.
FIGURE 2. GTPase activation assays in activated and azathioprine-treated primary T cells. A, CD4+ T lymphocytes were isolated from peripheral blood and stimulated with Abs to CD3, CD28, and rIL-2 in the presence or absence of indicated concentrations of azathioprine or its metabolites 6-MP and 6-TG for 3 days, as indicated. GTP-bound Rac1, Rac2, Ras, Cdc42, or RhoA was immunoprecipitated as specified in Materials and Methods followed by Western blotting to determine GTPase activation. Treatment with azathioprine or its metabolites led to a reduction of CD28-dependent Rac1 and Rac2 activation, whereas activation of Ras, Cdc42, and RhoA was virtually unaffected. One representative experiment of three to five per GTPase is shown. No marked change in T cell proliferation was noted by azathioprine treatment as compared with unstimulated cells in our experimental system (5432 cpm/10^6 cells vs 5713 cpm/10^6 cells). B, Densitometry of GTPase activation assays. Western blot signals for Rac1, Rac2, Ras, Cdc42, or RhoA were quantified by densitometry. Data are reported as percentage of expression as compared with the signal obtained in anti-CD3 plus anti-CD28-stimulated primary CD4+ T lymphocytes (100%). Data represent average values ± SD of three to five experiments per group. Statistically significant differences (*, p < 0.01; **, p < 0.001) are indicated. C, Azathioprine and its metabolites 6-MP and 6-TG induce Vav-1 accumulation in anti-CD3- plus anti-CD28-stimulated primary T cells. Purified CD4+ T lymphocytes were stimulated with Abs to CD3 plus CD28 in the presence or absence of azathioprine, 6-MP, or 6-TG for 3 days. Cells were immunostained with Rac1, Ras, RhoA, Cdc42, and Vav-1-specific Abs, as indicated. Confocal microscopy showed that CD28-induced accumulation of the Rac-associated GEF Vav-1 was increased by 6-MP, 6-TG, and azathioprine treatment, whereas the expression of the small GTPases Ras, Rac1, RhoA, and Cdc42 was unchanged. One representative experiment of three is shown. D, Expression of GTPases in T lymphocytes upon azathioprine treatment. Western blot analysis for Rac1, Rac2, Ras, RhoA, and Cdc42 was unchanged. For actin controls, blots were stripped and reincubated with an anti-actin Ab. E, 6-Thio-GTP specifically suppresses Rac1 and Rac2 activation but not activation of Cdc42. Purified CD4+ T lymphocytes were stimulated with Abs to CD3 plus CD28...
Azathioprine-generated 6-Thio-GTP causes a specific blockade of Vav guanosine exchange activity on Rac1 in primary T lymphocytes

As azathioprine blocks the Vav-Rac1 pathway controlling ERM phosphorylation and T cell-APC conjugation, we next studied the molecular mechanism of azathioprine-mediated blockade of the Vav-Rac1 pathway. Accordingly, we immunoprecipitated Rac1 and quantified the amount of coprecipitated Vav by Western blot analysis. Treatment of primary CD4 T cells with azathioprine, 6-MP, and 6-Thio-GTP led to reduced binding of Vav to Rac1, while...
FIGURE 3. Azathioprine and its metabolites suppress lamellipodia but not filopodia formation in primary CD4⁺ T lymphocytes. A, Azathioprine, 6-MP, and 6-TG do not suppress the formation of filopodial extensions enriched in F-actin in human CD4⁺ T lymphocytes. Purified CD4⁺ T lymphocytes were stimulated with Abs to CD3 plus CD28 in the presence or absence of azathioprine, 6-MP, and 6-TG for 5 days. After fixation and permeabilization, F-actin was visualized on cytospins by staining with phalloidin-Texas Red. Staining of cytoskeletal β-actin served as a control. Confocal microscopy showed that CD28-induced formation of filopodia (arrowheads) was not affected by azathioprine treatment. B, Effects of azathioprine on lamellipodia formation. CD4⁺ T lymphocytes were stimulated with anti-CD3 plus anti-CD28 Abs and cultured for 4 days in the presence or absence of azathioprine, 6-MP, 6-TG, or 6-Thio-GTP. Next, T cells were allowed to adhere to human fibronectin followed by staining for F-actin with Texas Red-X-phalloidin. Hoechst 33342 was used for nuclear counterstaining. One representative picture per group of three independent experiments is shown. Quantification of the number of lamellipodia (arrow) was performed on 10–15 high-power fields per condition. Statistically significant differences (*, p < 0.01) are indicated. Azathioprine, 6-MP, 6-TG, and 6-Thio-GTP led to a significant suppression of lamellipodia formation by anti-CD3 plus anti-CD28-stimulated T lymphocytes. C, Cytokine production by T cells. Purified CD4⁺ T lymphocytes were stimulated with Abs to CD3 plus CD28 in the presence or absence of 6-MP for 3 days. Culture supernatant were analyzed for content of IFN-γ and IL-4 by ELISA. The mean values ± SEM from five independent experiments are shown (*, p < 0.01).
these metabolites caused increased binding of Vav to RhoA (Fig. 5A) in the presence of augmented Vav-1 and pVav levels upon such treatment (Fig. 2). Consistent with this finding, transfection of a Vav expression vector led to partial reconstitution of Rac1 activity in T lymphocytes treated with physiological doses of 6-MP or 6-Thio-GTP (5 μM; Fig. 5, B and C), suggesting that azathioprine-generated 6-Thio-GTP modulates binding of Vav to the Rac1 GTPase. However, a further increase of 6-MP or 6-Thio-GTP to unphysiologic doses (10–50 μM) caused unchanged or even elevated Rac1 activation levels when Vav1 was overexpressed (Fig. 5C), possibly due to the generation of very high levels of 6-Thio-GTP-bound Rac1 with intact effector coupling and reduced levels of 6-Thio-GDP-bound Rac1.

To test directly the capacity of 6-Thio-GTP to block Vav guanosine exchange activity on Rac1, we coincubated recombinant Rac1, radiolabeled GTP, and recombinant Vav proteins in the presence or absence of 6-Thio-GTP followed by analysis of GTP incorporation. As shown in Fig. 6A, 6-Thio-GTP inhibited Vav

FIGURE 4. Effects of azathioprine and 6-MP on ERM phosphorylation and T cell-APC conjugation. A, Purified CD4+ T lymphocytes were stimulated with indicated stimuli in the presence or absence of azathioprine or 6-Thio-GTP for 3 days followed by Rap1 activation assays. Whereas a strong Rap1 activation signal was obtained with Jurkat T cells, no strong signal was found in our experimental system with primary T cells at day 3 of culture probably due to the fact that CD3 stimulation causes transient Rap1 activation only and that previous CD28 stimulation suppresses Rap1 activation, as previously shown by Reedquist and Bos (65). B, Purified CD4+ T lymphocytes were stimulated with Abs to CD3 and CD28 in the presence or absence of 6-MP for 3 days. Cells were stained with pERM-specific Abs before flow cytometry. One representative experiment of three is shown. CD28 costimulation led to down-regulation of pERM levels. Furthermore, 6-MP treatment inhibited CD28-induced down-regulation of pERM levels in primary CD4+ T lymphocytes. C, Analysis of T cell-APC conjugates upon azathioprine and 6-Thio-GTP treatment. Purified CD4+ T lymphocytes were stimulated with Abs to CD3 plus CD28 in the presence or absence of 6-Thio-GTP for 3 days. CellTracker green-labeled T cells were then placed in contact with CellTracker orange-labeled APCs prepulsed with superantigens (SEA, SEB) in the presence (upper panels) or absence (middle panels) of anti-CD3/CD28 Abs. The percentages of conjugates were analyzed by flow cytometry. One representative experiment is shown. To exclude increased 6-Thio-GTP-dependent T cell apoptosis at day 3 in same experiment, purified CD4+ T lymphocytes were analyzed for apoptosis by FACS using annexin-V/propidium iodide staining (lower panels). No apoptosis was seen at day 3 of cell culture. D, Quantitative analysis of the effects of azathioprine and 6-MP on T cell/APC conjugates in five experiments. The azathioprine- and 6-MP-dependent decrease of T cell-APC conjugates as compared with untreated cells was determined in all experiments and is shown as mean values ± SEM.
guanosine exchange activity on Rac1. Furthermore, Vav1 activity on hydrolyzed 6-Thio-GTP was strongly suppressed as compared with hydrolyzed GTP (Fig. 6B), suggesting that azathioprine-generated 6-Thio-GDP blocks the guanosine exchange activity of Vav1 and, therefore, leads to an accumulation of inactive, 6-Thio-GDP-loaded Rac1 molecules in T cells over time.

Taken together, these data suggested that azathioprine targets the guanosine exchange activity of Vav on Rac GTPases in primary T cells leading to blockade of Rac-dependent Th1 cytokine production as well as lamellipodia formation and T cell-APC conjugation.

Discussion
In the present study, we have identified a unique role for azathioprine and its metabolites in the control of T cell-APC conjugation by modulation of the Vav-Rac1 signaling pathway in T lymphocytes. Azathioprine suppressed activation of both Rac1 and Rac2 but had little effects on the activation of other small GTPases. Binding of azathioprine-generated 6-Thio-GDP to Rac1 led to specific blockade of Vav guanosine exchange activity, suggesting that the guanosine exchange activity of Vav on Rac GTPases is the molecular target of azathioprine. Consecutive blockade of Vav-dependent Rac1 activation caused an inhibition of lamellipodia and T cell-APC conjugation. Importantly, these findings also occurred in the absence of T cell apoptosis. Thus, azathioprine specifically blocks Ag presentation and Ag-specific immune responses by targeting the Vav signaling pathway in T cells. These findings may explain the beneficial immunosuppressive effects of azathioprine and have important implications for the design of novel specific therapies for organ transplantation and autoimmune diseases based on suppression of the Vav-Rac signaling pathway.

The Vav family (Vav1–3) of three highly conserved Rho GEFs was initially shown to regulate signaling events downstream of the T and BCR (43–45). In contrast to other Rho-GEFs, Vav proteins are activated via receptor-induced tyrosine phosphorylation allowing them to interact directly with several tyrosine kinases through Src homology 2 domain interactions (43, 46). In this context, tyrosine phosphorylation relieves Vav autoinhibition by exposing the GTPase interaction surface of the Dbl homology domain, which is obligatory for Vav activation (47). In T lymphocytes, Vav-1 is much more important than Vav-2, and its GEF activity regulates Rac proteins (48) thereby linking CD28 ligation to the activation of Rac1 (48–51). In the present manuscript, we demonstrate that the azathioprine metabolite 6-Thio-GTP directly blocks the GEF activity of Vav on Rac1 rather than the binding of Rac1 to its effector PAK. Immunoprecipitation studies showed that binding of Vav1 to Rac1 is inhibited in the presence of 6-Thio-GTP, whereas binding of Vav1 to RhoA was increased.

FIGURE 5. Azathioprine suppresses binding of Vav-1 to Rac1 but not RhoA. A, Immunoprecipitation of Rac1, RhoA, and Cdc42 in azathioprine-treated T cells. CD4+ T lymphocytes were stimulated with anti-CD3 plus anti-CD28 Abs and cultured for 3 days in the presence or absence of azathioprine, 6-MP, or 6-TG. Next, immunoprecipitation for indicated GTPases was performed followed by Western blotting for Vav-1 to determine GTPase-bound Vav-1 levels. Azathioprine and its metabolites led to a marked suppression of Vav-1 binding to Rac1. In contrast, binding of Vav-1 to RhoA was increased. Analysis of the Vav-1-independent, Db1-dependent GTPase Cdc42 served as negative control. A Western blot of β-actin on whole cell lysates before immunoprecipitation served as a loading control. B, Effects of Vav-1 overexpression on Rac1 activation in T cells. Vav-transfected and control-transfected primary CD4+ T lymphocytes were stimulated with anti-CD3 plus anti-CD28 Abs and cultured for 3 days in the presence or absence of 6-MP. Rac1 activation assays were performed with PAK as described above. C, Effects of increasing amounts of 6-MP and 6-Thio-GTP on Vav-1-mediated Rac1 activation in T cells. Vav-transfected and control-transfected primary CD4+ T lymphocytes were cultured with anti-CD3 plus anti-CD28 Abs for 3 days in the presence or absence of increasing amounts of 6-MP and 6-Thio-GTP. Rac1 activation assays were performed with PAK.
Hydrolysis of Rac1-bound 6-Thio-GTP augmented the inhibition of Vav GEF activity, suggesting an inability of Vav to exchange 6-Thio-GDP when bound to Rac1 (Fig. 7). This concept would suggest that clinical therapy with azathioprine results in an accumulation of 6-Thio-GDP-loaded inactive Rac proteins in T lymphocytes over time due to a functional inactivation of Vav GEF activity. Therefore, azathioprine therapy would require time to inhibit Rac activity indirectly via suppression of Vav guanosine exchange activity on Rac GTPases. Consistently, azathioprine exhibits a well-known delayed onset of therapeutic efficacy in autoimmune and chronic inflammatory diseases in humans (6, 7).

The concept that the guanosine exchange activity of Vav on GTPases in primary T lymphocytes is the molecular target of azathioprine predicts similarities between the clinical phenotype of azathioprine-treated patients and findings in Vav-deficient animals. Indeed, whereas Vav-1-deficient mice displayed reduced T cell numbers with leukopenia and suppressed Ag-specific B cell responses (52–54), azathioprine-treated patients have been shown to exhibit leukopenia and reduced T cell-dependent B cell responses as well (6, 7, 55, 56). Furthermore, azathioprine-treated patients are known to display reduced IFN-γ production (57), consistent with the observed down-regulation of Rac2 activation upon azathioprine treatment and the well known role of Rac2 in inducing IFN-γ production by T cells (18). Thus, azathioprine suppresses production of the proinflammatory cytokine IFN-γ that is known to play a key role in the pathogenesis of IBD (58–60). Interestingly, very recent experiments by Tanaka et al. (61) showed augmented IFN-γ and deficient IL-4 production in Vav-1-deficient T cells, while we find augmented IL-4 and reduced IFN-γ production in azathioprine-treated T cells. We believe that this difference could be due to the fact that azathioprine specifically blocks Vav activity on Rac proteins only but not overall Vav-1 activity. Therefore, other GTPases such as RhoA, that are regulated by Vav-1 remain functionally intact thereby explaining why azathioprine-treated T cells may not exhibit all features identical to Vav-1-deficient T cells. Interestingly, RhoA has been shown to regulate both IFN-γ and IL-4 production (62) raising the possibility that reduced IL-4 production in Vav-1-deficient T cells could be due to a defect of other GTPases than Rac1/2. Finally, Vav-1 deficiency causes a defect in lymphocyte development that may affect the capacity of T cells to produce certain cytokines.

Azathioprine and its metabolites suppressed dephosphorylation of ERM proteins that are critical for T cell activation in response to CD28 costimulation. Interestingly, Delon and coworkers (42) recently showed that inactivation of ERM proteins is mediated by the Vav-Rac1 signaling pathway in T lymphocytes. Taken together, these data suggest that 6-Thio-GTP-mediated suppression of Vav GEF on Rac1 leads to blockade of ERM dephosphorylation. Subsequently, ERM proteins would be blocked in their ability to create flexible regions in the plasma membrane and lamellipodia that are essential for weak ligands to help TCR signal generation. Because we have shown recently that azathioprine induces a caspase-9-dependent pathway of T cell apoptosis when T cells are stimulated with high concentration of anti-CD3 plus anti-CD28 Abs (15), it was important to determine whether blockade of ERM dephosphorylation depends on the induction of T cell apoptosis. Interestingly, the observed inhibition of ERM dephosphorylation occurred also in the absence of T cell apoptosis, when T cells were stimulated for short periods of time. These data suggest a dual

FIGURE 6. Exchange activity of oncogenic Vav3 (Δ1–144) protein on Rac1 in the presence or absence of 6-Thio-GTP. A, Exchange reactions in the presence of free 6-Thio-GTP. GDP-bound GST-Rac1 alone (4 pmol, squares) or in combination with Vav3 (Δ1–144) (3.0 pmol, triangles) was subjected to GDP/GTP exchange reactions with [35S]GTP-γS in the absence (filled symbols) or presence of 6-Thio-GTP (500 μM, open symbols). At each time point, exchange values were determined in duplicate after the indicated periods of time using a filter immobilization assay. B, Exchange reactions of Rac1 after preloading with 6-Thio-GDP. GST-Rac1 was loaded with either GTP or 6-Thio-GTP for 45 min at RT, the GTAPase incubated 60 min longer at 37°C to induce hydrolysis from GTP to GDP and from 6-Thio-GTP to 6-Thio-GDP, and then subjected to GDP/GTP exchange reactions with recombinant Vav1 and radiolabeled GTP as indicated above. After 45 min, exchange rates were determined in duplicate using a filter immobilization assay. 6-Thio-GDP loading led to a marked suppression of Vav guanosine exchange activity. Mean values ± SEM of three independent experiments are shown.

FIGURE 7. Model of azathioprine-mediated immunosuppression. Azathioprine is metabolized via 6-MP and 6-TG into 6-Thio-GTP. 6-Thio-GTP binds to the small GTAPase Rac1. Upon hydrolysis, 6-Thio-GDP bound to Rac1 inhibits Vav guanosine exchange activity leading to accumulation of 6-Thio-GDP-bound inactive Rac1 molecules, blockade of GTP incorporation into Rac1 and, consecutively, suppression of Rac1 functions on T cell survival and T cell-APC conjugation.
function of azathioprine on T lymphocytes; while long-term stimulation of T cells via anti-CD3/CD28 Abs for 5 days results in azathioprine-mediated T cell apoptosis via caspase-9, short-term stimulation over 3 days via anti-CD3/CD28 Abs blocks T cell-APC conjugation via azathioprine-mediated blockade of the Vav-Rac1 signaling pathway. The latter concept may be particularly relevant for the activated mucosal immune system in IBDs, where numerous bacterial Ags from the gut lumen are known to play a key role (60, 63) and where azathioprine is considered as the gold standard for maintenance of remission and prevention of postoperative relapse (64). In summary, our data provide novel insights into the molecular mechanism of action of azathioprine and suggest inhibition of the Vav-Rac signaling pathway in T lymphocytes as a molecular target for treatment of autoimmune and chronic inflammatory diseases.

Disclosures

The authors have no financial conflict of interest.

References


