Selective Inhibition of Inflammatory Gene Expression in Activated T Lymphocytes: A Mechanism of Immune Suppression by Thiopurines


Division of Gastroenterology and Hepatology (C.W.T., W.J.S., L.J.E.); Department of Molecular Pharmacology and Experimental Therapeutics (G.M.M., J.J.L., L.J.E.); Department of Immunology (D.J., D.J.M.); and Genomics Research Center (R.S.), Mayo Clinic, Rochester, Minnesota

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ABSTRACT

Azathioprine and 6-mercaptopurine are antimetabolite thiopurine drugs that play important roles in the treatment of leukemia and in the management of conditions requiring immunosuppression, such as inflammatory bowel disease. The biochemical pharmacology of these drugs suggests that inhibition of purine nucleotide formation through the 6-thioguanine nucleotide metabolites is their key molecular mechanism. However, it is unclear how these metabolites suppress immunity. We hypothesized that azathioprine produces a selective inhibitory effect on activated but not quiescent T lymphocytes. We first established a model system of T lymphocyte culture with azathioprine that produced pharmacologically relevant concentrations of 6-thioguanine nucleotides. Using genome-wide expression profiling, we identified a group of azathioprine-regulated genes in quiescent and activated T lymphocytes. Several genes involved in immunity and inflammation were selectively down-regulated by azathioprine in stimulated but not quiescent cells. Quantitative reverse transcription-polymerase chain reaction for three of these genes, tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor superfamily member 7, and α4-integrin, confirmed down-regulated expression of transcript levels. Tumor necrosis factor-related apoptosis-inducing ligand protein expression was further studied and found to be inhibited by azathioprine, 6-mercaptopurine, and 6-thioguanine, implying that the inhibitory effects of azathioprine on expression are mediated by 6-thioguanine nucleotides. These results therefore provide a previously unrecognized molecular mechanism for the immunosuppressive properties of thiopurine antimetabolite drugs.

The antimetabolite thiopurine medications azathioprine [AZA; 6-(1-methyl-4-nitroimidazol-5-yl)thiopurine] and 6-mercaptopurine (6-MP) are important therapies for both induction and maintenance of remission in patients with Crohn’s disease and ulcerative colitis (Pearson et al., 1995; Egan and Sandborn, 2004). Although originally developed for use in leukemia, AZA and 6-MP are now commonly used for their immunosuppressive properties in solid organ transplantation, autoimmune hepatitis, rheumatoid arthritis, and autoimmune dermatological diseases in addition to inflammatory bowel disease (Lake et al., 2000). Once ingested, these drugs enter a metabolic path-
DNA, purine nucleotides are also essential in RNA synthesis, and in phosphorylation and glycosylation reactions. This complexity raises the possibility that thiopurine drugs might have pleiotropic effects in cells, in addition to inhibiting proliferation. Currently, it remains unclear whether inhibition of lymphocyte proliferation is a relevant mechanism underpinning the immunosuppressive effect of thiopurine drugs. Indeed, overt inhibition of lymphocyte numbers in circulating blood is not required for the therapeutic effects of AZA or 6-MP, but it is considered an adverse effect that correlates with the occurrence of opportunistic infections (Connell et al., 1993).

Since the therapeutic immunosuppression of AZA and 6-MP is evident in the setting of chronic inflammatory diseases, but usually does not predispose to opportunistic infections, we reasoned that these drugs act by selectively activating but not resting immune cells. To test this possibility, we developed an in vitro system of AZA-treated T lymphocytes that models closely the effects of AZA therapy on those cells in vivo. Using a genome-wide expression profiling approach, we evaluated the effects of AZA on the expression of genes in both resting and stimulated T lymphocytes. Our results have identified several immune and inflammation-related genes whose up-regulated expression is potently inhibited by thiopurines in activated but not resting T lymphocytes, suggesting potential novel immunosuppressive mechanisms of these drugs.

Materials and Methods

Reagents. Unless otherwise stated, reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Ju.1 T lymphocytes that stably express the interleukin (IL)-1 receptor were used for these studies (McKean et al., 1995). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). For experiments, log-growth phase cells were cultured in medium supplemented with varying concentration of AZA, 6-MP, or 2-amino-6-mercaptopurine (6-TG). Cells were stimulated with 5 µM cross-linked OKT3 (Janssen, Titusville, NJ) plus 0.25 ng/ml recombinant human IL-1α, as described previously (McKean et al., 1995; Kalli et al., 1998). In some experiments, cells were stimulated using plate-bound OKT3 alone, prepared by incubation of plastic tissue culture plates with a 0.5 µg/ml solution of OKT3 before addition of cells, or in combination with IL-1 or 1:20,000 diluted monoclonal antibody ascesites 9.3 anti-CD28 (a gift of Carl June, University of Pennsylvania, Philadelphia, PA), which were added to culture medium.

Annexin V and Propidium Iodide Staining. Cells were collected, washed with cold phosphate-buffered saline, and resuspended in tubes containing fluorescein isothiocyanate-Annexin V (Beckman Coulter, Hialeah, FL). Cells were incubated for 20 min at 4°C and then were washed before resuspension in buffer containing propidium iodide (0.5 µg/ml). Flow cytometry was performed and analyzed using FACScalibur and CellQuest (BD Biosciences, San Jose, CA). Apoptotic cells were Annexin V-positive but propidium iodide-negative.

Human Peripheral Blood T Lymphocyte Studies. After obtaining approval from the Institutional Review Board of the Mayo Foundation, six inflammatory bowel disease patients taking AZA and two taking 6-MP provided 50-ml blood samples. The mean dose of AZA was 2.1 mg/kg/day for a mean duration of 30 months, and the mean dose of 6-MP was 1.15 mg/kg/day for a mean duration of 45 months. Fifty milliliters of whole blood was divided into two 50-ml conical tubes. Mononuclear cells were extracted from whole blood using Isolymph (Gallard-Schlesinger, Plainview, NY), according to manufacturer’s instructions. Phagocytic cells were removed by l-leucine methyl ester incubation, and then cells were filtered through nylon mesh to remove platelet clumps. T cell rosetting was performed using sheep red blood cells (Colorado Serum, Denver, CO) and ammonium chloride potassium lysis buffer (Cambrex, East Rutherford, NJ). The isolated T lymphocytes were resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum, and under these purification procedures have been found to contain less than 5% cells other than T lymphocytes.

6-TGN Assay. Intracellular 6-TGN concentration was measured as described previously (Pike et al., 2001).

Sample Preparation and Scanning for Affymetrix Gene Chip Expression Profiling. Total RNA was isolated from Ju.1 cells and purified using commercial affinity resin column kits (QIA-GEN, Chatsworth, CA). cDNA was prepared from a total of 10 µg of RNA pooled from three independent experiments to minimize the effect of random variation on the expression profiles. The purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). Labeled cRNAs were then fragmented and hybridized onto the U133A array. Appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, and BioD), herring sperm DNA, and bovine serum albumin to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 min followed by incubation at 45°C for 5 min before injecting the sample onto the microarray. Then, the hybridization was carried out at 45°C for 16 h with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed and the arrays were washed and then stained with streptavidin-phycocerythrin (Molecular Probes, Eugene, OR). After washes, probe arrays were scanned using the Genechip system confocal scanner (Hewlett Packard, Palo Alto, CA). The quality of the fragmented biotin labeled cRNA in each experiment was evaluated before hybridizing onto the U133A expression array by both gel electrophoresis and hybridizing a fraction of the sample onto test-3 array as a measure of quality control.

Gene Chip Data analysis. GeneChip 5.0 (Affymetrix) was used to scan and quantitatively analyze the scanned image. Once the probe array has been scanned, GeneChip software automatically calculates intensity values for each probe cell and makes a presence or absence call for each mRNA. Algorithms in the software use probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene, which directly correlates with the amount of mRNA. Spotfire (Spotfire, Cambridge, MA) and Microsoft Excel are also used for data analysis. Expression patterns for each group were compared with the control group. When assessing the difference between two different RNA samples, the -fold changes from side-by-side experiments on the same lot of microarrays were compared directly. In this analysis, we considered gene transcriptions altered ≥2-fold as significant. Gene expression profiles were analyzed at the Mayo General Clinical Research Center Genomics, Proteomics, and Metabolic Core Facility using these established protocols (Sreekumar et al., 2002a,b).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). cDNA was prepared using Superscript II (Invitrogen). Sequence-specific RT-PCR primers were designed using Affymetrix and Primer 3 algorithms (sequences available on request). Real-time PCR reactions were carried out using SYBR Green master mix on a 7700 Sequence Detector instrument (Applied BioSystems, Foster City, CA). Relative mRNA expression between samples was quantified using the ΔCt method where β-actin mRNA was used as an internal control, as described previously (Egan et al., 2004).

Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) and IL-2 ELISAs. A human TRAIL ELISA kit (BioSource International, Camarillo, CA), and a human IL-2 ELISA kit (BD Biosciences PharMingen, San Diego, CA) were used to quantify expression of those proteins, using the manufacturers’ instruc-
AZA-Treated Ju.1 T Cells: An in Vitro Model of AZA Therapy. Most studies of 6-TGN in patients receiving chronic thiopurine therapy have measured concentrations of this metabolite in erythrocytes, but not in lymphocytes, the likely target cells. Therefore, in preliminary experiments, we initially measured the concentration of 6-TGN in lymphocytes and in whole blood from eight inflammatory bowel disease patients receiving stable doses of AZA or 6-MP (Fig. 1). We found that the mean 6-TGN concentration in lymphocytes of 5590 pmol/8 × 10⁸ cells was 26-fold higher than the mean whole blood concentration, consistent with the notion of 6-TGN incorporation into DNA in target cells.

Next, we developed a model system in vitro of culturing Ju.1 cells with varying concentrations of AZA for varying time periods and assessed the accumulation of intracellular 6-TGN. We found that 6-TGN accumulated in cells in an AZA dose-dependent manner (Fig. 2A). After 48 h of culture in AZA at concentrations from 1 to 10 μM, Ju.1 cells contained 6-TGN in the concentration range of approximately 3000 to 30,000 pmol/8 × 10⁸ cells. Although 1 μM AZA did not significantly affect Ju.1 cell growth at 48 h, 10 μM AZA caused an approximately 70% reduction in Ju.1 cell number at this time (not shown). Based on these data, we treated Ju.1 cells with 1 μM AZA for 48 h for expression profiling experiments, since this concentration produced 6-TGN concentrations in cells that approximated pharmacologically observed values in relevant target cells in vivo, and it did not overtly inhibit cell proliferation. Additional experiments established that incubation with AZA at concentrations up to 10 μM for 48 h did not induce significant apoptosis or death in Ju.1 cells, judged by Annexin V (Fig. 2B) and propidium iodide staining. To model T lymphocyte activation, Ju.1 T lymphocytes were left unstimulated or stimulated by T cell receptor cross-linking, using biotinylated OKT3 in the presence of streptavidin, plus IL-1. Prior studies had established that such stimulation resulted in robust activation of Ju.1 cells as assessed by nuclear factor-κB activity, and IL-2 expression (McKean et al., 1995; Kalli et al., 1998).

Microarray Results: AZA Affects the Expression of a Subset of Genes in Ju.1 T Lymphocytes. For expression profiling experiments, Ju.1 cells were cultured until in exponential phase of growth and then divided into four subcultures.

Fig. 1. Whole blood and lymphocyte 6-TGN concentrations in eight patients receiving stable doses of AZA or 6-MP.

Two control subcultures were maintained in standard growth medium, and in two others, 1 μM AZA was added to standard growth medium. After 48 h, one control and one AZA-treated subculture were stimulated with OKT3 plus IL-1, whereas the other subcultures were left unstimulated. Twenty-four hours later, cells were collected and washed, and RNA was extracted. This experiment was conducted on three independent occasions, and equal amounts of RNA were pooled from the three experiments for each condition. The pooled RNA was then processed for gene chip hybridization.

Table 1 lists the genes whose expression was significantly (>2-fold) affected by AZA in unstimulated Ju.1 cells. Six genes, mainly involved in metabolism, were down-regulated by AZA treatment in unstimulated Ju.1 cells. Twenty genes were significantly up-regulated by AZA treatment in unstimulated cells and were mainly involved in metabolism and signal transduction. Table 2 lists the genes whose expression was significantly (>2-fold) affected by AZA in Ju.1 cells that were stimulated with OKT3 plus IL-1. We identified 11 genes that were significantly down-regulated by AZA treatment. Most of these genes were involved in immune functions. In eight of these 11 genes down-regulated by AZA, stimulation had induced an up-regulation of expression. Fifteen genes were up-regulated by AZA in stimulated Ju.1 cells, and most of these were involved in metabolism. The effect of stimulation on these genes was varied.

Real-Time RT-PCR Analysis of Candidate Gene Expression. Gene chip studies identified many genes whose expression was affected by AZA, including some with prom-
Zinc finger protein 42 Transcription factor 2.5 (0.4)
Protein phosphatase 1B2 Signal transduction 2.5 (0.0)
HPF1 protein Transcription factor 3.2 (0.8)

TABLE 2
Genes significantly affected by AZA in stimulated Ju.1 cells

<table>
<thead>
<tr>
<th>Function</th>
<th>Fold Change (Effect of Stimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium metabolism</td>
<td>-2.1 (0.2)</td>
</tr>
<tr>
<td>Immune regulation</td>
<td>-2.0 (3.1)</td>
</tr>
<tr>
<td>Immune regulation</td>
<td>-2.8 (4.0)</td>
</tr>
<tr>
<td>Immune function</td>
<td>-2.2 (0.9)</td>
</tr>
<tr>
<td>Immune regulation</td>
<td>-2.8 (3.3)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>-2.1 (0.1)</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>-2.3 (0.6)</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>-3.2 (0.2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>-4.3 (3.2)</td>
</tr>
<tr>
<td>Growth factor</td>
<td>-4.2 (2.2)</td>
</tr>
<tr>
<td>Cytokine</td>
<td>-2.1 (4.5)</td>
</tr>
<tr>
<td>Steroid metabolism</td>
<td>2.8 (1.1)</td>
</tr>
<tr>
<td>Protein metabolism</td>
<td>2.1 (0.4)</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>2.6 (0.6)</td>
</tr>
<tr>
<td>Protein metabolism</td>
<td>2.8 (0.4)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>2.7 (0.0)</td>
</tr>
<tr>
<td>Protein metabolism</td>
<td>2.5 (1.4)</td>
</tr>
<tr>
<td>Serine protease</td>
<td>2.5 (2.5)</td>
</tr>
<tr>
<td>Protein metabolism</td>
<td>2.8 (0.4)</td>
</tr>
<tr>
<td>Ion transport</td>
<td>4.0 (0.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3.9 (2.8)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>2.7 (1.4)</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>2.7 (1.3)</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>2.5 (0.4)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>2.5 (0.0)</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>3.2 (0.8)</td>
</tr>
</tbody>
</table>

inent immune and inflammatory functions. From the lists of genes in Tables 1 and 2, we chose for further study by real-time RT-PCR, two genes whose up-regulated expression was dampened by AZA in stimulated Ju.1 cells, TNF ligand superfamily member 10, also known as TRAIL, and TNF receptor superfamily member 7 (TNFRSF7). We also further studied α4-integrin, a gene whose expression was down-regulated by AZA in unstimulated Ju.1 cells. Expression of TRAIL mRNA after Ju.1 cell stimulation increased by 32.9-fold compared with unstimulated cells (Fig. 3A). In the presence of 10 μM AZA, the expression of TRAIL was blunted to an increase of 10.7-fold by stimulation. A similar phenomenon was noted in TNFRSF7 expression whereby a 27.7-fold increase was noted after stimulation, which was blunted in the presence of 10 μM AZA to 12.8-fold. Stimulation of Ju.1 cells had little effect on the expression of α4-integrin, but a
significant 6-fold down-regulation in α-4 integrin expression was noted in the 10 μM AZA-treated stimulated cells compared with untreated stimulated cells. In contrast to results obtained from the gene chip, by real-time RT-PCR, AZA did not significantly reduce α-4 integrin mRNA abundance relative to control untreated, unstimulated cells. Effect of AZA; t test, *P < 0.05. B, dose-dependent inhibition of TRAIL, TNFRSF7, and α-4 integrin by AZA in stimulated Ju.1 cells. Ju.1 cells were cultured in normal medium or in medium containing the indicated concentrations of AZA for 48 h. Cells were then stimulated with OKT3 plus IL-1. After a further 12 h, mRNA was extracted from cells and analyzed for the indicated targets by real-time RT-PCR. Results are expressed as (mean plus standard error) mRNA abundance relative to control untreated, unstimulated cells. Analysis of variance, P < 0.05, all three genes.

Effect of AZA on TRAIL Protein Expression. For further studies, we focused on TRAIL, since this cytokine shares many similarities with the proinflammatory cytokine TNF-α and plays important roles in immune and inflammatory functions (Smyth et al., 2003). We used a specific ELISA to quantify the effect of AZA on TRAIL protein production in Ju.1 cells. Stimulation with OKT3 plus IL-1 resulted in marked up-regulation of TRAIL expression, which could be blocked by AZA (Fig. 4A). AZA alone did not affect TRAIL expression, but produced a dose-dependent inhibition of stimulated TRAIL expression in Ju.1 cells (Fig. 4B). The inhibitory effect of AZA on TRAIL expression was progressive with increasing durations of pretreatment with AZA before stimulation, up to 48 h (Fig. 4C). This is consistent with the time-dependent accumulation of 6-TGN in AZA-treated cells. To extend the observations in Ju.1 cells, we also evaluated the effect of AZA on TRAIL expression in primary T lymphocytes. TRAIL was not detected in unstimulated primary T lymphocytes. Consistent with Ju.1 cell results, we observed a dramatic up-regulation of TRAIL expression by stimulation of primary T lymphocytes with OKT3 plus IL-1. AZA treatment was effective in partially blocking the up-regulated expression of TRAIL by stimulation (Fig. 4D), a finding which supports the importance of results obtained in Ju.1 cells.

AZA Selectively Abrogates Ju.1 Cell TRAIL Expression Irrespective of Costimulatory Signal. The activation of T lymphocytes requires a signal from the ligated T cell receptor, which is usually greatly amplified by a second, or costimulatory signal. To determine whether the effect of AZA on TRAIL expression in Ju.1 cells was sensitive to the costimulatory stimulus, we measured TRAIL production under varying conditions of costimulation. The addition of either soluble IL-1 or anti-CD28, both established T lymphocyte costimulatory signals, to a T cell receptor stimulus in the form of plate-bound OKT3, greatly increased TRAIL production in Ju.1 cells (Fig. 5A). AZA almost completely abolished TRAIL expression under all of these conditions. In contrast, AZA had only a relatively minor, although statistically significant effect on IL-2 secretion by Ju.1 cells under the same conditions (Fig. 5B). These results indicate that AZA blocks TRAIL expression induced by T cell activation under a variety of stimulation conditions and further show the specificity of the effect since Ju.1 cells, despite almost complete shut-down of TRAIL expression, can still mount a robust IL-2 response to stimulation.

6-MP and 6-TG Inhibit TRAIL Expression in Stimulated Ju.1 Cells. Current concepts of thiopurine antimetabolite drugs’ mechanism of immunosuppression invoke 6-TGN as common effector entities that affect the target cells. We therefore reasoned that 6-MP and 6-TG would produce effects on TRAIL production similar to what we observed with AZA. To assess this possibility, we pretreated Ju.1 cells with varying concentrations of 6-MP or 6-TG and measured the expression of TRAIL after stimulation with OKT3 plus IL-1. We observed a dose-dependent inhibition by both 6-MP and 6-TG of TRAIL expression in stimulated cells (Fig. 6). This strongly suggests that the inhibitory effect of thiopurines on inflammation-induced gene expression in T lymphocytes depends on 6-TGN metabolic products.

Discussion

The mechanism of action of AZA has remained poorly understood despite decades of use in the treatment of malignancy and conditions requiring immunosuppression. We hypothesized that the immunosuppression caused by AZA results from selective effects on the expression of specific genes in activated lymphocytes. Using a genome-wide expression profiling approach, we identified a number of can-
didate genes whose up-regulated expression in T lymphocytes was inhibited by AZA. Those genes identified with this approach included some with prominent immune and inflammatory functions, such as \( \beta 2 \)-integrin, TNFRSF7, and TRAIL. We further established that, in the case of TRAIL, 6-MP and 6-TG produced similar inhibitory effects on expression. This indicates that thiopurine antimetabolite drugs such as AZA selectively inhibit the expression of immune and inflammation-related genes in activated T lymphocytes, effects that are likely mediated through 6-TGN. These findings provide a previously unrecognized molecular mechanism of immunosuppression by AZA, which might underlie the beneficial actions of thiopurines in inflammatory disease states.

Through a complex metabolic pathway (Aarbakke et al., 1997), thiopurine drugs lead to the intracellular accumulation of 6-TGNs, which interfere with the synthesis of DNA, affect the expression of certain genes, and inhibit cell proliferation. For these reasons, 6-TGNs are likely to be the active metabolites of thiopurines that mediate their therapeutic effects. In some studies, circulating 6-TGN levels have been proposed for therapeutic drug monitoring during thiopurine treatment of inflammatory bowel disease (Cuffari et al., 1996b; Dubinsky et al., 2000; Cuffari et al., 2001; Dubinsky, 2003). This is based on the observation that 6-TGN concentration in erythrocytes correlates with the dose of AZA or 6-MP, and in some series 6-TGN correlates with clinical response to these drugs. However, the clinical utility of measuring 6-TGN levels in inflammatory bowel disease patients has been challenged by studies that found no correlation with clinical response when thiopurine dose had been adjusted for thiopurine methyltransferase activity (Lowry et al., 2001). Nevertheless, the importance of 6-TGN is further supported by the apparent clinical benefit of 6-TG therapy in inflammatory bowel disease patients (Dubinsky et al., 2003a). Unfortunately, 6-TG seems to cause significant hepatotoxicity, seriously limiting its clinical utility (Dubinsky et al., 2003b).

Although most studies have measured the concentration of 6-TGN in erythrocytes since these cells are easily obtained, we wished to measure 6-TGN concentration in relevant target cells. We therefore purified lymphocytes from peripheral blood of patients receiving standard doses of AZA or 6-MP and measured 6-TGN in those cells and in erythrocytes. We found a much higher concentration in lymphocytes, which probably reflects incorporation of 6-TGN into cellular DNA. Our results of 6-TGN accumulation in leukocytes are consistent with prior reports (Cuffari et al., 1996a).

We used a genome-wide expression-profiling approach to identify genes in a T lymphocyte model, whose up-regulated expression was inhibited by AZA at concentrations that are pharmacologically relevant and result in 6-TGN levels that are seen in vivo. Using this approach, we identified eight genes whose up-regulated expression was inhibited more than 2-fold by AZA. Among these AZA-regulated genes were...
two that are known to possess important immune and inflammation-related functions, TRAIL and TNFRSF7. TRAIL, also known as Apo-2 ligand, is a member of the TNF superfamily of cytokines. TRAIL is expressed widely within the immune system, especially on lymphocytes, and its production is induced by many stimuli that are associated with inflammation, in part through the action of transcription factor nuclear factor-κB (Rivera-Walsh et al., 2001). Similar to TNF, TRAIL is expressed by many cells in a membrane-bound form, or it can be secreted. Many cells possess plasma membrane receptors for TRAIL, of which there are at least five distinct forms. Only DR4 ligation by TRAIL results in transmission of a signal into cells, with the other receptors lacking known functions, other than potentially acting as “decoys” to buffer the effects of TRAIL (Sheridan et al., 1997). TRAIL, acting through DR4, initiates caspase activation that results in apoptosis, primarily of transformed cells (Smyth et al., 2003). Considering the established potential for AZA and 6-MP to induce non-Hodgkins lymphomas, it is intriguing to speculate that inhibition of TRAIL expression, an important inducer of apoptosis in transformed lymphocytes, might represent a molecular mechanism for lymphomagenesis in thiopurine-treated patients. The actions of TRAIL on transformed cells include induction of nuclear factor-κB, which tends to counterbalance caspase activation, in a manner similar to that observed with TNF. Recent reports have highlighted the function of plasma membrane-associated TRAIL as a receptor for DR4. In this model, TRAIL transmits a signal into the cells on which it is expressed, for example, lymphocytes, when ligated by DR4 that is expressed on other cells (Chou et al., 2001). In lymphocytes, this results in proliferation and interferon-γ secretion, markers of activation. Thiopurine-mediated interference in such a mechanism could dampen overactive immune responses, such as occur in inflammatory bowel disease.

We also identified TNFRSF7 (CD27) as a gene up-regulated by T lymphocyte activation that is significantly inhibited by AZA. TNFRSF7 is member of the TNF receptor superfamily that is involved in the regulation of T lymphocyte activation, as assessed by nuclear factor-κB activation and interferon-γ secretion (Yamamoto et al., 1998). Recent evidence suggests that T lymphocyte activation after ligation of the T cell receptor can be significantly augmented by signaling from TNFRSF7 when this receptor is activated by its ligand CD70 (Prasad et al., 1997; Hendriks et al., 2000). Along with the inhibitory effect on TRAIL expression, down-regulation of TNFRSF7 by AZA would decrease the responsiveness of T lymphocytes to activation, an immunomodulating effect consistent with this drug’s clinical effects.

The expression profiling studies also identified α4-integrin as an AZA-regulated gene. T lymphocyte stimulation had little effect on the expression levels of α4-integrin, but AZA resulted in a significant down-regulation of its expression on quiescent cells. Although real-time RT-PCR studies did not confirm lower expression of α4-integrin in AZA-treated unstimulated cells, we did observe significantly lower expression of this gene in AZA-treated cells after stimulation. These discrepancies highlight the need to confirm gene chip data.
with an independent technique. We chose α4-integrin for further study because of compelling recent evidence that implicates this adhesion molecule in the pathogenesis of two important immunological diseases, Crohn's disease and multiple sclerosis (Ghosh et al., 2003; Miller et al., 2003). α4-Integrin is one component of heterodimeric adhesion molecules that are expressed on the surface of activated lymphocytes and monocytes (von Andrian and Engelhardt, 2003). α4-Integrin, along with one of two β subunits, interacts with selectin ligands expressed on endothelium, thereby mediating eflux of activated lymphocytes and monocytes from the vasculature into sites of inflammation (Berlin et al., 1995). Down-regulated expression of α4-integrin by AZA might therefore act to inhibit the emigration of T lymphocytes, or monocytes, from blood vessels at sites of inflammation. Such an effect on leukocyte recruitment would serve to decrease injury and inflammation, potentially relevant mechanisms.

A number of recent reports have highlighted the ability of thiopurines to induce apoptosis of activated T lymphocytes. Such an effect could rationally result not only in the antineoplastic effects of AZA and 6-MP but also in immunosuppression since it might reduce the numbers of activated cells causing disease. One report showed that 6-MP could block cell cycle progression of T lymphocytes and could induce apoptosis, but only if the cells were actively proliferating (Quemeneur et al., 2003). In another recent publication, AZA or 6-MP, acting through their 6-thio-GTP metabolites, was found to block Rac1 (Tiede et al., 2003). In activated lymphocytes, this resulted in apoptosis but only after prolonged incubation over several days. Our results point toward an alternative anti-inflammatory or immunosuppressive effect of thiopurine drugs, in which the stimulus-induced expression of specific genes by T lymphocytes is greatly inhibited. AZA-treated Jurkat cells did not undergo apoptosis significantly more than control cells under the conditions of our experiments. However, our results are not inconsistent with those of Quemeneur et al. (2003) and Tiede et al. (2003), since we used different cell types, different concentrations of drugs, and shorter durations of incubation.

Based on our studies, a model of the immunosuppressive properties of thiopurines is presented in Fig. 7. AZA or 6-MP therapy results in accumulation of 6-TGN in lymphocytes. In the presence of T cell activation, as occurs in immunologically driven diseases, 6-TGN blocks the expression of TRAIL, TNFRSF7, and α4-integrin, effects that functionally decrease inflammation. These findings provide a new framework for understanding thiopurine-mediated immunosuppression, and further studies will establish the specific roles of these gene products in mediating the beneficial effects of AZA and 6-MP in immune diseases.

References


Address correspondence to: Dr. Laurence J. Egan, Mayo Clinic, 200 1st St. SW, Rochester, MN 55905. E-mail: egan.laurence@mayo.edu