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Tipifarnib-mediated suppression of T-bet-dependent signaling pathways

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Abstract

Large granular lymphocyte (LGL) leukemia is a chronic lymphoproliferative disease in which T-bet [T-box transcription factor 21 gene (*tbx21*)] overexpression may play a pathogenic role. T-bet orchestrates the differentiation of mature peripheral T-cells into interferon- γ (IFN- γ) and tumor necrosis factor- α producing CD4⁺ T-helper type I (Th1) and CD8⁺ T cytotoxic cells that are necessary for antiviral responses. When IL-12 is produced by antigen-presenting cells, T-bet expression is induced, causing direct stimulation of *ifng* gene transcription while simultaneously acting as a transcriptional repressor of the *IL4* gene, which then leads to Th1 dominance and T-helper type 2 differentiation blockade. Additionally, T-bet has been shown to regulate histone acetylation of the *ifng* promoter and enhancer to loosen condensed DNA, creating greater accessibility for other transcription factor binding, which further amplifies IFN γ production. We found that treatment with a farnesyltransferase inhibitor tipifarnib reduced Th1 cytokines in LGL leukemia patient T-cells and blocked T-bet protein expression and IL-12 responsiveness in T-cells from healthy donors. The mechanism of suppression was based on modulation of histone acetylation of the *ifng* gene, which culminated in Th1 blockade.

Keywords

Farnesyltransferase inhibitors; Immunomodulatory; T-bet; Large granular lymphocyte leukemia; Interferon; Histone acetylation

Introduction

LGL leukemia is a rare CD8⁺ clonal T-lymphoproliferative disorder of unknown etiology with primary clinical manifestations characterized by chronic neutropenia, anemia, arthritis, hypergammaglobulinemia, and occasionally associated with pulmonary artery hypertension [1–3]. A simple discrete model, called a Boolean model, predicted that the T-box expressed in T-cells (T-bet) transcription factor would be constitutively activated and critical for survival in T-LGL cells [4]. In agreement with the model's prediction, T-bet mRNA, protein and DNA binding activity was found to be significantly elevated in T-LGL leukemia PBMCs compared with healthy peripheral blood mononuclear cells (PBMCs) [4].

T-bet is a transcription factor that orchestrates multiple coordinated events involved in differentiation into Th1 and Tc-type cells including transcriptional activation of *ifng* and *TNF α* gene expression in response to IL-12, while simultaneously acting as a transcriptional

repressor of the *IL4* gene [5–7]. T-bet has also been shown to regulate histone acetylation [8]. DNAase I hypersensitive sites and regions of histone acetylation have been identified within the promoter and enhancer regions of both the *ifng* and the *IL4* genes [9, 10]. The fact that ectopic overexpression of T-bet alone is sufficient to mediate Th1 lineage commitment suggests that it is the “master regulator” of these events [11, 12].

T-cells possess the genetic differentiation program for several distinct functional lineages based on the type, dose, and strength of the antigen stimulus; and the nature of cytokines present in the microenvironment [13]. Many studies have focused on understanding this differentiation process since pathogenesis can be mediated by overrepresentation of any one of these T-cell lineages. For example, protection from graft-versus-host disease (GVHD) [14] and experimental encephalomyelitis (EAE) [15] was attributed to Th1 blockade and promotion of Th2 differentiation in mice treated with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitors of the statin family (atorvastatin, simvastatin, and lovastatin). This drug class reversibly inhibits cholesterol biosynthesis, leading to a reduction in both farnesylation and geranylgeranylation [14–16]. Unlike the relatively nonspecific effects of statins on prenylation, specific inhibitors of farnesylation (farnesyltransferase inhibitors, FTIs) [17] and geranylgeranylation (geranylgeranyltransferase inhibitors, GGTI) [18] have been developed for cancer therapy. These inhibitors were rationally designed to inhibit Ras-family proteins [19], which are frequently mutated or activated oncogenes. FTIs and GGTIs also block other GTPases and some cell cycle regulatory proteins that require prenylation for function [20–27]. Tipifarnib is a highly selective FTI with activity in hematologic malignancies such as acute myeloid leukemia (AML), T-cell leukemias and lymphomas, multiple myeloma, and in solid tumors such as non-small-cell lung cancer [21–30]. Improvement in pulmonary artery hypertension was observed after 4 months of therapy with tipifarnib in a patient with LGL leukemia, suggesting that the drug has some activities in this disease [3].

The role of FTIs in modulating cytokine polarization is controversial, but Marks et al. [31] previously showed that both Th1 and Th2 cytokines are inhibited in murine and human T-cells using three farnesyltransferase inhibitors (FTIs) (CP390392, Pfizer, SCH66336, Schering Plough and tipifarnib). Due to the overexpression of T-bet in LGL leukemia and the possible role of this drug in modulating cytokine response, we examined the effect of tipifarnib on cytokine production in T-cells from patients and healthy donors.

Materials and methods

Reagents and drugs

Ras activation assay biochemical kit (GST-RBD) for immunoprecipitation was purchased from Cytoskeleton. FTI-2153 and GGTI-4350 were provided by Dr. Andrew Hamilton, Yale University.

Patients and healthy controls

Samples from six patients with T-LGL leukemia were obtained from patients at the H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL or the Penn State Cancer Center,

Hershey PA. Diagnoses were confirmed by centralized pathology review at Moffitt Cancer Center based on phenotype analysis and T-cell receptor (TCR) restriction of the complementary-determining region-3 of the V β chain and total lymphocyte count (Table 1) [32]. After obtaining written informed consent, 40 ml of peripheral blood was collected in heparinized tubes from each patient. The study protocol was approved by the University of South Florida Institutional Review Board in accordance with the Declaration of Helsinki.

Buffy coats from healthy blood donors were obtained from the Southwest Florida Blood Services (SFBS) in St. Petersburg, FL, and a waiver of the informed consent process was granted by the University of South Florida IRB. To limit the difference in naïve and memory cells per donor, all experiments were conducted in healthy individuals under the age of 60 years.

Peripheral blood mononuclear cells (PBMCs) were isolated from LGL patients and healthy donors by Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) gradient centrifugation, as previously described [33]. CD3⁺ T-cells were isolated by negative selection (T-cell RosetteSep[®], Cell Separation Systems, Vancouver, BC) using methods suggested by the manufacturer [34]. In some experiments, CD4⁺ T-cells were purified using negative selection and used as indicated in the text (Miltenyi Biotec, Auburn, CA).

T-cell activation

T-cells were activated with plate-bound anti-CD3 and soluble anti-CD28 antibody (BD Pharmingen, San Jose, CA USA). Tissue culture plates were coated with anti-CD3 antibody at a concentration of 10 μ g/ml for 24 h at 4°C. Cells (1×10^6 /ml) were then added in the presence of soluble anti-CD28 antibody (1 μ g/ml) (BD Pharmingen, San Jose, CA USA) in a 200 μ l volume at 37°C. For activation with PMA and ionomycin (PMA/Ion), we used a concentration of 5 and 250 ng/ml, respectively (BD BioSciences, San Jose, CA, USA). In some experiments, purified T-cells were polarized under Th1 or Th2 conditions for 72 h prior to stimulation. For Th1 and Th2 polarization, 10 ng/ml of IL-12 plus anti-IL-4 antibody (10 μ g/ml) or 10 ng/ml IL-4 plus anti-IL-12 antibody (10 μ g/ml) were used, respectively.

Intracellular cytokine staining and flow cytometry

For intracellular cytokine staining, cells were cultured with brefeldin A (10 μ g/ml, Sigma, CA USA) during the last 4 h of activation to prevent extracellular secretion. Triple staining was carried out with monoclonal antibodies directed against the surface markers CD3 (with PE Cy5 conjugated antibody), CD4 or CD8 (FITC-conjugated antibody) and intracellular cytokines IFN γ and IL-4 (both PE-conjugated antibodies obtained from BD Biosciences, San Jose, CA USA). Subsequently, cells were washed, resuspended in staining buffer, and analyzed with a FAC-Scan flow cytometer (BD Biosciences). Data analysis was conducted using the CELLQuest software program (BD Immunocytometry Systems, San Diego, CA). Quadrant markers were positioned to include >99% of immunoglobulin control stained cells in the negative quadrant. LGL leukemia phenotype and clonal expansion was performed

using previously described methods [32, 35]. All experiments were performed in duplicate or triplicate, and the standard deviation of the assay was determined.

Ras activity

Ras activation biochemical kits were used to precipitate GTP-bound Ras using methods described previously as recommended by the manufacturer (GST-RBD immunoprecipitation, Cytoskeleton) [33].

Western blot analysis

All Western blots were performed on purified CD3+ or CD4+ T-cells isolated by negative selection, as previously described [33]. Proteins were separated by SDS–polyacrylamide electrophoresis, and Western blot analysis was performed with anti-T-bet antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HDJ-2 (Neomarkers, Fremont, CA), anti-RAP1A (specific for unprenylated RAP1, Santa Cruz Biotechnology), anti-Ras (BD Transduction Laboratories), and anti- β -actin antibodies (Sigma).

Quantitative RT-PCR

Total RNA was prepared from purified T-cells using the RNeasy mini kit (Qiagen, Valencia, CA), and contaminating DNA was removed using the DNA-free kit (Ambion, Austin, TX). Reverse transcription (RT) reactions were performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR (qRT-PCR) reactions for IFN γ and EEF1A1 were performed using Taqman gene expression assays (Applied Biosystems) and analyzed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). A negative control without cDNA template was run with every assay. Transcript copy number per individual was calculated by normalization to EEF1A1 expression.

Chromatin immunoprecipitation

ChIP assays were performed, as described previously [36]. All steps were carried out at 4°C. Purified primary CD4+ T-cells were cultured in 100-mm dishes and incubated for 10 min with 1% formaldehyde (Sigma, under gentle agitation. Cross-linking was stopped by the addition of 0.125 M glycine for 5 min. Then, cells were washed with 10 ml of PBS, harvested in the same volume of PBS, and collected by centrifugation at 1,000g for 5 min. The cell pellet was resuspended in lysis buffer (50 mM HEPES [pH 7.8], 20 mM KCl, 3 mM MgCl₂, 0.1% NP-40, and a cocktail of protease inhibitors) and incubated for 10 min on ice. The cell extract was collected by centrifugation at 1,000g for 5 min, resuspended in a sonication buffer (50 mM HEPES [pH 7.9], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a cocktail of protease inhibitors), and incubated for 10 min on ice. To reduce the length of the chromatin fragments to approximately 500 bp (confirmed by electrophoretic analysis and PCR), the extract was sonicated using a Bioruptor™ from Diagenode. Eight pulses of 30 s with 30 s rest time were repeated six times at a frequency of 20 kHz. After centrifugation at 16,000g, the supernatant was collected, frozen in liquid nitrogen, and kept at –80°C. An aliquot was used for A260 measurements. Cross-linked extracts (6 U A260) were resuspended in a sonication buffer to a final volume of 500 μ l. The samples were pre-cleared

by incubation with normal mouse IgG plus protein A/G agarose (Santa Cruz Biotechnology) for 2 h at 4°C with agitation. After centrifugation at 1,000g for 5 min, the supernatant was collected and immunoprecipitated with specific antibodies as detailed for each experiment. The immunocomplexes were recovered with the addition of 30 µl of protein-A agarose beads and subsequent incubation for 2 h at 4°C with agitation. The complexes were washed twice with sonication buffer plus 500 mM NaCl, twice with LiCl buffer (20 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, and 0.5% Triton X-100), and twice with TE buffer (2 mM EDTA and 50 mM Tris-HCl [pH 8.0]). The solution was incubated at each washing for 5 min at 4°C. The protein–DNA complexes were eluted by incubation with 100 µl of elution buffer (50 mM NaHCO₃ and 1% SDS) for 15 min at 65°C. After centrifugation at 1,000g for 5 min, the supernatant was collected and incubated with 10 µg of RNase A per ml for 1 h at 42°C. NaCl was added to the mixture to a final concentration of 200 mM and incubated at 65°C to reverse the cross-linking. The proteins were digested with 200 µg/ml of proteinase K for 2 h at 50°C. The DNA was recovered using Qiagen Quiaquick™ columns. The primers used were as follows: *ifng* (promoter region) sense 5'-GTTTCACTGCCAGTCTGG-3' and antisense 5'-ACT ACACCCTATCAGGCTTTA-3', *ifng* (intron region) sense 5'-GGTGGGAATCATTG-3' and antisense 5'-GCAACGAAGTTCCGGAATAC-3' [37]. All samples and inputs were quantified using MyIQ single-color quantitative real-time PCR detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). Single product amplification was confirmed by melting curve analysis, and primer efficiency was near or close to 100% in all experiments performed. Quantification is expressed in arbitrary units, and target sequence levels were normalized to the input signal using the method of Pfaffl [38]. All ChIP experiments were repeated twice starting from the cross-linking, and final quantitative real-time PCR was done in triplicate.

Results

T-bet overexpression and tipifarnib immune modulation in LGL leukemia

Using primary purified T-cells from six LGL leukemia patients, we confirmed previous results showing that T-bet is overexpressed in patients compared to healthy controls by Western blot analysis (Fig. 1a) [4, 33]. Using intracellular cytokine staining on gated viable T-cells, a higher percentage of CD4+ ($P = 0.02$) and a trend for higher IFN γ production in CD8+ ($P = 0.06$) T-cells were observed in patients compared to controls after 6 h of TCR stimulation with anti-CD3/anti-CD28 plate-bound antibodies (Fig. 1b). As shown in Fig. 1c, treatment of LGL leukemia T-cells for 72 h during TCR stimulation resulted in a reduction in IFN γ and TNF α , both Th1-CD4+/Tc1-CD8+ cytokines, and enhanced IL-4 and IL-10, both Th2-CD4+/Tc2-CD8+-type cytokines, consistent with a change in cytokine signaling.

We have shown previously that Ras is constitutively activated and important for survival in T-LGL leukemia cells, and likewise, T-bet was predicted to be important for survival in these cells. Therefore, we determined whether the T-cells from LGL patients or controls apoptosed in response to the drug. Using tipifarnib 5 µM for 72 h, the percentage of apoptotic cells was significantly higher in drug-treated compared to DMSO(vehicle)-treated LGL T-cells (Fig. 1d, $P = 0.03$), suggesting that this agent in vitro may be directly toxic to

the cells. In contrast, tipifarnib-treated cells in T-cells from healthy controls showed no significant increase in apoptosis compared to vehicle control (Fig. 1d).

Although our results are consistent with a previous report, Western blots do not indicate whether an increased proportion of T-cells express T-bet or whether T-bet is overexpressed on a per cell basis in LGL leukemia [4]. Moreover, the distribution of cells may be different in cases and controls potentially impacting the results. To examine this possibility, we performed phenotyping and clonality studies of T-cells in these 6 cases compared to controls. Consistent with our previous data, the peripheral compartment in LGL leukemia is dominated by CD8⁺ T-cells with a CD45RA⁺/CD62L[–] terminal effector memory phenotype (Fig. 1e) TCR repertoire with overrepresentation of specific V β families by flow cytometry, as shown in one representative case in Fig. 1e [34, 39]. Because the T-cell compartment is highly skewed and apoptosis occurred in tipifarnib-treated LGL T-cells, we decided that it was more appropriate to study the mechanism of tipifarnib-mediated cytokine modulation in cells from healthy donors to control for the distribution of cells. To limit the inter-sample variability in naïve and memory cells, all experiments were conducted in healthy individuals under the age of 60 years. Using T-cells from healthy donors, tipifarnib (5 μ M) reduced the percentage of IFN γ -positive cells in a time-dependent manner (Supplemental Figure 1). The fact that similar drug effects were seen in patients, with such a dramatically skewed repertoire, and in controls argues that repertoire changes do not contribute to the drug response.

Primary peripheral blood CD3⁺ T-cells from healthy donors were treated with tipifarnib for 72 h at doses ranging from 1.25 to 10 μ M and found to have a dose-dependent reduction in the percentage of IFN γ -positive CD4⁺ cells relative to IL-4-producing CD4⁺ cells (Fig. 2a). These results suggest that tipifarnib, like statins, skews the Th1-Th2 profile (Fig. 2a) under non-polarizing conditions. This skewed pattern of cytokine production was evident after 12 h of tipifarnib treatment based on kinetic analysis (data not shown).

Th1 differentiation is blocked selectively by tipifarnib, but not GGTIs

IFN γ and IL-4 cytokine secretion was next compared in the presence of tipifarnib and a geranylgeranyltransferase 1 (GGTase) inhibitor (GGTI-4350). GGTase catalyzes the addition of a 20-carbon geranylgeranyl group based on the amino acid sequence present in the conserved “CAAX” box, prenylation domain [17]. Most prenylated proteins display preference for farnesylation or geranylgeranylation, but some can be promiscuously prenylated in response to pharmacological inhibitors through a salvage pathway [17, 40]. In these experiments, purified CD3⁺ T-cells were isolated by negative selection and then exposed to these drugs either alone or in combination and compared to DMSO-treated cells in the presence of anti-CD3/anti-CD28 antibodies for 72 h. Increasing doses of GGTI-4350 alone failed to reduce the percentage of IFN γ -producing cells within the CD4⁺ flow cytometry gate (Fig. 2b). In the presence of low-dose tipifarnib (1.25 μ M), with only minimal effects alone, GGTI-4350 treatment resulted in a dose-dependent reduction in the percentage of IFN γ -producing cells, suggesting that the farnesylated protein targeted in this response may be promiscuously geranylgeranylated (Fig. 2b).

Aliquots of these CD3⁺ purified T-cells were then examined for Ras activation using immunoprecipitation with glutathione-S-transferase-Ras binding domain (GST-RBD) protein expressed in *E. coli*. Tipifarnib reduced the amount of activated Ras in precipitates compared to DMSO (Fig. 2c). Precipitated protein from Jurkat T-cells was used as a positive control. The specificity and sensitivity of tipifarnib and GGTI-4350 were determined by monitoring the prenylation status of an exclusively farnesylated protein HDJ-2 and the appearance of unprenylated Rap1A, which is exclusively geranylgeranylated. Accumulation of the unfarnesylated (Un-processed, UnProc) HDJ-2 protein (Fig. 2d, panel i) with minimal or no induction of unprenylated Rap1A (Unproc-Rap1A) (panel ii) confirms the pharmacologic specificity of tipifarnib. Appearance of unproc-Rap1A confirmed the specificity of GGTI-4350 for its target. Equal expression of β -actin (Fig. 2c, d) was used to confirm uniform protein loading in each lane. Therefore, we conclude that IFN γ production is selectively modulated by a farnesylated protein intermediate with the ability to be geranylgeranylated under sub-optimal farnesyltransferase inhibition.

Farnesylation is necessary for T-bet expression and STAT6 phosphorylation in Th1 polarized CD4⁺ T-cells

T-bet, IL-12, and STAT4 are vital for inheritable Th1 lineage commitment by CD4⁺ T-cells, whereas Th2 is dependent on IL-4 and STAT6 signaling [41, 42]. To determine the effect of tipifarnib on polarized differentiation, purified CD4⁺ T-cells from healthy donors were cultured under Th1 (IL-12 plus anti-IL-4) conditions and the percentage of IFN γ -positive cells determined after secondary stimulation for 6 h with anti-CD3/anti-CD28 antibodies. As shown in Fig. 3a, the drug effectively reduced IFN γ production in both unpolarized and polarized cells. We determined that T-bet is maximally induced by Th1 polarizing cytokines and antibodies (as indicated in the “Materials and methods”) at 72 h (Supplemental Figure 2). As shown in Fig. 3b using purified CD4⁺ T-cells polarized under Th1 conditions and then either treated with a secondary stimulus (Ac) or not (Un), tipifarnib treatment reduced the expression of T-bet protein under both conditions. Efficacy of the drug was shown by an increase in unprocessed (U) HDJ-2 protein (Fig. 3b, panel ii) and equal protein expression in each sample was shown by blotting for β -actin (Fig. 3b, panel iii).

Since the pathway of IFN γ production may differ after phorbol 12-myristate 13-acetate plus ionomycin (PMA/ ionomycin) and TCR stimulation with anti-CD3/anti-CD28 mAb [43], we examined Th1- and Th2-mediated activation of STAT4 and STAT6, respectively, in the presence of both modes of stimulation. Purified CD4⁺ T-cells were Th1 polarized with IL-12 plus anti-IL-4 and Th2-polarized with IL-4 plus anti-IFN γ in the presence of tipifarnib or DMSO for 72 h and then re-stimulated with either anti-CD3/anti-CD28 or PMA/Ion. Unpolarized cells that received no secondary stimulation were used as a negative control (Fig. 3c, lane 1). Interestingly, treatment with tipifarnib selectively suppressed STAT4, but not STAT6 phosphorylation under all conditions tested (Fig. 3c). With non-polarizing conditions (lanes 2, 3 and lanes 8, 9) and with Th2 polarization, phospho-STAT6 was activated equally in tipifarnib-and DMSO-treated cells showing selective Th1 signal suppression (Fig. 3, panel ii). In these experiments, analysis of β -actin was used to ensure equal protein loading in each sample (Fig. 3, panel iii).

Inhibition of *ifng* mRNA expression and histone acetylation by tipifarnib

To determine whether tipifarnib alters *ifng* mRNA expression, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted. Tipifarnib-treated cells were compared to DMSO (DM) vehicle control-treated cells, and *EEF1A1* gene expression was used as the normalizer since this gene is unaltered by both the TCR stimulation and the drug and has been accepted as the best house-keeping gene for tipifarnib studies [22, 23]. Unpolarized, unstimulated cells were used as a negative control (first bar) and tipifarnib significantly reduced the expression of *ifng* mRNA in unpolarized (U) and in Th1-polarized cells (Th1) (Fig. 4a) that received a secondary stimulus with anti-CD3/anti-CD28.

Aliquots of these cells were then used for chromatin immunoprecipitation (ChIP) assays to assess the amount of histone H3 and histone H4 acetylation at the regulatory regions of *ifng* including the promoter (a) and intron (i.e., enhancer [44]) (c). As shown in Fig. 4b, unstimulated and unpolarized cells were used as the negative control for these ChIP assays (first bar in each graft). In purified CD4⁺ T-cells cultured with Th1-polarizing conditions prior to receiving a secondary stimulus, and to a lesser degree in unpolarized cells that received a secondary stimulus, the *ifng* promoter and enhancer showed an increase in histone H3 and H4 acetylation, as expected from previous studies [8] (Fig. 4b, c). Pre-treatment with tipifarnib, in most cases, blocked *ifng* acetylation (Fig. 4b, c, panels i, ii, v, and vi). H3 and H4 acetylation of the *IL4* promoter was reduced in Th1-polarized cells, but unchanged by tipifarnib treatment (Fig. 4b, c, panels iii and vii). Acetylation of the *IL4* enhancer, which may invoke more access for transcription factor binding in response to Th2-differentiating cytokines, was increased significantly by tipifarnib (Fig. 4b, c, panels iii, iv, vii, and viii), demonstrating the mechanism for differential cytokine regulation.

Discussion

Distinctive patterns of gene expression regulate Th1/Tc1 and Th2/Tc2 differentiation. While Th1-type cytokines are linked to T-bet via transgenic and murine knockout experiments, less is known about early signaling events regulating T-bet expression and the role of histone acetylation in this process [45]. The current model of Th1 differentiation suggests that chromatin is first remodeled through the actions of T-bet during a biphasic response initiated by TCR stimulation followed by cytokine-dependent lineage commitment [8]. Here, we show that Th1 cytokines during the first 72 h of activation are reduced by tipifarnib. Based on our results, the target protein is primarily farnesylated, but contains a “CAAX” sequence that can be alternatively geranylgeranylated in suboptimal FTI treatment. Farnesyltransferase blockade reduced not only *ifng* mRNA expression, but also T-bet expression during Th1 differentiation, STAT4 phosphorylation, and *ifng* promoter and enhancer H3 and H4 acetylation. These results confirm, on several levels, that Th1 differentiation is blocked by tipifarnib. Both K- and N-Ras, but not H-Ras, would meet the predicted “CAAX” box specification and should be further investigated as potential targets [17]. While the mechanism could be mediated by the direct action of a farnesylated protein, like K- or N-Ras, it is also possible that the drug may limit TCR signal strength, which may ultimately control the Th1/Th2 outcome.

Since overexpression of T-bet is linked to autoimmunity in LGL leukemia and in other diseases, it is important to understand molecular events controlling its expression [46–52]. T-bet expression is known to be controlled by IFN γ through the signal transducer and activator of transcription-1 (STAT1) signaling pathway, but recent results using Jak3/STAT5 $^{-/-}$ mice indicated that a third cytokine signal independent of T-bet is also involved in Th1 regulation [12, 53]. Regardless of the precise mechanism, tipifarnib treatment in vivo was shown to improve pulmonary artery hypertension and increased bone marrow colony formation in LGL leukemia in a small pilot study [3]. In a related disorder known as autoimmune lymphoproliferative syndrome (ALPS), a somatic activating mutation in N-Ras was found to be important for lymphocyte expansion and autoimmune pathology so this drug may also be effective in this disease [54]. Because excessive production of Th1 cytokines and overexpression of T-bet play central roles in autoimmunity and in GVHD, tipifarnib may be useful as an alternative to HMG-CoA inhibitors where Th1/Tc1 cytokine-dependent pathways and T-bet play a pathological role [14–16]. The role of tipifarnib as an immunomodulator may also prove important in T-cell malignancies, AML, and MM where it is used for anti-leukemia therapy [22, 27, 30, 55].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Reduced Th1 cytokine production in LGL leukemia T-cells after tipifarnib treatment. **a** Lysates from patients with LGL leukemia were analyzed by Western blot analysis after fractionation on polyacrylamide gels and stained for the expression of T-bet and β -actin. Jurkat cells were used as a negative control since they are predominantly Th2-type cells. **b** T-cells in the peripheral blood from 6 patients with LGL leukemia (circles) and 9 healthy controls (triangle) were stimulated with anti-CD3/anti-CD28 mAb for 6 h and the % of IFN γ -producing CD4 $^{+}$ and CD8 $^{+}$ T-cells determined by intracellular staining. **c** T-cells

from 6 LGL leukemia patients were treated in vitro with DMSO (drug vehicle control) or tipifarnib 5 μ M for 72 h followed by anti-CD3/anti-CD28 mAb stimulation for 6 h and intracellular cytokine analysis by flow cytometry. Each sample was measured in duplicate, and results indicate the mean % difference between DMSO and tipifarnib-treated cells. **d** Results of apoptosis assays performed in duplicate are shown from 6 patients (Pt) and 3 healthy donors (NL) using annexin-V-FITC/7-actinomycin D staining and analysis by flow cytometry. Statistical analysis was performed using a Wilcoxon matched-pairs signed rank test comparing DMSO and tipifarnib-treated cells. *ns* Not significant. **e** Flow phenotyping in an exemplary LGL patient out of 6 examined. Forward and side scatter was used to establish the lymphocyte gate (*R1*), the T-cell population (*R2*) and CD4+ and CD8+ subpopulations (*R3*) in healthy controls and LGL patients (data not shown). LGL patients have significantly lower numbers of CD4+ T-cells due to leukemic CD8+ T-cell expansion (data not shown). Clonal expansion was determined in terminal effector memory (CD45RA+/CD62L-) T-cells from age-matched patients and controls with one exemplary case shown out of 6 analyzes performed. V β -repertoire distribution was shown using antibodies to V β 3, 7.1, 5.3, 16, 17, 9, 20, 5.1, 18, 8, 13.6, 13.1, 12, 2, 5.2, 21.3, 1, 23, 14, 22, 11, 12, 4, and 13.2. Expansion of V β 13.1-positive cells representing 83% of all peripheral blood CD8+ T-cells occurred in this patient. Each patient was found to have positive V β expansion that was greater than $3 \times \text{mean} \pm \text{SD}$ of controls in one or more family

Fig. 2.

Farnesyltransferase inhibitors reduce IFN γ production in cells from healthy donors. **a** Purified CD3 $^{+}$ T-cells from the peripheral blood of healthy donors were activated with increasing doses of tipifarnib that included 1.25, 2.5, 5.0, and 10 μ M for 72 h. DMSO was added as a control at the highest volume. After the culture period, cells were then stimulated with plate-bound anti-CD3/anti-CD28 mAb for 6 h and then stained for intracellular cytokine analysis. The percentage of cells that produced IFN γ and IL-4 $\pm$ standard deviation (SD) and the ratio of IFN γ /IL-4-producing cells is shown. Results are representative of 5

individual experiments. Results are shown in gated populations of CD4⁺ T-cells and confirmed in CD8⁺ T-cells (data not shown). **b** Flow cytometry was used to determine the percentage of CD4⁺ T-cells that express IFN γ after treatment for 72 h and then with 6 h anti-CD3/anti-CD28 cross-linking in the presence of DMSO (*box* showing control), tipifarnib alone (1.25, 2.5, 5, 10, and 20 μ M, *solid line, circles*), GGTI-4350 alone (1.25, 2.5, 5, 10, and 20 μ M, *dashed line, squares*), or a constant amount of tipifarnib 1.25 μ M causing minimal reduction in IFN γ (see *box*) + escalating GGTI-4350 (1.25, 2.5, 5, 10, and 20 μ M, *hashed line, triangles*). Results exemplify three independent experiments with the mean and SD of duplicate samples. **c** Purified CD3⁺ T-cells were cultured with DMSO, or tipifarnib 2.5, 5.0 and 10 for 72 h and then activated with anti-CD3/anti-CD28 mAb for 6 h. The cells were lysed and lysates examined for expression of activated Ras by immunoprecipitation with GST-Ras-RBD beads and Western blot analysis with anti-total Ras antibody. β -actin shows equal protein in each sample used for precipitation. **d** Specificity of the inhibitors were shown by examining unprenylated/unprocessed (UnProc) and processed (Proc)-HDJ2 (high-migrating band is unprenylated, U-HDJ2) (*i*), unprenylated-Rap1A (gergeranylated protein, *ii*) and β -actin (*iii*) by Western blot analysis. Results are shown for exemplary experiments from 5 experiments performed

Fig. 3.

Deficiency in Th1 differentiation and signaling in FTI-treated cells. **a** Purified CD4⁺ T-cells were cultured for 72 h in culture media or with Th1 polarization with IL-12 plus anti-IL-4 mAb as described in the “Materials and methods” section in the presence of DMSO or 5 μ M tipifarnib. Flow cytometry was then performed to determine the percentage of IFN γ -positive cells after secondary stimulation with anti-CD3/anti-CD28. Unstimulated cells (Un) were used to establish the negative gate (data not shown). **b** Aliquots of these purified CD4⁺ Th1 polarized cells that received the secondary TCR stimulation with anti-CD3/anti-CD28 (Ac)

or were unstimulated (Un) were lysed and examined for T-bet expression by Western blot analysis (i). Specificity of the inhibitor was shown by examining unprocessed (U) and processed (P)-HDJ2 (ii). β -actin was examined to show equal protein loading in each lane (iii). Inhibition of T-bet expression was evident in T-cells treated with tipifarnib (5 μ M) compared to DMSO. Re-activation for 6 h with anti-CD3/anti-CD28 showed no difference in T-bet expression in this assay compared to unstimulated cells (Un). **c** Phosphorylation/activation of STAT4 and STAT6 was determined by phosphorylation-specific antibodies (PO₄-STAT4 and PO₄-STAT6) in unstimulated and after stimulation with anti-CD3/anti-CD28 mAb and PMA/Ionomycin (PMA/Ion) in the presence of tipifarnib (5 μ M, tip) or DMSO (drug-solvent control). Secondary stimulation was performed for 6 h with anti-CD3/anti-CD28 antibodies or PMA/Ion to induce STAT phosphorylation. Cells were either unpolarized (Unpol), polarized with Th1 conditions (same as a) or were pre-cultured in the presence of Th2-polarization (IL-4 plus anti-IFN γ mAb) in the presence of DMSO (vehicle control) or with tipifarnib 5 μ M for 72 h prior to the secondary stimuli to determine if tipifarnib modulates STAT signaling. β -actin was used to determine that equal protein was loaded in each *lane*. Results are exemplary of three independent experiments performed

Fig. 4.

Effect of tipifarnib on chromatin remodeling of the *ifng* and *IL4* genes. **a** Bar graphs represent the amount of *ifng* mRNA normalized to *EEF1A1* mRNA. CD4⁺ T-cells that were unpolarized and received no secondary stimulation were used as a negative control (–, *first bar*). Induction of *ifng* mRNA was also determined in unpolarized cells (U) or Th1 polarized cells (Th1) (same as in Fig. 3) in the presence of DMSO (DM) or tipifarnib (TIP, 5 μM) for 72 h. Both the unpolarized and the Th1 polarized cells received the same secondary stimulation with anti-CD3/anti-CD28 as indicated (+). Results were normalized to *EEF1A1* expression due to previous results showing that alternative house-keeping genes such as *GAPDH* are regulated by tipifarnib and/or by TCR stimulation [23]. Th1 polarization was carried out for 72 h with IL-12 plus anti-IL-4 mAb (IL-12). **b** Immunoprecipitation with AcH3 antibody and **c** with AcH4 antibody was performed on purified unpolarized, unstimulated CD4⁺ cells as a negative control (*first bar*) and under all conditions with polarization and without and with secondary stimulation as described above. PCR was

examined for sites in the *ifng* locus with known histone acetylation sites indicated as the promoter and enhancer. Results of CHIP assays of the *ifng* gene are shown, and *bar grafts* represent the amount of DNA (mean $\pm$ SD) of triplicates after quantitative PCR out of an exemplary experiment of three performed

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Characteristics of LGL leukemia patients

Table 1

Study no.	Demographics (sex/years)	Subtype (Dx criteria) ^a	Cytopenia	Prior therapies	Lymphocyte count (1 × 10 ⁹ cells/l)
PSU-02-002	M/19	T-cell (αβ + CDR3 clonality)	Anemia/neutropenia	MTX ^b	11.2
MCC-02-002	F/65	T-cell (αβ + CDR3 clonality)	Neutropenia	MTX ^b	4.87
MCC-02-003	M/67	T-cell (αβ + CDR3 clonality)	Anemia	None	3.1
MCC-02-005	F/67	T-cell (αβ + CDR3 clonality)	Anemia	None	1.16
PSU-02-003	M/38	T-cell (αβ + CDR3 clonality)	Anemia	MTX ^b , CyA ^c , CT ^d	1.7
PSU-02-004	M/79	T-cell (αβ + CDR3 clonality)	Anemia/neutropenia	MTX ^b , CyA ^c , CT ^d	10.8

^aDiagnosis of LGL leukemia was conducted by central pathology review to rule out other possible malignancies, >300 cells/μl of abnormal T-cell with a phenotype expressing CD57, αβ-TCR, lowCD5, lowCD7, CD8 and T-cell receptor (TCR) repertoire restriction of the complementary-determining region of the β-chain gene from the Laboratory of Esoteric Testing at Tampa General Hospital, which is a certified reference laboratory

^bTreated previously with 10 mg/M² per week of methotrexate

^cTreated previously with cyclosporine A

^dTreated previously with cyclophosphamide