Trabectedin Reveals a Strategy of Immunomodulation in Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is a B-cell neoplasia characterized by protumor immune dysregulation involving nonmalignant cells of the microenvironment, including T lymphocytes and tumor-associated myeloid cells. Although therapeutic agents have improved treatment options for CLL, many patients still fail to respond. Some patients also show immunosuppression. We have investigated trabectedin, a marine-derived compound with cytotoxic activity on macrophages in solid tumors. Here, we demonstrate that trabectedin induces apoptosis of human primary leukemic cells and also selected myeloid and lymphoid immunosuppressive cells, mainly through the TRAIL/TNF pathway. Trabectedin modulates transcription and translation of IL6, CCL2, and IFNα in myeloid cells and FOXP3 in regulatory T cells. Human memory CD8+ T cells downregulate PD-1 and, along with monocytes, exert in vivo antitumor function. In xenograft and immunocompetent CLL mouse models, trabectedin has antileukemic effects and antitumor impact on the myeloid and lymphoid cells compartment. It depletes myeloid-derived suppressor cells and tumor-associated macrophages and increases memory T cells. Trabectedin also blocks the PD-1/PD-L1 axis by targeting PD-L1+ CLL cells, PD-L1+ monocytes/macrophages, and PD-1+ T cells. Thus, trabectedin behaves as an immunomodulatory drug with potentially attractive therapeutic value in the subversion of the protumor microenvironment and in overcoming chemoresistance.

Introduction

Introduction of therapeutic agents including kinase and BCL-2 inhibitors has improved treatment options for patients with chronic lymphocytic leukemia (CLL), although some patients fail to respond, become resistant or relapse during treatment (1–3). Patients with lymphoid malignancies receiving the Bruton tyrosine kinase inhibitor ibrutinib are at risk of infection, including lethal invasive fungal infections (4), likely due to the inhibition of innate immune surveillance (5). Fludarabine-based chemoimmunotherapy remains the first-line therapy for many patients, although the cytotoxic activity that fludarabine has against leukemic cells is accompanied by damage to normal immune cells, which leads to immune dysfunction and opportunistic infections (6–10). Alternative strategies are needed to bypass mechanisms that contribute to CLL cell resistance and immune dysfunction and to improve CLL patients' quality of life.

Leukemic cells depend on survival signals provided by microenvironment nonneoplastic cells (11). As in solid tumors, these signals enhance the frequency of immunosuppressive cells, including regulatory T cells (Treg; ref. 12), tumor-associated macrophages (13), and myeloid derived suppressor cells (14). In CLL, T lymphocytes exhibit exhaustion and functional defects (ref. 15; e.g., impaired immunologic synapse formation and reduced cytotoxic capacity).

Clinical trials of CD19 chimeric antigen receptor (CAR) T-cell therapy revealed resistance in CLL patients likely due to T-cell defects that are characteristic of CLL and worsen with disease progression (16).

The CLL microenvironment is characterized by an excess of CD4+ CD25+ Foxp3+ Tregs that are resistant to apoptosis likely due to high Bcl-2 expression (17). CD4+ T cells seem to provide a niche that favors development and maintenance of the
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disease (18). Another component of the immune microenvironment is represented by nurse-like cells (NLC) that in vitro promote CLL cell survival mainly through SDF1 (19). NLCs resemble macrophages and have been identified as CLL-specific tumor-associated macrophages (TAM; ref. 20).

We demonstrated in CLL mouse models that TAMs support survival and proliferation of CLL and can be therapeutically targeted by CSFIR signaling blockade to restore leukemic cells' apoptosis sensitivity. Macrophage targeting via CSFIR inhibition reprograms the tumor microenvironment toward an anti-apoptosis phenotype by activating effector memory T cells and reducing immunosuppressive cells of the lymphoid and myeloid lineage such as Tregs and monocyctic-myeloid derived suppressor cells (M-MDSC; ref. 13). These findings indicate bidirectional cross-talk between CLL cells, MDSCs, and Tregs (14).

These observations led us to investigate ET-743/trabectedin, a DNA binding sea squirt-derived compound, known to target TAMs in solid tumors. Trabectedin is approved for the treatment of soft-tissue sarcoma (21) and ovarian cancer (22). Unlike conventional chemotherapeutic agents (23), trabectedin binds the minor groove of DNA, blocks the cell cycle, affects gene transcription, and DNA-repair pathways (24, 25), and is a transcription-coupled nucleotide excision repair (TC-NER) agent. The targeting of TC-NER induces selective CLL cell death, regardless of genotype or preceding therapies (26). Trabectedin affects transcription regulation of cancer cells and some normal cells of the tumor microenvironment, which produce cytokines and factors promoting tumor growth (25). In solid tumors, trabectedin shows activity against tumor-associated myeloid cells (23, 27) and other immune cells (25, 28). In sarcoma, trabectedin targets cancer cells, shows selective TRAIL-mediated cytotoxicity against human monocytes, and inhibits the production of cytokines such as CCL2 and IL6 (29). We therefore hypothesized that trabectedin synergistically targets both leukemic cells and nonmalignant microenvironmental cells. This study uncovered the immunomodulatory function of trabectedin in CLL. We showed that at very low doses, this drug repairs the immune system and kills leukemic cells.

Materials and Methods

Cells and reagents

For cell viability studies, human primary samples were obtained from CLL patients (RAI stage 0–2), who provided informed consent as approved by the Institutional Ethical Committee (protocol VIVI-CLL) of San Raffaele Scientific Institute in accordance with the Declaration of Helsinki. For all the other studies with human blood samples, specimens were obtained from CLL patients (any RAI stages) referred to the Leukemia Department at MD Anderson Cancer Center with the approval of MD Anderson’s Institutional Review Board (protocol 2014-0678), in accordance with the Declaration of Helsinki. Written informed consent was obtained from the donors. The clinical and biological features of CLL patients analyzed are described in Supplementary Tables S1 and S2.

MEC1 cells are a CD5low– CD20 þ/– CLL cell line established from a CLL patient in prolymphocytoid transformation to B-PLL, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) and cultured in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum and gentamicin (15 μg/mL; Sigma-Aldrich). MEC1 cells have been obtained in 2014 from DMSZ; they were cultured for 1 to 2 weeks and frozen at low passages (5–10). For in vitro and in vivo experiments, MEC1 cells were thawed, cultured for 1 to 2 weeks, and used at 15 to 20 passages. MEC1 cell lines regularly tested negative for Mycoplasma contamination (PCR mycoplasma detection kit, Applied Biological materials Inc.) and have not been reauthenticated in the past year. Trabectedin (Yondelis) was provided by Pharma Mar, S.A., Sociedad Unipersonal. For in vitro studies, trabectedin as pure powder was dissolved in DMSO to 1 mmol/L and kept at −20°C. For in vivo studies, trabectedin was provided as sterile lyophilized powder (including sucrose, potassium dihydrogen phosphate, phosphoric acid, and potassium hydroxide) and dissolved in physiologic solution, following the preparation guide for patient infusion of Yondelis. A monoclonal antibody (mAb) to human CD20 (GA101; ref. 30) was provided by Roche Innovation Center Zurich, Switzerland.

Cytotoxicity assay

Human primary CD19+ cells and MEC1 cells were seeded in 96-well plates at a concentration of 3 × 104 cells/mL in 0.2 mL of RPMI. Vehicle (DMSO) as control and increasing concentrations of trabectedin (0.001 μmol/L, 0.01 μmol/L, 0.1 μmol/L, 1 μmol/L, 10 μmol/L) were added, and cell viability was assessed at 24, 48, and 72 hours using CellTiter-Glo chemoluminescence assay (Promega).

In vitro cultures and quantitative flow cytometry-based cell-depletion assay from CLL patient samples

Depending on the experiments, fresh peripheral blood mononuclear cells (PBMC) or CD19+ cells from untreated CLL patients were seeded, in triplicate, at 3 × 106 cells/mL in culture medium and treated with trabectedin (0.01 μmol/L) or DMSO vehicle for 24 hours, in the presence or absence of anti-TRAIL-R2 (human, 1 μg/mL) mAb (HS201) from Adipogen AG. Specific percentages of remaining cells in the treated samples were calculated as (the absolute number of cells in treated samples/the absolute number of cells in control samples) × 100. For each condition, the absolute number of remaining cells was calculated as the total number of viable cells (trypan blue exclusion determination) × the percentage of viable cells (flow-cytometry analysis determination).

For transcriptional studies, fluorescence-activated cell sorting was performed after 15 hours of trabectedin treatment (described below and in Supplementary Table S8).

Human cell purification, flow cytometry, and cell sorting

For cytotoxicity studies, leukemic cells were purified immediately after blood withdrawal, by negative depletion, using a B-lymphocyte enrichment kit (RosetteSep, STEMCELL Technologies). The purity of all preparations was more than 99%, and the cells coexpressed CD19 and CD5 on their cell surfaces as assessed by flow cytometry; preparations were virtually devoid of natural killer cells, T lymphocytes, and monocytes. Phenotype analysis of human MEC1 leukemic cells in xenotransplanted mice was performed with PE-Cy7 Mouse Anti-Human CD19 (J3-119) purchased by Beckman Coulter. Surface expression was analyzed using CytoFex FC500 (Beckman Coulter).

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For in vitro cell-depletion assays, 8-color flow-cytometry phenotype analysis of human live myeloid cells and 11-color flow-cytometry phenotype analysis of human live lymphoid cells were performed using LSRFortessa X-20 (BD Biosciences). PBMCs were first incubated with LIVE/DEAD fixable Aqua dye (Thermo Fisher Scientific); then, after the blocking of Fc receptors, cells were stained with surface antibodies described in Supplementary Tables S3 and S4. Finally, cells were incubated with ammonium chloride solution (STEMCELL Technologies) to lyse red cells. For lymphoid cell Foxp3 detection, surface-stained cells were further fixed and permeabilized using a Treg detection kit (Miltenyi Biotec) and finally stained with an anti-Foxp3 antibody.

For transcriptional and patient-derived xenograft studies, live myeloid cells were isolated by four-way fluorescence-activated cell sorting on a BD FACSARia II (BD Biosciences), after surface staining with the following antibodies: Alexa Fluor 700 mouse anti-human CD66b (G10F5), APC mouse anti-human Lineage Cocktail (CD3/CD19/CD20/CD56; ref. UCHT1, HIB19, 2H7, and 5.1H11), Brilliant Violet 786 mouse anti-human CD14 (Mep99) purchased by BD Biosciences, PE mouse anti-human CD16 (3G8) purchased by BioLegend, APC-Cy7 mouse anti-human HLA-DR (L243).

Live lymphoid cells were isolated by four-way fluorescence-activated cell sorting on a BD FACSARia II after surface staining with the following antibodies: eFluor 450 mouse anti-human CD8a (SK1) purchased by eBioscience, PerCP mouse anti-human CD45RO (UCHL1) purchased by BioLegend, PE-Cy7 mouse anti-human CD45RA (L48) purchased by BD Biosciences, Brilliant Violet 605 mouse anti-human CD62L (DREG-56), PE-Dazzle 594 mouse anti-human CD4 (SK3), PE mouse anti-human CD25 (4E3) purchased by Milteny Biotec, APC mouse anti-human CD19 (J3-119) purchased by Beckman Coulter, FITC mouse anti-human CD127 (HIL-7R-M21) purchased by BD Biosciences. Live/Dead Fixable Aqua (Thermo Fisher Scientific) staining was first performed to allow the discrimination of Live/Dead cells. Samples were analyzed with FCS Express 6 Flow-Cytometry Software (De Novo Software). Monocyte subsets have been identified by a negative exclusion gating strategy (31, 32). We excluded CD66b+ neutrophils, then, using a lineage (Lin) cocktail including mAbs to CD3, CD19, CD20, and CD56, we excluded T cells, B cells, and NK cells, respectively. CD14 and CD16 expression was then used to identify CD14+CD16++ nonclassic (NC), CD14++CD16+ intermediate (I), and CD14++CD16+ classic (C) monocytes. T cells have been identified as follows: naïve hCD8+CD45RO+CD45RA+CD62L+ T cells, central memory hCD8+CD45RA+CD45RO−CD62L+ TCM, and effector memory hCD8+CD45RA−CD45RO+CD62L− TEM.

Intracellular cytokine detection on human myeloid cells

For the detection of IFNγ, TNFα, IL12, IL6, and CCL2 on human myeloid cells, fresh PBMCs from CLL patients were seeded at 1 × 10⁶ cells/mL in culture medium and treated with trabectedin (0.01 μmol/L) or DMSO vehicle for 15 or 38 hours. Then, they were stimulated in vitro with ionomycin and Brefeldin A, and stained with the following surface antibodies: Alexa Fluor 700 mouse anti-human CD66b (G10F5), APC mouse anti-human Lineage Cocktail (CD3/CD19/CD20/CD56; UCHT1, HIB19, 2H7, and 5.1H11), Brilliant Violet 786 mouse anti-human CD14 (Mep99) purchased by BD Biosciences, BV421/37 mouse anti-human CD16 (3G8) purchased by BD Biosciences, PE mouse anti-human TRAIL-R2 purchased by BioLegend. The Intraprep Permeabilization Kit (Beckman Coulter) was used for the intracellular detection of the cytokines described in Supplementary Table S5. Live/Dead Fixable Aqua (Thermo Fisher Scientific) staining was first performed to allow the discrimination of Live/Dead cells. Samples were acquired using an LSRFortessa X-20 and analyzed with FCS Express 6 Flow-Cytometry Software.

Violet proliferation dye–based phagocytosis assay

MEC1 cells were labeled with Calcein-violet proliferation dye (VPD; BioLegend) and cocultured with monocytes at an effector-target ratio of 1:1 in the presence or absence of 0.01 μmol/L trabectedin for 16 hours. To distinguish between phagocytosed VPD-positive MEC1 cells and free MEC1 cells, samples were counterstained with the following surface antibodies: anti-human CD66b, anti-human Lineage Cocktail (CD3/CD19/CD20/CD56), anti-human CD14, and anti-human CD68 antibody. The Intraprep Permeabilization Kit (Beckman Coulter) was used for the intracellular detection of the CD68 macrophage marker. After the exclusion of neutrophils (through CD66b molecule), and then the exclusion of NK/NKT cells, T cells, and B cells (through Lineage Cocktail including CD56, CD3, CD19, CD20 molecules), VPD+CD14+ cells and VPD+CD68− cells were identified by flow-cytometric analysis. The absolute number of surviving VPD+CD68− target cells and of VPD+CD14+ target cells were calculated. The percentage of trabectedin-mediated phagocytosis by CD68− macrophages was calculated using the previously described (33) formula: % trabectedin-mediated phagocytosis = 100 − [(absorbance number of surviving VPD−CD68−MEC1 cells in the presence of trabectedin/absolute number of surviving VPD+CD68−MEC1 cells in the absence of trabectedin) × 100%]. The percentage of trabectedin-mediated phagocytosis by CD14+ monocytes was calculated using the formula: % trabectedin-mediated phagocytosis = 100 − [(absorbance number of surviving VPD+CD14−MEC1 cells in the presence of trabectedin/absolute number of surviving VPD+CD14−MEC1 cells in the absence of trabectedin) × 100%]. The antibodies used in this assay are described in Supplementary Table S6.

Human T-cell in vitro functional assay and cytokine detection

CD8+ T lymphocytes (including hCD8+TEMg and hCD8+TEMc) were separated by fluorescence-activated cell sorting, coincubated in vitro at an effector–target ratio of 2:1 with MEC1 cells heated at 47°C for 1 hour to induce tumor-antigen-specific T-cell response (Ag-experienced; ref. 34), in the absence or presence of 0.01 μmol/L trabectedin for 15 hours. The cells were then stimulated in vitro with Brefeldin A (4 hours, 4 μg/mL) and stained with the surface antibodies described in Supplementary Table S7, after LIVE/DEAD fixable Aqua dye incubation and Fc receptor blocking. Surface-stained cells were further fixed and permeabilized using a Staining Buffer kit (Miltenyi Biotec) and finally stained with an anti-HFNY antibody.

RNA extraction, RT-PCR amplification, and quantitative PCR

RNA extraction from sorted myeloid and lymphoid cells was performed using Mini Kit (Qiagen). RNA sample quality and quantity checks were performed using an Agilent RNA 6000 Pico Assay (Agilent Technologies) at MD Anderson’s Sequencing and Non-Coding RNA Program. Reverse-transcription reaction from 50 to 200 ng RNA template was done using the RevertAid H Minus First-Strand cDNA
experiments, xenotransplanted mice were intravenously injected
75% of the originally treated mice were still surviving. For survival
leukemia. Peritoneal exudate (PE), peripheral blood (PB), spleen
the manufacturer’s instructions. The sequences of primer pairs
NCBI-Primer Designing Tools (Supplementary Table S8).
Real-time PCR was performed using the SYBR Green PCR
Fast-Real-Time PCR System (Applied Biosystems) was used,
and NCBI-Primer Designing Tools (Supplementary Table S8).

Mice
All mice were housed and bred in specific pathogen-free
San Raffaele Scientific Institute and at The University of Texas MD Anderson Cancer Center. Depending on
animal experiment, mice were treated in accordance with the
European Union guidelines and with the approval of the San
Raficella Scientific Institute Institutional Ethical Committee (pro-
and the approval of the Institutional Animal Care and Use Committee of The University of Texas MD
on day 0. Once the mice developed palpable subcutaneous
tumors, they were i.v. injected with 0.15 mg/kg trabectedin or
were i.v. injected with 0.15 mg/kg trabectedin every week starting when the frequency of CD19+CD5– leukaemic
in the PB was 15% to 20%, compared with C57BL/6 wild-
type mice. Mice were monitored weekly for weight and leukemia
development by flow-cytometric analysis of the PB samples and,
depending on the last trabectedin injection, humanely killed at
different time points. PB, PE, and organs [SP, lymph node (LN),
femoral BM] were collected and analyzed.

Murine cell preparations and flow cytometry
PB, PE, SP, and femurs were collected from mice, and cells were
isolated. Erythrocytes from BM, PE, SP, and PB samples were lysed by
incubation in ammonium chloride solution (NH₄Cl, 0.15 M, KHCO₃ 10 mmol/L, Na₂EDTA 0.1 mmol/L, pH 7.2–7.4) for 5 minutes at room temperature. After blocking
fragment crystallize (Fc) receptors with Fc block (BD Biosciences) for 10 minutes at room temperature, cells from PB, BM, PE, SP, and LNs were stained with the antibodies (15 minutes at 4°C) listed in Supplementary Tables S9 and S10. Depending on
the experiments, cells were analyzed with a Beckman Coulter
Flow cytometry
The statistical analysis of the data was performed using the
GraphPad Prism 5.0 Software. Data were expressed as means ±
standard deviations (SD), and comparison of growth curves
between experimental groups were assessed with an
unpaired two-tailed Student t test (95% confidence interval)
and considered statistically significant for P values less than
0.05. Survival curves were compared with the use of the log-
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and considered statistically significant for P values less than
0.05. Survival curves were compared with the use of the log-
rank test.

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12 and 19 as single agents or in combination settings. For patient-
derived xenograft studies, MEC1-transplanted Rag2–/–γc–/– mice
were injected intravenously on day 18 with patient-derived CD8+ memory T cells (treated with 0.01 μmol/L trabectedin for 16 hours in vitro or left untreated) from patients 41 to 43 (Supplementary Table S2) or with patient-derived monocytes (treated with 0.01 μmol/L trabectedin for 16 hours in vitro or left untreated) from patients 41 to 44 (Supplementary Table S2). Mice were humanely
killed 5 days after the adoptive transfer. PB, SP, and BM were
collected and analyzed.

Transgenic studies
Eight-week-old male syngeneic immunocompetent C57BL/6 mice were injected i.p. (day 0) with 10 × 10⁶ cells purified from
the SP of leukemic male Eμ-TCL1 transgenic mice using the
EasySep mouse B-cell enrichment kit (STEMCELL Technologies).
The purity of the transplanted CD19+CD5–Igκ+ cells was assessed
by flow cytometry. Mice were monitored weekly for weight and leukemia development by flow-cytometric analysis of PB samples. Mice were i.v. injected with 0.15 mg/kg trabectedin
every week starting when the frequency of CD19+CD5– leukemic
cells in the PB was 15% to 20%, compared with C57BL/6 wild-
type mice. Mice were monitored weekly for weight and leukemia
development by flow-cytometric analysis of the PB samples and,
depending on the last trabectedin injection, humanely killed at
different time points. PB, PE, and organs [SP, lymph node (LN),
femoral BM] were collected and analyzed.

Murine cell preparations and flow cytometry
PB, PE, SP, and femurs were collected from mice, and cells were
isolated. Erythrocytes from BM, PE, SP, and PB samples were lysed by
incubation in ammonium chloride solution (NH₄Cl, 0.15 M, KHCO₃ 10 mmol/L, Na₂EDTA 0.1 mmol/L, pH 7.2–7.4) for 5 minutes at room temperature. After blocking
fragment crystallize (Fc) receptors with Fc block (BD Biosciences) for 10 minutes at room temperature, cells from PB, BM, PE, SP, and LNs were stained with the antibodies (15 minutes at 4°C) listed in Supplementary Tables S9 and S10. Depending on
the experiments, cells were analyzed with a Beckman Coulter
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unpaired two-tailed Student t test (95% confidence interval)
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and considered statistically significant for P values less than
0.05. Survival curves were compared with the use of the log-
rank test.

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In cell-depletion experiments that involved different human myeloid and lymphoid cell types, normality was tested with a Shapiro–Wilk normality test. We applied the t test to normally distributed data, otherwise the Mann–Whitney–Wilcoxon test. A box-and-whisker plot [box plot represents first (lower bound) and third (upper bound) quartiles, whiskers represent 1.5 times the interquartile range] was used to visualize the data. The y-axis was plotted on a logarithmic scale for convenience of representation. The analysis and graphical representation were performed in R (version 3.0.1; http://www.r-project.org/) and the statistical significance was defined as a P value less than 0.05.

The outliers at end-point were never excluded. The number of biological and technical replicates for each experiment is detailed in the figure legends. All the experiments were repeated independently and are described in the figures and figure legends. Investigators were not blinded when assessing the analysis of the experimental outcome.

Results

Trabectedin targets both CLL cells and their microenvironment

We first evaluated whether trabectedin affects human primary leukemic cells. As demonstrated previously (26), cytotoxicity studies performed on the MEC1 CLL cell line (Supplementary Fig. S1A) and purified human primary leukemic cells (Fig. 1A; Supplementary Fig. S1B; n = 3) revealed that trabectedin has a toxic effect on leukemic B cells in vitro. We selected the lowest trabectedin dose that reduced leukemic B-cell viability but did not completely kill them (e.g., 0.01 μmol/L; dotted line, Fig. 1A). The direct cytotoxic activity of trabectedin on CLL cells was confirmed in a flow-cytometry cell-depletion assay (Fig. 1B, empty circles). When unfractionated PBMCs of CLL patients (which contained both leukemic and normal hematopoietic cells) were treated with 0.01 μmol/L trabectedin, we observed depletion of both leukemic B cells and CD14+ myeloid cells (Fig. 1B, black circles). This confirmed the drug’s cytotoxicity to mononuclear phagocytes demonstrated in solid tumors (23). Leukemic cell depletion induced by trabectedin increased significantly in the PBMCs as compared with purified leukemic cells, suggesting a role for CD14+ cells and other mononuclear cells (Fig. 1B; Supplementary Table S1).

We next evaluated the effect of trabectedin on monocyte subtypes, identified by flow cytometry with a negative exclusion gating strategy (31, 32). CD14 and CD16 expression was used to identify CD14+CD16− nonclassic (NC), CD14++CD16− intermediate (I), and CD14++CD16+ classic (C) monocytes (Fig. 1C, PB; Supplementary Fig. S2A, PBMC). This gating strategy enabled us to better characterize myeloid cells and to avoid nonspecific cell inclusion (31, 32, 36). Monocyte subsets were also screened for the surface expression of HLA-DR protein, because of the demonstrated immunosuppressive activity of HLA-DR+ classic monocytes in non-Hodgkin lymphoma patients (ref. 37; Supplementary Fig. S2A). Moreover, M-MDSCs were analyzed (38, 39). PBMCs from 10 CLL patients were treated with trabectedin: after 24 hours, I monocytes and M-MDSCs were depleted, whereas NC and C monocytes (Fig. 1D and E; Supplementary Table S2), including their fractions with increased HLA-DR expression, were not depleted (Supplementary Figs. S2B and S3A and S3B). Trabectedin also increased the absolute number of CD14++CD16+ HLA-DR+ classic monocytes (Supplementary Fig. S4), high frequency of which has been found to correlate with improved overall survival in metastatic melanoma patients (36). These results led us to conclude that trabectedin affects myeloid cells.

We asked whether trabectedin also affects T lymphocytes. Human naïve, effector (TCM), and central memory (TCM) CD8+ T cells were identified based on the differential surface expression of CD45RO, CD45RA, and CD62L. CD4+ regulatory T lymphocytes expressing CD25 and Foxp3 were also analyzed. In addition to depleting the CD19+CD5+ leukemic clone, trabectedin reduced CD4+ Tregs. However, trabectedin did not deplete CD8+ TCM and TCM cells (Fig. 1D and F; Supplementary Tables S11 and S12).

Trabectedin triggers apoptosis of selected immune cells

Based on our findings, we hypothesized that trabectedin directly kills leukemic cells and tumor-supporting immune cells including CD4+ Tregs and some myeloid cell subtypes (M-MDSCs and I monocytes), and favors the activation of cytotoxic T lymphocytes.

Trabectedin’s effects on transcription regulation affect both cancer cells and normal cells of the malignant microenvironment (25), but the effect of the drug on immune cell types from leukemic patients is unexplored. Therefore, we analyzed RNA from CLL patient lymphoid and myeloid cell types after 15 hours of trabectedin treatment. By means of two independent four-way fluorescence-activated cell-sorting strategies, we separated myeloid subsets including C, NC, I, M-MDSCs, and some of B-T cell types described in Fig. 1D, including leukemic CD19+CD5+ B cells, CD8+ TCM, and TCM cells and Tregs. For RNA studies, CD4+ CD25+ Tregs were identified and sorted based on low-negative expression of CD127.

We then evaluated by RT-PCR the expression of RNA encoding effector molecules involved in TRAIL-, TNF-, and FAS/FASL-regulated death pathways on cell types depleted by trabectedin (Fig. 1D), including leukemic B cells, regulatory CD4+ T cells, I monocytes, and M-MDSCs. As described in Fig. 2, we observed upregulation of molecules involved in TRAIL and TNF pathways, including TRAIL-R2, TNFR1, BAX, BID, CASP3 transcripts in all the trabectedin-targeted cells types but not in memory CD8+ T cells (Supplementary Fig. S5). We confirmed the involvement of the TRAIL pathway by using a blocking anti-human TRAIL-R2 mAb. In 7 of 8 samples (Supplementary Fig. S6), the leukemic cell depletion induced by trabectedin was reduced when TRAIL signaling was blocked. Consistent with reported TRAIL-mediated activity of trabectedin in monocytes in solid tumors (23), we confirmed by flow cytometry that trabectedin modulated TRAIL-R2 receptor expression on monocytes and M-MDSCs (Supplementary Fig. S7A). We also observed the involvement of TRAIL-mediated cell death pathway in T cells treated with trabectedin (Fig. 2; Supplementary Fig. S6).

Trabectedin has immunomodulatory activity in vitro

We next evaluated the immunomodulatory activity of trabectedin on myeloid and lymphoid cell types. Trabectedin modified RNA expression of proinflammatory cytokines that promote the classic (M1) antitumor activation of macrophages (e.g., IFNα and TNFα), induce T-cell cytotoxic response (e.g., IFNγ and IL12a), and control B lymphocytes (e.g., IL6; Fig. 3A). Expression of TNFα RNA was also upregulated in M-MDSCs (Fig. 3B).

Expression of RNA encoding CCL2, a monocyte chemotactic, was reduced in C, I, and NC monocytes (Fig. 3A, C, and D), as demonstrated in solid tumors (23, 27). In a different set of CLL patient samples, we confirmed altered production of IL6, CCL2,
Trabectedin Induces Immunomodulation in CLL

Trabectedin depletes selected human primary lymphoid and myeloid cells. A, Human primary CLL cells obtained from CLL patients (n = 5, patients 1–5; Supplementary Table S1) were incubated with increasing concentrations of trabectedin (or with vehicle (DMSO v1, v2)) and subjected to a luminescent assay at 24 and 48 hours to assess the cells’ sensitivity to the drug. Cell viability of each sample was normalized to its control. Dot lines indicate CLL cell viability at 0.01 μmol/L trabectedin. B, The percentage of depletion of hCD19+ CD5+ cells (plated alone, empty circles; plated with total PBMCs, black circles) and hCD14+ cells (plated with total PBMCs, black circles) after 24 hours of treatment (n = 7, patients 6–12; Supplementary Table S1) with 0.01 μmol/L of trabectedin was analyzed by flow cytometry. Purple and orange dots identify 2 representative CLL patients. C, Monocytes were identified as CD14+ nonclass (green), CD14++ CD16+ intermediate (red), and CD14+++ CD16+ classic (blue) subsets. A representative PB sample from CLL patient 24 (Supplementary Table S2) is described. D, Classic monocyte, nonclassic monocyte, intermediate monocyte, and hCD14+ HLA-DRlow/M-MDSC cell depletion and CD8+ T cell, naive T cell, hCD8+ TCM, hCD8+ TEM, regulatory hCD4+ CD25+ Foxp3+, Treg, and hCD19+, hCD19+ CD5+, and hCD19+ CD5+ CD23+ lymphoid cell depletion (n = 10; patients 20–26, 45, 48, and 49; Supplementary Table S2) after 24 hours of treatment with 0.01 μmol/L of trabectedin were analyzed by multicolor flow cytometry and calculated by the formula described in Materials and Methods. The statistical analysis is described in Supplementary Tables S11 and S12. E and F, An alternate presentation of the distribution of data from D showing selected paired-cell type comparisons is depicted by box-and-whisker plots. P value is given by Mann–Whitney–Wilcoxon test; *, P < 0.05; **, P < 0.01.

Figure 1.

Trabectedin-induced IFN-α protein upon 38 hours of trabectedin treatment (Supplementary Fig. S7A–S7C). Patients 39 and 40 carried a 17p deletion and patient 40 (in red) was in Richter transformation, resistant to ibrutinib and venetoclax (Supplementary Table S2). In some patient PBMC samples, M-MDSCs disappeared after trabectedin treatment. In Tregs, trabectedin downregulated FOXP3, the transcription factor that modulates the functionality of these cells and abolishes
their suppressor activity (Fig. 3E). Unlike leukemic B lymphocytes (26), we did not observe TC-NER and homologous recombination (HR) pathways involvement in the selective targeting of Tregs by trabectedin (Supplementary Fig. S8).

The reduced immunosuppression was accompanied by upregulation of RNA encoding cytolytic factors in CD8\(^+\) TEM and TCM, including IFN\(\gamma\) and GRANZYM E B (GZMB; Fig. 3F and G).

Thus, trabectedin enhances the antitumor phenotype of monocytes and reprograms the tumor microenvironment toward immune effector T-cell cytotoxicity.

Trabectedin has therapeutic efficacy in CLL mouse models

We next performed a time-course experiment in which mice, transplanted intravenously with MEC1 cells, were sacrificed 1 or 10 days after the last of three trabectedin injections (days 27 and 36, respectively; Fig. 4D). The treatment reduced the leukemic expansion and stabilized the disease in the PB and lymphoid tissues, including SP and BM (Fig. 4E–G). Trabectedin also conveyed a survival benefit as both a single agent (Fig. 4H and I; \(P = 0.0009\), trabectedin vs. untreated) and in combination with GA101, a glycoengineered type II CD20 mAb, GA101 (Fig. 4J and K).

Trabectedin affects immune cells of the tumor microenvironment in vivo

We tested whether trabectedin affected myeloid cells in CLL mouse models, including the xeno- and the TCL1 transgenic-transplantation systems (13). The antileukemic effect observed in xenotransplanted mice sacrificed at day 27, the day after the last injection of the drug (Fig. 4D–G), was associated with a significant

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**Figure 2.**

Trabectedin-induced cell death mechanisms in selected primary lymphoid and myeloid cells from CLL patients. Relative mRNA expression of TRAILR2, TNFR1, BAX, BID, FADD, CASP3, CASP8, CASP9, and MCL1 in hCD19\(^+\)CD5\(^+\) human primary CLL cells, CD14\(^+\)HLADRlow/C0 M-MDSCs, intermediate monocytes, and CD4\(^+\)CD25\(^+\)CD127low/C0 Tregs separated by FACS from PBMCs (\(n = 6\); patients 173, 538, 665, 915, 089, and 642; Supplementary Table S2) and plated in 6-well plates alone or with 0.01 \(\mu\)mol/L of trabectedin for 15 hours. Three technical replicates were analyzed for each sample. Data were normalized to β-actin expression. Gene expression was determined by calculating the difference (\(\Delta\Delta\text{Ct}\)) between the threshold cycle (Ct) of each gene and that of the reference gene, and was expressed as the mean of three replicates ± SEM. Then the relative quantification values were calculated as the fold change expression of the gene of interest over its expression in the selected cell type reference sample (i.e., the untreated sample [considered as the calibrator sample]), by the formula \(2^{-\text{\(\Delta\Delta\text{Ct}\)}}\). Finally, the treated sample relative mRNA expression was normalized to the vehicle. Samples with undetermined Ct values are not included.

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**Figure 3.**
Figure 3.
Immunomodulatory activity of trabectedin in human myeloid and lymphoid cell types in CLL in vitro. A, Relative mRNA expression of IFNα, TNFα, IL12α, IL6, and CCL2 in classic monocytes; B, TNFα in M-MDSCs; C, CCL2 in intermediate monocytes; D, CCL2 in nonclassic monocytes; E, FOXP3 on CD4+ CD25+ CD127low/− Tregs; F, IFNγ in hCD8+ Tep; and G, IFNγ and GZMB in hCD8+ TCM. All cells were separated by FACS from CLL patient PBMCs (n = 6; patients 173, 538, 665, 915, 089, and 642; Supplementary Table S2), and plated in 6-well plates alone or with 0.01 mmol/L of trabectedin for 15 hours. Three technical replicates were analyzed for each sample. Data were normalized to β-actin expression. The gene expression was determined by calculating the difference (ΔΔCt) between the threshold cycle (Ct) of each gene and that of the reference gene, and was expressed as the mean of three replicates ± SEM. Then the relative quantification values were calculated as the fold change expression of the gene of interest over its expression in the selected cell type reference sample, i.e., the untreated sample (considered as the calibrator sample), by the formula $2^{-\Delta\Delta Ct}$. Finally, the treated sample relative mRNA expression was normalized to the vehicle. Samples with undetermined Ct values are not included.
reduction of CD11b⁺ F4/80⁺ TAMs in the SP and PE, with selective depletion of MRC1⁺ M2-like protumor TAMs in the PE (Supplementary Fig. S9A–S9E).

We next used a CLL immunocompetent mouse model, based on the transplantation of leukemic cells purified from the SP of Eμ-TCL1 transgenic (tg) mice into syngenic C57/B6 recipients (13).
To evaluate the immune cell dynamics induced by trabectedin and their persistence over time, we performed three sets of experiments in which mice transplanted with leukemic cells from Eμ-TCL1 tg donors #1, #2, #3 were sacrificed 6, 9, or 13 days after the last trabectedin injection. When donor #1-transplanted mice were treated with trabectedin (starting at day 40) and sacrificed 6 days after the last injection of the drug (day 53), a significant reduction of the leukemic clone in the SP, BM, PE but not in the PB was observed (Supplementary Fig. S10A). This reduction was associated with decreased frequencies of CD11b+ CSF1R+ monocytes and F4/80+ macrophages and with selective depletion of PD-L1+ monocytes and MRC1+ and PD-L1+ M2-like TAMs in the PE (Supplementary Fig. S10E–S10I). Among monocytes, we observed a reduction of the Ly6Clow cell component expressing PD-L1 in the SP (Supplementary Fig. S10J): such a reduction suppresses T-cell immunity in solid tumors (41). Overall, these data are in line with findings on the PD-L1–mediated functional remodeling of monocytes and macrophages in colon and mammary carcinoma (42). We observed a decrease in the whole pool of Gr1+ MDCSs in the PB, BM, and SP (Supplementary Fig. S10K–S10M), an increase of CD8+ T lymphocytes in the BM and SP and an increase of CD8+ memory T cells in the PB and BM (Supplementary Fig. S10N–S10Q).

To clarify the long-term cell dynamics induced by trabectedin in vivo, we transplanted mice with splenic leukemic cells from TCL1 tg donor mouse #2 or donor mouse #3 and sacrificed them 9 (Fig. S5A, donor #2 recipients) and 13 days (Fig. S5B, donor #3 recipients) after the last trabectedin injection, respectively. In donor #2 recipient mice treated with trabectedin starting on day 25 (Fig. S5A), a significantly lower percentage of leukemic B cells in the PB was observed over time (Fig. 5C). Upon necropsy, comparative analysis of the treated and untreated mice confirmed that in both donor #2 and donor #3 recipient mice (Fig. S5A and B) leukemic cell expansion was reduced in the PE, SP, BM, and LN (Fig. 5D–G). Trabectedin treatment selectively depleted CD19+CD5+ leukemic cells expressing PD-L1 in the lymphoid tissues and PE (Supplementary Fig. S11A–S11C) of donor #3 recipient mice killed 13 days after the last trabectedin injection.

As for the myeloid cells, in mice sacrificed 9 days after the last trabectedin injection (Fig. S5A, donor #2 recipients), numbers of CSF1R+ monocytes (Fig. 5H) and CD11b+ PD-L1+ (Fig. 5I) monocytes were reduced in the BM, and the Ly6Glow subset was the most affected and significantly reduced in the BM and PB (Fig. S5J and K). Conversely, Ly6Chi high monocytes increased in the SP of treated mice (Supplementary Fig. S12A and S12B). In mice sacrificed 13 days after the last trabectedin injection (Fig. S5B, donor #3 recipients), the whole pool of monocytes (Fig. 5L and M), CD11b+ PD-L1+ monocytes (Fig. S5N and O), and Ly6Cminim subset (Fig. 5P and Q) was reduced in the BM and PE of treated mice.

As for M-MDSCs and TAMs, we confirmed (Fig. 6A, donor #2 recipients) the reduced frequency of M-MDSCs in the PB, BM, SP, LN, and PE (Fig. 6B–F) and that of MRC1+ PD-L1−, and IAβlow protumor TAMs in the BM (Fig. 6G–I). M-MDSCs remained reduced in the SP 13 days after the last trabectedin injection (Fig. 6K and L, donor #3 recipients), as did CD11b+ F4/80+ TAMs and MRC1− PD-L1+, and IAβlow protumor TAMs in the BM (Fig. 6M–P). SP (Supplementary Fig. S13A–S13E), and PE (Supplementary Fig. S13F and S13G).

In treated mice, myeloid cell targeting by trabectedin was associated with increased frequencies of CD8+, CD4+, CD8+ TEM, CD4+ TEM, and TEM cells in the PB (Supplementary Fig. S14A–S14E) and increased frequency of potentially regulatory CD4+ CD25+ Tregs in the mesenteric LN (Supplementary Fig. S14F). Thirteen days after last trabectedin treatment (Supplementary Fig. S14G, donor #3 recipients), CD8+ and CD4+ TEM cells were maintained at higher frequencies in circulation (Supplementary Fig. S14H–S14I). and these frequencies were associated with a significant reduction of CD4+ CD25+ T cells in the PE, SP, and LN (Supplementary Fig. S14K–S14M). These results are in line with the reduction of PD-1+ CD8+ memory T cells we observed in the BM and LN of trabectedin-treated mice sacrificed 9 (Supplementary Fig. S15A, donor #2 recipients) or 13 (Supplementary Fig. S15B, donor #3 recipients) days after the last treatment (Supplementary Fig. S15C and S15D).

**Trabectedin repairs immune dysfunction of CLL patient-derived cells**

To conclusively delineate the direct effect of trabectedin on nonleukemic immune cells, we transplanted patient-derived monocytes or CD8+ memory T cells into leukemic MEC1-transplanted Rag2−/−, Il2rg−− mice. Using fluorescence-activated cell sorting, we separated monocytes (including NC, I, and C) from patients 41 to 44 (Supplementary Table S2) and T cells (including CD8+ TEM and TCM) from patients 41 to 43 (Supplementary Table S2). After 16 hours of *in vitro* trabectedin treatment (0.01 μmol/L), monocytes or CD8+ T cells were adoptively transferred into xenotransplanted mice (Fig. 7A and B). Compared with mice injected with untreated T cells and monocytes, mice injected with trabectedin-treated T cells and monocytes benefited from significant antileukemic effects in the SP (Fig. 7C).

The functionality of CLL patient-derived CD14+ monocytes and CD68+ macrophages upon trabectedin was confirmed *in vitro* in a different set of patient samples by means of a VPD-based assay. The number of not-phagocytosed VPD-labeled CD14+ VPD− MEC1 target cells or CD68+ VPD− MEC1 target cells treated or not with trabectedin was quantified by flow cytometry. In most patient samples tested, trabectedin did not impair the phagocytic ability of monocytes and macrophages (Fig. 7D).

To confirm T-cell functionality upon trabectedin treatment *in vitro*, CD8+ memory T cells, in the absence or presence of antigen (Ag) stimulation with heated-MEC1 cells, were treated with 0.01 μmol/L trabectedin and then assessed for their expression of PD-1 checkpoint markers and ability to produce cytolytic proteins. Upon trabectedin treatment, absolute numbers of total CD8+ and CD8+ TEM cells were not affected (Supplementary Fig. S16A and S16B), whereas PD-1+ CD8+ and PD-1+ TEM cells were reduced significantly (Fig. 7E–H). CD8+ TEM cells retained their capacity for IFNγ and GRANZYME B production in both the presence and absence of trabectedin (Supplementary Fig. S16C and S16D). Indeed, T cells from CLL patients have increased expression of the PD-1 exhaustion marker but retain their ability to produce cytokines (43). We demonstrated that trabectedin restores T-cell functionality, likely interfering with PD-1 checkpoint molecule.

Overall, these findings indicate that trabectedin inhibits leukemia progression with a cascade of immunomodulatory effects in immune cell types of the malignant microenvironment (Supplementary Fig. S17).
Figure 5.

Long-term impact of trabectedin on leukemic cells and on monocytes in the TCL1 tg transplantation system. **A**, C57BL/6 mice intraperitoneally transplanted with leukemic B cells from $E_{m}$-TCL1 transgenic mouse donor #2, left untreated ($n=6$, black circles) or treated (days $+25$, $+32$) with 0.15 mg/kg i.v. of trabectedin ($n=5$, white circles), were killed on day 41 (9 days after the last injection of trabectedin) and analyzed by flow cytometry. **B**, C57BL/6 mice intraperitoneally transplanted with leukemic B cells from $E_{m}$-TCL1 transgenic mouse donor #3, left untreated ($n=4$, black circles) or treated (days $+19$, $+26$) with 0.15 mg/kg i.v. of trabectedin ($n=4$, white circles), were killed on day 39 (13 days after the last injection of trabectedin) and analyzed by flow cytometry. **C**, The mean value ± SD of the relative contribution of CD19$^+$ CD5$^+$ cells to the whole B-cell pool over time in PB of mice described in **A** is shown in the graph. The mean value ± SD of the absolute number of CD19$^+$ CD5$^+$ cells gated on CD45$^+$ in BM of mice described in **A** is shown in graph **I**. The mean value ± SD of the absolute number of CD11b$^+$ CSL1R$^+$ cells gated on CD45$^+$ in BM of mice described in **A** is shown in graph **J**. The mean value ± SD of the absolute number of the CD11b$^+$ Ly6C$^{lo}$ monocyte subset gated on CD45$^+$ in the BM of mice described in **A** is shown in graph **K**. The mean value ± SD of the absolute number of CD11b$^+$ CSF1R$^+$ cells gated on CD45$^+$ in BM ($L$) and PE ($M$) of mice described in **B** is shown in graphs. The mean value ± SD of the absolute number of CD11b$^+$ PD-L1$^+$ cells to the whole monocyte pool (CD11b$^+$ CSF1R$^+$) gated on CD45$^+$ in BM ($N$) and PE ($O$) of mice described in **B** is shown in graphs. The mean value ± SD of the absolute number of the CD11b$^+$ Ly6C$^{hi}$ monocyte subset gated on CD45$^+$ in the BM ($P$) and PE ($Q$) of mice described in **B** is shown in graphs. *P < 0.05; **P < 0.01; Student t test. abs. number, absolute number; Trab, trabectedin.

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Figure 6.
Long-term impact of trabectedin on leukemic cells, M-MDSCs, and TAMs in the TCL1tg transplantation system. A, C57BL/6 mice intraperitoneally transplanted with leukemic B cells from Em-TCL1 transgenic mouse donor #2, left untreated (n = 6, black circles) or i.v. injected (days +25, +32) with 0.15 mg/kg of trabectedin (n = 5, white circles), were killed on day 41 (9 days after the last trabectedin injection) and analyzed by flow cytometry. The mean value ± SD of the relative contribution of monocytic Ly6C+ Ly6G- gated on CD11b+ F4/80low cells in the PB (B) and of the absolute number ± SD of mononuclear Ly6C+ Ly6G- gated on CD11b+ F4/80high cells in BM (C), in SP (D), in LN (E), and in PE (F) of mice described in A is shown in graphs. G, The mean value ± SD of the absolute number of CD11b+ F4/80+ cells gated on CD45+ in BM is shown in the graph. The mean value ± SD of the absolute number of CD11b+ F4/80+ cells gated on CD45+ in BM to the whole macrophage pool (CD11b+ F4/80+) gated on CD45+ in BM of mice described in A is shown in graphs. K, C57BL/6 mice intraperitoneally transplanted with leukemic B cells from Em-TCL1 transgenic mouse donor #3, left untreated (n = 4, black circles) or i.v. injected (days +19, +26) with 0.15 mg/kg of trabectedin (n = 4, white circles), were killed on day 39 (13 days after the last trabectedin injection) and analyzed by flow cytometry. L, The mean value ± SD of the absolute number of mononuclear Ly6C+ Ly6G- gated on CD11b+ F4/80high cells in SP of mice described in K is shown in the graph. M, The mean value ± SD of the absolute number of CD11b+ F4/80+ cells gated on CD45+ in BM is shown in the graph. The mean value ± SD of the absolute number of CD11b+ MRC1+ cells (N), of CD11b+ PD-L1+ cells (O), and of CD11b+ IA- low cells (P) to the whole macrophage pool (CD11b+ F4/80+) gated on CD45+ in BM of mice described in K is shown in graphs. *P < 0.05; **P < 0.01; ***P < 0.001, Student t test. abs. number, absolute number; Trab, trabectedin.
In vivo and in vitro antitumor activity of patient-derived T cells and monocytes/macrophages. A and B, Rag2−/−/γc−/− mice intravenously injected with MEC1 cells (day 0) were adoptively transferred on day 18 with monocytes (i.v.: 35,000–156,000 cells depending on the patient, including NC, C, and I subsets) or CD8+ memory cells (i.v.: 36,000–226,000 cells depending on the patient, including TEM and TCM cells) separated from the PBMCs of patients 41–44 (Supplementary Table S2) and in vitro pretreated with 0.01 μmol/L trabectedin for 15 hours. Mice injected with the same patient sample cells (either treated or untreated, n = 4) received the same number of cells and were killed at day 23. C, The mean value ± SD of the absolute number of hCD19+ in the SP from mice transplanted with monocytes (left) and T cells (right) is shown in the graph. *, P < 0.05, Student t test. Trabe, trabectedin; Unt, untreated.

D, Monocytes (including NC, C, and I) from patient 43 and patients 45–49 (n = 6) were separated by cell sorting and coincubated with VPD-labeled MEC1 cells at an effector/target ratio of 1:1 in the presence or absence of 0.01 μmol/L trabectedin for 16 hours. The percentage of viable MEC1 target cells was measured by flow cytometry to determine absolute numbers of surviving MEC1 cells. The percentage of trabectedin-mediated phagocytosis by CD68+ macrophages and CD14+ monocytes was calculated as described in Materials and Methods. E–H, CD8+ T lymphocytes (including TEM and TCM) from PBMCs of patients 45–49 (n = 5) were separated by cell sorting and coincubated in vitro with heated-MEC1 cells (at an effector/target ratio of 2:1) to induce tumor antigen-specific T-cell response (Ag-experienced) in the absence (C) or presence (Trab) of 0.01 μmol/L trabectedin for 15 hours. E, The mean value ± SD of the absolute number of PD-1+ T cells to the whole CD8+ T-cell pool gated on live cells is shown in the graph. *, P < 0.05; ***, P < 0.001, Student t test. F, Flow-cytometry detection of PD-1 by CD8+ T cells from representative patient 46. The percentage of PD-1-expressing cells is indicated. G, The mean value ± SD of the absolute number of PD-1+ cells to the TEM cell pool gated on CD8+ T cells is shown in the graph. H, Flow-cytometry detection of PD-1 on CD8+ TEM cells from representative patient 46. The percentage of PD-1-expressing cells is indicated. abs. number, absolute number; Trab, trabectedin.
Trabectedin Induces Immunomodulation in CLL

Discussion

In this study, we demonstrated that in CLL trabectedin interfered with leukemic cells and nonleukemic myeloid and lymphoid cells of the microenvironment.

In vitro, trabectedin was directly cytotoxic to human primary leukemic cells. It upregulated mRNAs for several cell death pathway elements such as CASP3 (supporting the caspase-3 activity seen in CLL, ref. 26), while downregulating the mRNA for antiapoptotic MCL1.

Trabectedin depleted selected human myeloid cell subtypes by rapidly engaging CASPASEs and other cell death pathway elements, corroborating previous findings with CASP8 (23). The murine counterpart of human CD16+ monocyte subsets is represented by Ly6Chi monocytes (44), which were depleted by trabectedin in our CLL mouse studies, in a T cell–independent manner. In mice, trabectedin depleted Ly6Chi monocytes expressing PD-L1, which inhibits T-cell proliferation and cytotoxic potential in solid tumors (41). In contrast, trabectedin has a selective activity on Ly6Chig monocytes in murine fibrosarcoma (23), implicating a different leukemia-dependent cell mechanism (45). Patient-derived xenograft and in vitro phagocytosis studies showed that trabectedin enhanced immunomodulatory activity of monocytes and macrophages. These trabectedin-dependent effector cell mechanisms are reminiscent of the immunomodulatory activity of lenalidomide, a regulator of T-cell and macrophage function in CLL (33, 46).

Trabectedin had selective activity against human and mouse M-MDSCs, which inhibit T-cell activity (39) and limit antitumor activity through immunosuppression in CLL (14). In human in vitro studies and in vivo mouse models, targeting M-MDSCs was associated with reduced expression of transcription factor FOXP3 mRNA and the depletion of immunosuppressive Tregs, which often accumulate in CLL patients and are linked to worse prognosis and advanced disease (47). Trabectedin can break the immunosuppressive biological cross-talk among CLL cells, MDSCs, and Tregs.

Consistent with data in fibrosarcoma (23), long-term targeting of murine M2-like protumor TAMs was another determinant of in vivo trabectedin efficacy in CLL mouse models.

Effects other than tumor-associated myeloid cell depletion were involved in the antitumor activity of trabectedin, including downregulation of chemokine CCL2 RNA and protein expression in all monocyte subtypes. The CCL2/CCR2 axis is involved in the recruitment of myeloid cells at the tumor sites and several anti-tumor strategies interfere with this axis (48). Another effect of trabectedin was the rapid increase in mRNA expression of proinflammatory cytokines that promote the classic M1 antitumor activation of macrophages, inducing T-cell responses and interfering with B-cell activity (49). TNFα, IFNγ, IL12a, and IL6 RNA and protein were differentially expressed by human classic monocytes and M-MDSCs. Cytokines, including IFNγ, might be induced as a byproduct of the DNA-repair mechanisms (50). These transcriptional changes correlated with the induction of mRNA transcripts for IFNγ and GZMB cytolytic factors in human memory T lymphocytes. Supporting our in vitro findings in human PBMCs, we observed induction of circulating memory CD4+ and CD8+ T cells in the TCL1 tg immunocompetent mouse system. Trabectedin effects on T cells were consistent with osteosarcoma showing that recruitment and expansion of adaptive T cells were induced by trabectedin (28). However, in our immunocompetent mouse system, trabectedin interfered with the PD-1/PD-L1 axis in the lymphoid tissues by reducing CLL cells as well as monocytes/macrophages expressing PD-L1, and also memory CD8+ T cells expressing PD-1. Unlike for osteosarcoma and pancreatic adenocarcinoma (28, 51), in primary CLL patient cells, trabectedin reduced PD-1 and restored CD8+ T-cell antitumor function. Alterations in DNA damage response and repair genes are associated with response to PD-1/PD-L1 blockade in patients with metastatic solid tumors (52). In addition, the downregulation of PD-L1 we observed on myeloid cells is in line with evidence on the role of PD-1/PD-L1 checkpoint in monocyte/macrophage remodeling (42) and indicates that trabectedin has immunomodulatory activity.

Trabectedin, besides having a cytotoxic effect in leukemic cells, has immunomodulatory activity on several cell types of the microenvironment. Our findings suggest a therapeutic strategy in which one drug hits both the leukemic cell compartment and the protumor microenvironment, repairing CLL immune dysfunction. Future studies should aim to clarify the molecular mechanisms by which trabectedin functions in the distinct immune cell types.

Disclosure of Potential Conflicts of Interest

M. Aracil is a project leader, R&D, at PharmaMar. C. Klein is a department head, has a family member who is an employee of, and has ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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Trabectedin Reveals a Strategy of Immunomodulation in Chronic Lymphocytic Leukemia

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