Inhibition of Integrin αVβ3 Signaling Improves the Antineoplastic Effect of Bexarotene in Cutaneous T-Cell Lymphoma

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ABSTRACT

Bexarotene is a specific retinoid X receptor agonist that has been used for the treatment of cutaneous T-cell lymphoma (CTCL). Because bexarotene causes hypothyroidism, it requires the administration of levothyroxine. However, levothyroxine, in addition to its ubiquitous nuclear receptors, can activate the αVβ3 integrin that is overexpressed in CTCL, potentially interfering the antineoplastic effect of bexarotene. We thus investigated the biological effect of levothyroxine in relation to bexarotene treatment. Although in isolated CTCL cells levothyroxine decreased, in an αVβ3-dependent manner, the antineoplastic effect of bexarotene, levothyroxine supplementation in preclinical models was necessary to avoid suppression of lymphoma immunity. Accordingly, selective genetic and pharmacologic inhibition of integrin αVβ3 improved the antineoplastic effect of bexarotene plus levothyroxine replacement while maintaining lymphoma immunity. Our results provide a mechanistic rationale for clinical testing of integrin αVβ3 inhibitors as part of CTCL regimens based on bexarotene administration.

Teaser: Inhibiting αVβ3 integrin improves the antineoplastic effect of bexarotene while maintaining lymphoma immunity.

Introduction

Cutaneous T-cell lymphoma (CTCL) is a heterogenous group of non-Hodgkin lymphomas caused by the initial homing of malignant T cells to the skin. The most common subtypes include the mycosis fungoides and the more aggressive Sezary syndrome. Bexarotene is a retinoid X receptor (RXR)-selective agonist that is clinically used for the treatment of patients with CTCL that fail to respond to at least one systemic therapy (1). In addition, bexarotene in combination with other treatments for CTCL have been extensively evaluated in clinical trials (1). The mechanism of bexarotene activity relies on the binding and activation of RXR that, in turn, dimerize with other nuclear receptors to activate gene expression (2). In CTCL cells this could lead to cell-cycle arrest, apoptosis, and impaired chemotaxis (3–5). In addition, bexarotene has an immunomodulatory effect particularly in the activity of normal T cells (6). However, the relative contribution of these effects to the overall antineoplastic activity of bexarotene has not been completely addressed (2).

The clinical use of bexarotene is associated with development of hypothyroidism in more than 95% of patients as consequence of the central suppression of thyroid-stimulating hormone and the increase of the clearance of thyroid hormones in peripheral tissues (7). The almost universal prevalence and fast development of this side effect requires the concurrent administration of levothyroxine when initiating a bexarotene treatment (7). The demonstration that physiologic levels of thyroid hormones can influence the biology of T-cell lymphomas in preclinical models (8) as well as their influence on the immune system (9) provides the rationale to investigate a possible interference of levothyroxine replacement therapy in the antineoplastic activity of bexarotene.

The cellular activity of thyroid hormones (TH) is mediated by the binding of triiodothyronine (T3) to the nuclear receptors TRα or TRβ, generally as heterodimers with RXR, leading to the transcriptional expression of target genes containing TH-response elements (10). In addition, T3 and its prohormone thyroxine (T4) can also activate membrane-bound receptors, such as the integrin αVβ3, to mediate signaling events in many cell types (11). Specifically, in malignant T cells, we have demonstrated that TH-dependent activation of integrin αVβ3 increases cell proliferation and secretion of VEGF (8). Accordingly, pharmacologic inhibition of this integrin dimer with cilengitide, induces tumor apoptosis and anti-angiogenesis in xenograft mice models of T-cell lymphoma (8). On the other hand, TH are also important modulators of the cellular immunity (12, 13), an effect likely mediated by the activity of TH on nuclear receptors since normal lymphocytes do not express integrin αVβ3 dimers (8). This is a potential important aspect of the bexarotene-induced hypothyroidism, because the immune system plays a key role in CTCL progression as exemplified by the association of tumor-infiltrating

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$CD8^+$ T cells with good prognosis (14, 15) as well as by the increasing presence of Th2 cells in the tumor microenvironment during disease progression (16).

The consequences of levothyroxine administration to the antilymphoma effect of bexarotene are unknown. Given the above considerations, it is possible that physiologic concentrations of TH decrease the activity of rexinoids administered locally or systemically. However, not replacing TH in patients receiving bexarotene, in addition to cause symptoms of hypothyroidism, could risk decreasing the lymphoma immune response ultimately compromising the efficacy of the treatment. On the basis of our previous work (8), we hypothesized that inhibition of TH-dependent integrin αVβ3 could circumvent the problem of levothyroxine replacement, allowing the optimization of bexarotene treatment in CTCL. Here, we report that the antilymphoma effect of bexarotene results from decreasing cell proliferation and motility in cancer cells while improving the lymphoma immune response. We identified the molecular mediators of these effects in CTCL cells. We described that levothyroxine replacement decreases the effect of bexarotene in CTCL cells, however improves lymphoma immunity. Using a mouse model of T-cell lymphoma, we found that mice treated with bexarotene and levothyroxine replacement had improved antineoplastic responses and preserved lymphoma immunity when this treatment was combined with the integrin αVβ3 inhibitor cilengitide.

Materials and Methods

Cell culture

Human CTCL cells HuT78 and MJ, and murine TCL EL4 cells were obtained from the ATCC and cultured in RPMI1640 medium, supplemented with 10% FBS, 2 mmol/L glutamine and antibiotics (all from Invitrogen). We conducted monthly tests for mycoplasma sp. and other contaminants and yearly cell identification by single-nucleotide polymorphism. To maintain a stable physiologic concentration of thyroid hormones in the culture medium, we added levothyroxine (T4) and triiodothyronine (T3) to thyroid-hormone depleted serum (10%) in RPMI1640. The final concentrations of T4 and T3 were 100 and 1 nmol/L, respectively. Bexarotene and cilengitide were purchased from MedChemexpres.

Transient transfections and gene knockdown

For transfection, 1×10^7 HuT78 cells in RPMI1640 medium were mixed with a final concentration of 50 nmol/L of each siRNA against *THRA, ITGAV*, and *ITGB3* or to a noncoding sequence (L-004565, L-004124, and D-001810, respectively, ON-TARGETplus SMART pool; Thermo Fisher Scientific) and electroporated at 250 V for 13 milliseconds in a BTX ECM 830 electroporator. After transfection, cells were plated in RPMI with 10% FBS for 24 hours and then treated with the different conditions shown in the results.

Cell viability and apoptosis assays

Cell viability was evaluated at 48 hours with the fluorometric resazurin reduction method (CellTiter-Blue; Promega). The percentage of viable cells was calculated by using the linear least-squares regression of the standard curve. Fluorescence was determined for six replicates per treatment condition, and cell viability in treated cells was normalized to their respective controls. Apoptosis was determined at 72 hours by caspases 7 and 3 activation using the caspase-Glo 3/7 assay (Promega) following the manufacturer's instructions.

RT-PCR

Cell samples were homogenized in Tri-Reagent (Genbiotech) and total RNA was isolated following the manufacturer's instructions. RNA pellets were dissolved in RNase-free water and stored at −80°C. The RNA concentration was quantified by measuring the absorbance at 260 nm. The purity of the RNA preparations was assessed by spectrophotometry at 260/280 nm ratios and on a 1% agarose gel stained with ethidium bromide. The samples were used for RT-PCR analysis. Complementary DNA (cDNA) was synthesized by retrotranscription using the M-MLV reverse transcriptase (Biodynamics SRL). cDNA amounts present in each sample were determined using a commercial master mix for real-time PCR containing SYBR Green fluorescent dye (Biosystem SA). qPCR reactions were carried out in an Applied Biosystems 7500. The primer sequences (Biodynamics SRL) were designed using Primer Express software version 3.0 (Applied Biosystems) with a melting temperature of 60 to 61°C. Quantification of the target gene expression was performed using the comparative cycle threshold (Ct) method according to the manufacturer's instructions (Applied Biosystems). An average Ct was obtained from the duplicate reactions and normalized to *RPL13A* and the ΔΔCt was calculated.

RNA sequencing

RNA was isolated from HuT78 cells transfected with siRNA for integrins (si-ITGAV/B3); TRA (si-TR) or nontarget sequences (si-CT) followed by treatment with bexarotene in the presence of physiologic concentrations of TH, for 6 hours using Tri-reagent (Genbiotech). Genomic DNA was eliminated, and RNA concentration and purity were determined by the Nanodrop (Thermo Fisher Scientific). RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA integrity number values were greater than 8 for all samples. Sequencing libraries were generated with polyA+ RNA using the TruSeq RNA Sample Prep Kit (Illumina). Sequencing was performed at the Epigenomics Core Facility of Weill Cornell Medicine, using the Illumina HiSeq2000. Raw-sequencing reads were quality checked using Fastp and summarized with MultiQC. Transcripts were quantified against Genocode v29 using Salmon. Transcript abundances were summarized with tximport in R version 3.6.2. Gene expression changes were assessed with DESeq2. Pathway enrichment scores per sample were calculated with GSVA.

Human samples and single-cell RNA sequencing

Pathology discarded human samples were obtained with written informed consent from patients according to the Helsinki's protocol and under the approval of the Weill Cornell Medicine—New York Presbyterian Hospital IRB. Library preparation, sequencing and post-processing was performed at the Weill Cornell Medicine Epigenomics Core. Briefly, T-cell receptor (TCR) repertoire and 5′ gene expression sequencings were performed on PBMC obtained from two different patients before and after 30 days of bexarotene treatment using the Chromium (10× Genomics) Kit. Targeted number of cells per sample was set at 4,000. Libraries were sequenced using the Illumina NovaSeq 6000. Fastq files were aligned to human genome (GRCh38) using the CellRanger v3.1 pipeline according to the manufacturer's instructions. Processing was performed in R using the Seurat v2 library. Cells were filtered on the basis of gene number (min. features = 200) and mitochondrial read content (<15%). PCA was performed using 2,000 highly variable genes. Differential gene expression analyses on the malignant cells were run with FindMarkers function in Seurat using DESeq2 test.

Mouse experiments

All experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals, School of Pharmacy and Biochemistry, University of Buenos Aires. A murine model of TCL was developed using male C57BL/6J mice 6 to 8 weeks old implanted subcutaneously with 3×10^6 EL4 cells (17). Tumor volume was measured every other day for the duration of the experiment, and the area under the tumor growth curve (AUC) was calculated. When EL4 tumors reached a palpable size (75–100 mm^3) mice were randomized into three treatment arms to receive orally: vehicle (5% DMSO in corn oil), bexarotene (5 mg/day) with or without levothyroxine (150 ng/day, Sigma). In other experiments, tumor bearing mice were randomized into four treatment arms: vehicle (35% PEG300, 5% Tween-80, and 65% dextrose 5% in water), bexarotene + levothyroxine (as indicated above), cilengitide (1 mg/day), and bexarotene + levothyroxine + cilengitide. Plasma levels of T4 were determined using commercial RIA kits with specific antibodies according to the manufacturer's instructions (Immunotech).

Immune cells quantification and activity

Lymphoid organs and solid tumors were removed and disrupted through a 1-mm metal mesh. The red blood cells were lysed using a buffer containing 150 mmol/L NH_4Cl, 10 mmol/L K_2CO_3, and 0.1 mmol/L EDTA. The resulting cell suspensions were filtered through a 40-μm cell strainer (BD Bioscience) and resuspended in PBS. Single-cell suspensions obtained from tumors, TDLN and spleens were stained with antibodies against various cell surface markers using standard staining methods. The following panel of commercially available and fluorochrome conjugated anti-mouse mAbs were used in the study: PE-anti-mouse CD8, FITC-anti-mouse CD44, PE-anti-mouse Gr1, FITC-anti-mouse CD11b (BD Bioscience). At least 20,000 cells were acquired using a BD Accuri TM C6 flow cytometer. Cytokine production was measured in supernatants obtained from tumor explants or cell suspensions of TDLN. From one side, seven pieces (1 mm × 1 mm) of tumors were cultured in RPMI medium and after 24 hours supernatant were collected. On the other side, 2×10^6 cell suspensions obtained from TDLN were cultured with 2×10^5 EL4 irradiated cells (EL4i), and after 24 hours supernatants were collected. IFNγ concentration was quantified using a mouse Th1/Th2 ELISA ready SET-go (Thermo Fisher scientific). TNFα concentration was quantified using Bead-Based Immunoassays from BD Biosciences. Single-cell secretomics were done in unstimulated peripheral monocytes from a CTCL patient (before and after bexarotene treatment) using IsoLight single-cell human immune adaptive secretome arrays (Isoplexis).

Cell proliferation in CTCL organoid cultures

Maleimide functionalized 4-arm polyethylene glycol (PEG-MAL; 20,000 Da, 99% functionalized) was purchased from Laysan Bio, Inc., and cross-linker peptide used was NH2-GCRDVPMSMRGGDRCG-COOH. Hydrogels were prepared as reported previously (8). Briefly, 4-arm PEG-MAL were functionalized with cell adhesive ligand RGD peptide (NH2-GRGDSPC-COOH) and cross-linked with collagenase degradable peptide (VPM) to form hydrogels. In each 10 μL hydrogel, HuT78 CTCL cells and human tonsil-derived HK follicular dendritic cells were encapsulated at 2(CTCL):1(HK) ratio. The hydrogels were then incubated with vehicle (DMSO), 1.5μmol/L bexarotene, and/or 1.5μmol/L cilengitide in the presence of thyroid hormones (1 nmol/L T3 and 100 nmol/L T4). Cells were then encapsulated within 3D hydrogels by mixing with polymers prior to gelation. After 96 hours of incubation, hydrogels were photographed using a Nikon Biostation CT and quantify using Cell Titer Glo 3D (Promega).

3D cell motility assays

HuT78 CTCL cells were embedded into type I collagen gels, as described previously (18, 19). Briefly, 10^5 cells were suspended into a 1:1 volume mixture of cell culture media and reconstitution buffer, then mixed with the appropriate volume of soluble rat-tail collagen-1 (Corning) to obtain a final concentration of 1 mg/mL. After mixing, a calculated amount of NaOH was quickly added to obtain a final pH of ∼7. Then, 250 μL of the collagen containing cell-suspension was added per well in 48-well plates and incubated under physiologic conditions for 30 minutes to allow collagen polymerization. Once polymerized, 300 μL of fresh media, or drug-containing media was added to the top of the gels and allowed to acclimate in the cell culture incubator for 1 hour prior to imaging. In the case of Cilengitide-treated conditions, cells were incubated in a 5 μmol/L solution of drug for 1 hour prior to embedding into the collagen gels, where the supernatant contained either equimolar concentrations of cilengitide or bexarotene, respectively. Following the 1-hour incubation of cell-impregnated gels with compounds, the plate was mounted onto an EVOS FL auto (Thermo Fisher Scientific), equipped with a live cell environment control (temperature, humidity, and CO_2). Phase-contrast images (at 10×) were acquired every 5 minutes for 16 hours per condition. The image recognition software (MetaMorph, Molecular Devices) was used to track 12.5 continuous hours of single-cell movements, based on the displacement of the cell centroids. For evaluating single cell movements, only cells located at least 100 μm from the plate's surface in the z-axis direction was tracked to diminish edge effects. Furthermore, cells that either moved out of the image frame, divided, had long contact time with other cells, and cells that underwent cell death during the 16-hour tracking window were omitted from the analysis. From the movement trajectories per single cell, the x–y coordinates were extracted and analyzed using the anisotropic persistent random walk model described previously (20). Parameters to quantify cell motility include: mean squared displacements (MSD) as the average squared distances of cell movements per 20-minute intervals; time lag as multiples of the image interval time of 5 minutes; average speed as the distance moved per unit time computed as the square-root of the 20-minute MSD divided by time; total diffusivity as the spatio-temporal exploration of single cells per unit time; and anisotropy as the spatial persistence computed as the ratio of diffusivities in the primary and secondary axes of migration based on the APRW model.

Statistical analysis

Means of the different experimental groups were analyzed for statistical significance with the software GraphPad PRISM 4.0 (GraphPad Software), using unpaired two-tailed Student t test or two-way analysis of variance followed by Tukey analysis. Differences between means were considered significant if $P < 0.05$. Results are expressed as mean ± SEM.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials and Methods. RNA-sequencing Gene Expression Omnibus (GEO) with the accession number: GSE119345.

Results

The antineoplastic effects of bexarotene in CTCL are affected by TH levels

To understand the pharmacologic effect of bexarotene in CTCL cell lines, we first exposed them to clinically achievable concentrations of bexarotene (21) and determined proliferation, apoptosis, and motility. CTCL cell lines HuT78 and MJ were grown in complete tissue culture medium with physiologic level of TH to mimic conditions in patients. We found that bexarotene decreased cell proliferation and induced apoptosis (**Fig. 1A** and **B**). In addition, it decreased the spontaneous motility as determined using a 3D motility assay in which HuT78 cells were embedded into a type I collagen extracellular matrix in complete culture medium and four cell-motility parameters were computed (22). We found that bexarotene (vs. vehicle) significantly impacted the distance covered (mean square displacement, $P < 0.0001$), the speed of displacement ($P < 0.0001$), and the extent of spatial exploration (total diffusivity, $P < 0.01$); whereas the directionality was not significantly affected (**Fig. 1C** and **D**). To determine the effect of TH replacement on bexarotene activity, we conducted the above experiments without TH administration. We found that bexarotene suppressed proliferation and increased apoptosis more profoundly in cells grown in TH-depleted conditions (**Fig. 1E** and **F**). The spontaneous motility of

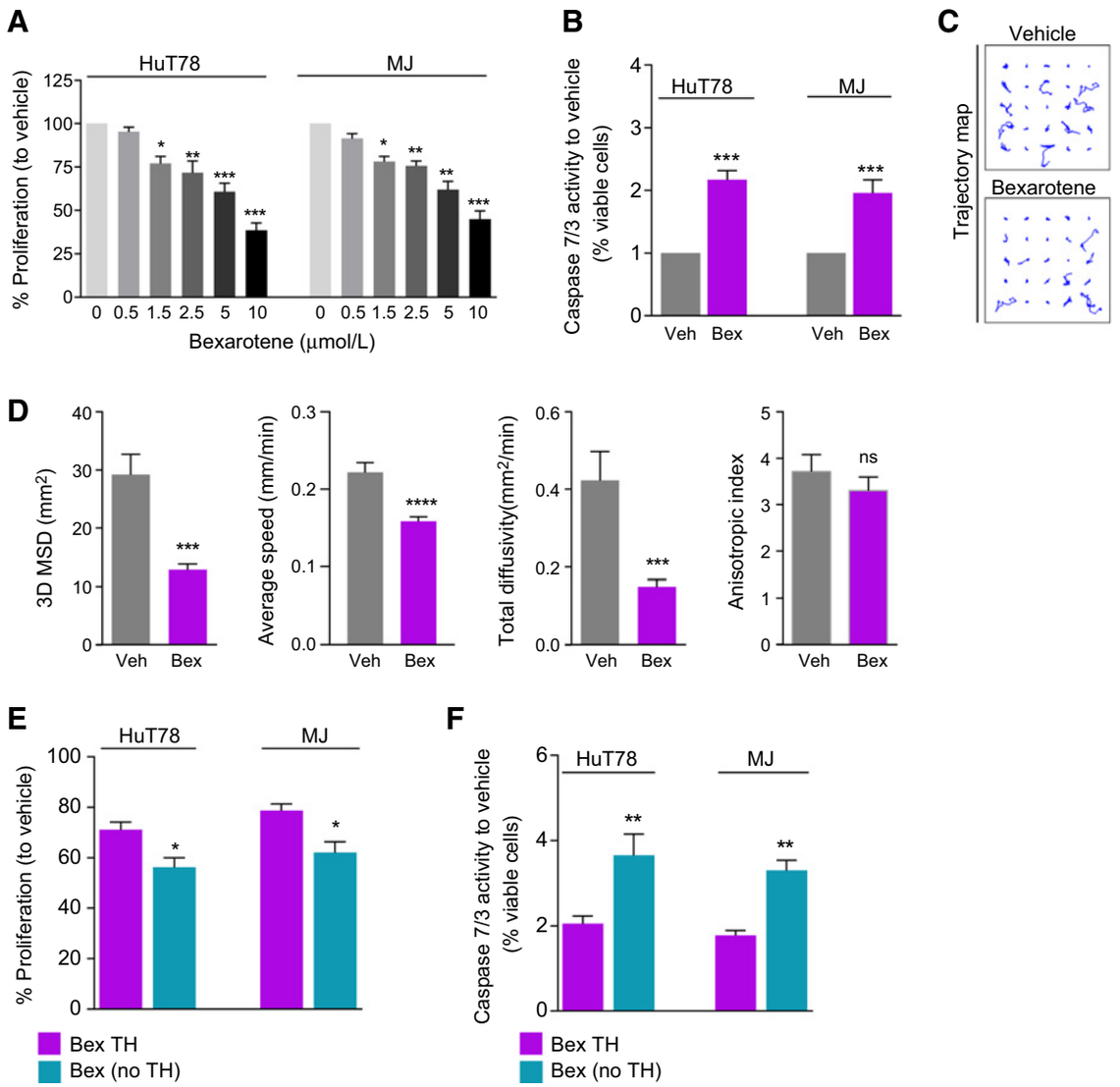

Figure 1.

Effect of thyroid hormones on the *in vitro* activity of bexarotene. **A,** Cell proliferation in HuT78 and MJ CTCL cells upon bexarotene treatment for 48 hours. **B,** Apoptosis induction determined by caspase 3/7 activation in HuT78 and MJ CTCL cells upon bexarotene (Bex, 1.5 μmol/L) or vehicle (Veh) treatment for 72 hours. **C,** Grid showing the trajectories of 25 randomly selected HuT78 cells exposed to vehicle control (DMSO) and 5 μmol/L bexarotene. **D,** Quantification of cellular displacements (MSD at 20 minutes), average speed of displacement, degree of cellular exploration (diffusivity), and spatial persistence (anisotropy) in HuT78 cells treated with bexarotene (vs. vehicle) as in **C**. **E,** Cell proliferation in HuT78 and MJ cells upon bexarotene treatment (48 hours, 1.5 μmol/L) with or without physiologic concentration of TH (T4 = 100 nmol/L plus T3 = 1 nmol/L). **F,** Apoptosis determined by activation of caspases 3/7 in HuT78, and MJ treated as in **E** but for 72 hours. ns, not significant.

HuT78 cells was severely affected by lack of TH impeding the evaluation of this effect under these experimental conditions.

The above experiments indicate that TH may decrease antineoplastic effects of bexarotene; however hypothyroidism may be associated with decrease lymphoma immunity (23). To further investigate this effect, we used the murine EL4 TCL cell line that expresses αVβ3 (Supplementary Fig. S1A) and, like HuT78 and MJ cells, are also sensitive to bexarotene *in vitro* (Supplementary Figs. S1B and S1C). EL4 cells were implanted orthotopically in immunocompetent syngeneic mice to obtain an CTCL-like model suitable to study the lymphoma microenvironment (17). Once tumors reached palpable size, mice were randomized to receive vehicle, bexarotene without levothyroxine, and bexarotene with levothyroxine replacement (**Fig. 2A**). We measured plasma levels of T4 to ascertain that bexarotene induced hypothyroidism and that levothyroxine replacement restored its level within physiological range (**Fig. 2B**). Although bexarotene significantly decreased EL4 growth (**Fig. 2C**, $P = 0.001$, vs. vehicle), its antitumoral effect diminished upon levothyroxine replacement (**Fig. 2C**, $P = 0.002$, vs. no replacement). The analysis of the lymphoma microenvironment showed that mice with no levothyroxine replacement had lower infiltration of cytotoxic $CD8^+$ T cells in tumors and tumor draining lymph nodes (TDLN; $P = 0.0048$, vs. levothyroxine replacement, TDLN; Supplementary Figs. S2D and S1D). The TDLN of these mice also had lower number of activated T cells ($CD8^+CD44^+$; $P = 0.0061$, vs. levothyroxine replacement, **Fig. 2E**). Moreover, mice with no levothyroxine replacement had higher number of splenic myeloid-derived suppressor cells ($Gr1^+CD11b^+$; $P = 0.0008$, vs. levothyroxine replacement, **Fig. 2F**). These results suggest that, in the context of bexarotene treatment, levothyroxine replacement allows for the restoration of a favorable microenvironment for lymphoma immunity. To determine whether this effect was associated with Th1 activity, we measured IFNγ and TNFα secretion from lymphoma explants grown as organotypic cultures (LOC) and in TDLN lymphocytes exposed to irradiated EL4 cells. Consistent with the previous results, we found that although bexarotene decreased IFNγ and TNFα production in the TDLNs and LOCs (**Fig. 2G**), except for IFNγ in TDLNs, the effect was completely restored by levothyroxine replacement (**Fig. 2G**). These indicate that upon bexarotene treatment, the levothyroxine replacement allows for the reestablishment of a cytotoxic Th1-based lymphoma immunity that is critical for the long-term control of the disease.

Bexarotene activates immune transcripts in CTCL cells

We determined the molecular mediators of bexarotene effect by conducting single-cell RNA sequencing of peripheral blood cells from 2 patients with disseminated disease (CTCL1 and CTCL2) before and after 4 weeks of bexarotene therapy. Bexarotene was administered with prophylactic levothyroxine replacement. In the malignant clones, identified by TCR sequencing (**Fig. 3A**), we found 548 and 91 genes differentially expressed upon bexarotene treatment in CTCL1 and CTCL2, respectively (**Fig. 3B**). To associate these changes more precisely with the bexarotene treatment, we exposed HuT78 to bexarotene (with TH) or vehicle (with TH) for a short period (6 hours) and conducted RNA sequencing. We found 6,168 differentially expressed genes. A subset of genes related to CTCL proliferation, apoptosis, motility, and immune response pathways were independently validated in HuT78 and MJ cells by qRT-PCR (Supplementary Fig. S1E; Supplementary Table S1). We then compared genes differentially expressed in bexarotene-treated HuT78 cells with the genes differentially expressed from both bexarotene-treated patients. We found an enrichment in the upregulated genes in these conditions (**Fig. 3C**), consistent with the pharmacological mechanism of bexarotene as an RXR agonist and transcriptional activator. Among the upregulated genes in the three datasets (Supplementary Table S1), we identified *IL32* (**Fig. 3B**), that was reported in association with immune activation via TNFα (24). We then investigated whether the bexarotene-associated expression of *IL32* as well as *INFG* and its regulator *TBX21* in CTCL cell lines was dependent on TH exposure. We found that by bexarotene and TH cotreatment increased the expression all three genes in HuT78 and MJ cells (**Fig. 3D**) and of *Tbx21* and *Ifng* in EL4 cells (**Fig. 3D**, IL32 is not present in mouse), indicating that TH is necessary for upregulating these immune genes in CTCL cells. With variations among cell lines, a similar effect of TH on bexarotene was demonstrated for the downregulated genes *CCR7, CCR4, REL,* and *BCL2* (Supplementary Fig. S1F). Because IL32 can induce TNFα production (24), we investigated TNFα secretion in the monocyte fraction of CTCL2 and found that, indeed, bexarotene treatment increased the proportion of TNFα secreting monocytes (**Fig. 3E**). In addition, other cytokines including IL8 and IFNγ also increased in this patient (**Fig. 3E**). Overall, these data indicate that bexarotene induces the expression of immune genes in CTCL cells and this, in part, requires physiologic levels of TH.

Integrin αVβ3 signaling counteracts the transcriptional effects of bexarotene

Our previous studies (8) demonstrated that TH replacement may stimulate the proliferation of human TCL cells through the activation of both integrin αVβ3 and TRA receptors. To determine their individual contributions to the bexarotene effect, we conducted RNA sequencing in HuT78 cells transfected with siRNA for integrins αVβ3 (si-ITGAV/B3), TRA (si-TRA), or nontarget sequences (si-CT); followed by treatment with bexarotene in all three conditions (**Fig. 4A**). All these experiments were conducted in complete standard tissue culture medium containing physiologic levels of TH. The expression of *ITGAV, ITGB3,* and *THRA* after siRNA treatment was analyzed by qRT-PCR (Supplementary Fig. S1G). To obtain the genes that were mobilized by bexarotene in absence of integrins αVβ3, we conducted a two-way comparison as follows. After normalization by si-CT (+bexarotene), differentially expressed genes in si-ITGAV/B3 (+bexarotene) versus si-TRA (+bexarotene) were obtained from the comparison of bexarotene versus vehicle-treated HuT78 cells (**Fig. 4A**).

We found that majority of bexarotene up- and downregulated genes maintained or increased their mobilization in cells lacking integrins αVβ3 (**Fig. 4A**). Among the upregulated that further increased their expression were *IL26, IL32, IFNG, TBX21*, and *CD74*, among the downregulated that maintained their expression were *CCR7* and *ITGAL*, whereas *CD5, CD6*, *BCL2, BCL2L1*, and *REL* decreased their expression in the second comparison as well (**Fig. 4A**; Supplementary Table S1). We independently confirmed some of these results by qRT-PCR (**Fig. 4B** and **C**). These result suggest that si-ITGAV/B3 may potentiate some antilymphoma effects of bexarotene such as apoptosis and cell proliferation, while sparing (or activate further) most of the immune genes. Accordingly, we found a higher effect of bexarotene in decreasing cell proliferation and inducing apoptosis in HuT78 cells transfected with si-ITGAV/B3 versus si-TRA (**Fig. 4D** and **E**).

Pharmacologic inhibition of integrin αVβ3 improves bexarotene activity in CTCL

To analyze whether the pharmacologic αVβ3 inhibitor cilengitide can mimic the effect of si-ITGAV/B3 in sensitizing CTCL cells to

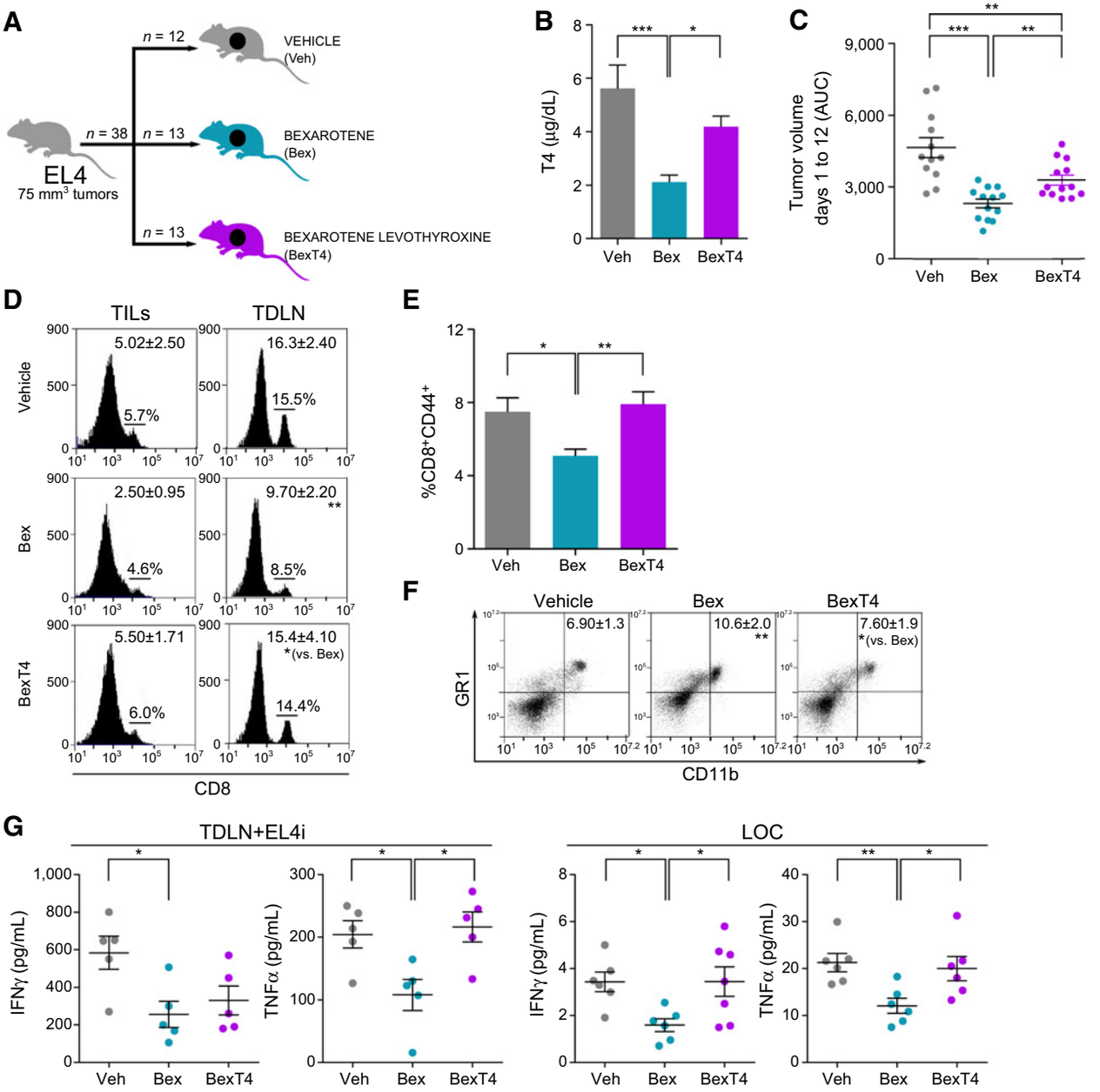

Figure 2.

Effect of TH replacement on the immune effect of bexarotene. **A,** Schematic representation of the experimental design. Once tumors reached a palpable size, mice were randomized in groups to receive treatment with vehicle (Veh), bexarotene with no levothyroxine replacement (Bex), and bexarotene with levothyroxine replacement (BexT4). **B,** Plasma levels of thyroxine (T4) on the three treatment groups measured by radioimmunoassay. **C,** Tumor volume (represented by AUC from day 1 to 12 of treatment) of EL4 cells injected subcutaneously in C57BL/6 mice. Data from three experimental replicates. **D,** $CD8^+$ T cells in tumor (TIL) and TDLN from the mice in **A** analyzed by flow cytometry (**, $P < 0.01$). **E,** Activated $CD8^+CD44^+$ T cells in TDLN cell suspensions. **F,** $Gr1^+CD11b^+$ splenic myeloid-derived suppressor cells (MDSC). **G,** IFNγ and TNFα concentration (both in pg/mL) in the culture medium of TDLN exposed to irradiated EL4 cells (EL4i) and in lymphoma explants grown as organotypic cultures (LOC).

bexarotene, we first analyzed its effects on the transcriptomics. We treated HuT78, MJ, and EL4 cells with vehicle, bexarotene, cilengitide, or the combination of bexarotene and cilengitide for 24 hours and analyzed the expression of *BCL2, BCL2L1, CCR4*, and *CCR7* by qRT-PCR. We found that in most instances, the combination impacted the expression of these genes more profoundly that each drug alone (**Fig. 5A**). Accordingly, the combination of cilengitide and bexarotene decreased the proliferation of HuT78 cells in 3D cultures (**Fig. 5B** and **C**) and in all the cell lines in standard culture conditions (Supplementary Fig. S1H). Also, we found increased caspases 7 and 3 levels in HuT78, MJ, and EL4 cells in regular culture conditions to a greater extent than each drug alone (**Fig. 5D**). Regarding spontaneous

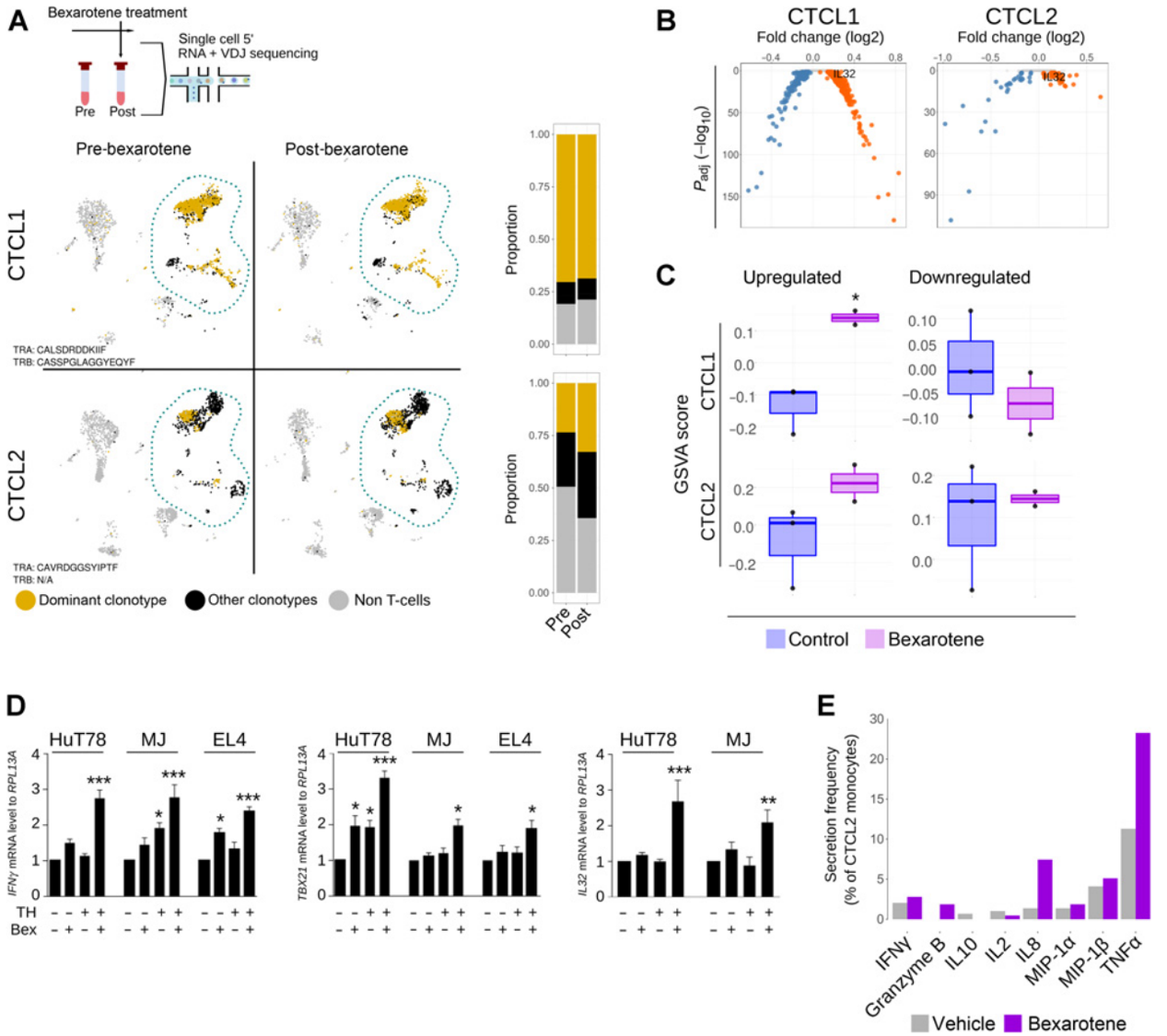

Figure 3.

Bexarotene upregulates immune signaling genes in CTCL. **A,** Isolation process and sequencing of PBMCs from patients with CTCL before and after bexarotene treatment. UMAP plot depicting the T-cell population and identification of the malignant clonotypes in dark yellow (dominant) and black (others) in the four samples analyzed. On the right, proportion of malignant and nonmalignant T cells in these samples. **B,** Volcano plots of differential gene expression analyses (post- vs. pre-bexarotene) for the malignant clones in the patients with CTCL1 and CTCL2. **C,** Boxplots of GSVA scores from HuT78 cells treated with bexarotene versus up- and downregulated genes form the malignant T cells from CTCL1 and CTCL2 patient samples [*, $P < 0.05$ (Wilcoxon test)]. **D,** Gene expression level (RT-qPCR) of IFNG, TBX21, and IL32 after 6 hours of treatment with vehicle or bexarotene (1.5 μmol/L) in the presence or absence of physiologic levels of TH. Data from four independent samples. **E,** Single-cell secretome of a panel of cytokines in CTCL2 monocytes comparing pre- and post-bexarotene treatment.

motility in 3D cultures, the combination behaved similarly to bexarotene alone in distance covered, speed of displacement, and extent of spatial exploration (**Fig. 5E**). However, the directionality (i.e., spatial persistence along the primary axes of migration) was impaired at higher degree with the combination ($P = 0.04$, **Fig. 5F**), indicating that the inhibition of the integrin αVβ3 impairs an additional aspect of the cell motility. These results indicate that the combination of bexarotene and cilengitide decreases cell proliferation and migration while increasing the apoptosis of CTCL cells. Regarding immune transcripts, the combination increased to a higher level (HuT78) or similar level than bexarotene alone (MJ) the expression of *IL32* (**Fig. 5G**). This provides another rationale for the combination, because IL32, that we have demonstrated is induced by on-target activity of bexarotene in patients and cell lines, is also a soluble ligand of integrin αVβ3 (25).

To elucidate the effect of the combination on the lymphoma immunity, we used the syngeneic EL4 subcutaneous TCL mice model. When the implanted tumors reached 75 mm^3, mice were randomized in four treatment groups to receive vehicle, bexarotene, cilengitide, and their combination according to the scheme shown in **Fig. 6A**.

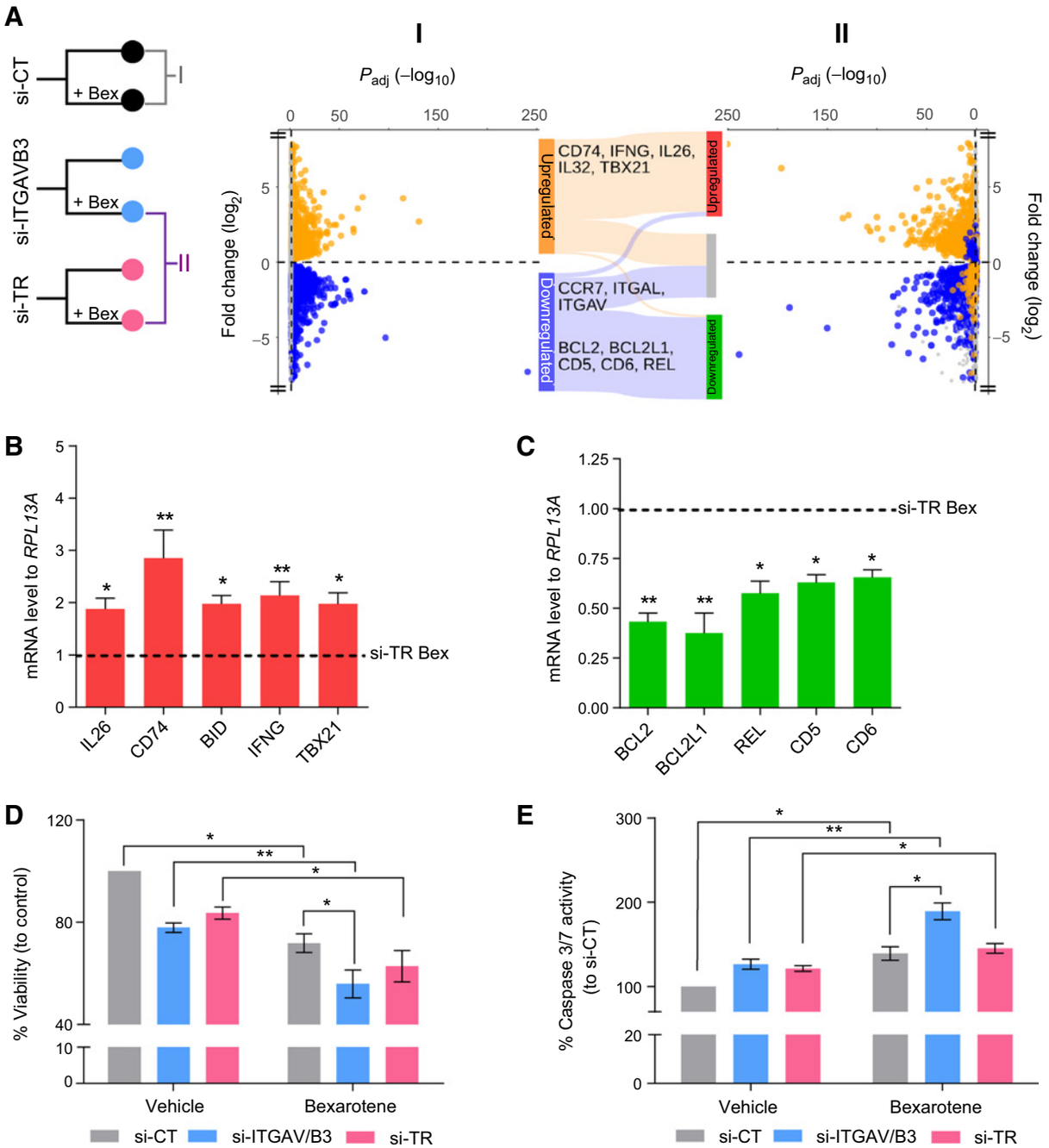

Figure 4.

Individual contributions of distinct TH receptors in decreasing the effect of bexarotene. **A,** Sankey plot of differentially expressed genes shared between two analyses, depicted in the volcano plots on the left (control vs. bexarotene; **I**) and si-RNA against integrins alpha-V and beta-3 versus si-RNA against TRA and TRB on the right (siINTAVB3 vs. siTRA/B; **II**). **B** and **C,** Gene expression level (RT-qPCR) of selected upregulated (**B**) and downregulated (**C**) genes in si-ITGAV/B3 + bexarotene versus si-TRA + bexarotene (dotted line, si-TR Bex) in HuT78 cells. **D** and **E,** Cell proliferation and apoptosis induction (caspase 7/3 activity) after bexarotene or vehicle treatment in HuT78 cells transfected with siRNA against ITGAV/B3 (si-ITGAV/B3), TRA (si-TR), or noncoding sequence as control (si-CT; *, $P < 0.05$; **, $P < 0.01$; bexarotene = 1.5 μmol/L).

Mice receiving bexarotene were treated with levothyroxine to maintain an euthyroid state (**Fig. 6B**). We found that the addition of cilengitide did not alter the levothyroxine-dependent restoration of the lymphoma immunity upon bexarotene treatment (**Fig. 6C**; Supplementary Figs. S2A and S2B). In fact, the infiltration of tumors and TDLN with $CD8^+$ and activated T cells ($CD8^+CD44^+$) and the number of splenic MDSCs ($Gr1^+CD11b^+$) in mice receiving the combination was like those receiving bexarotene supplemented with

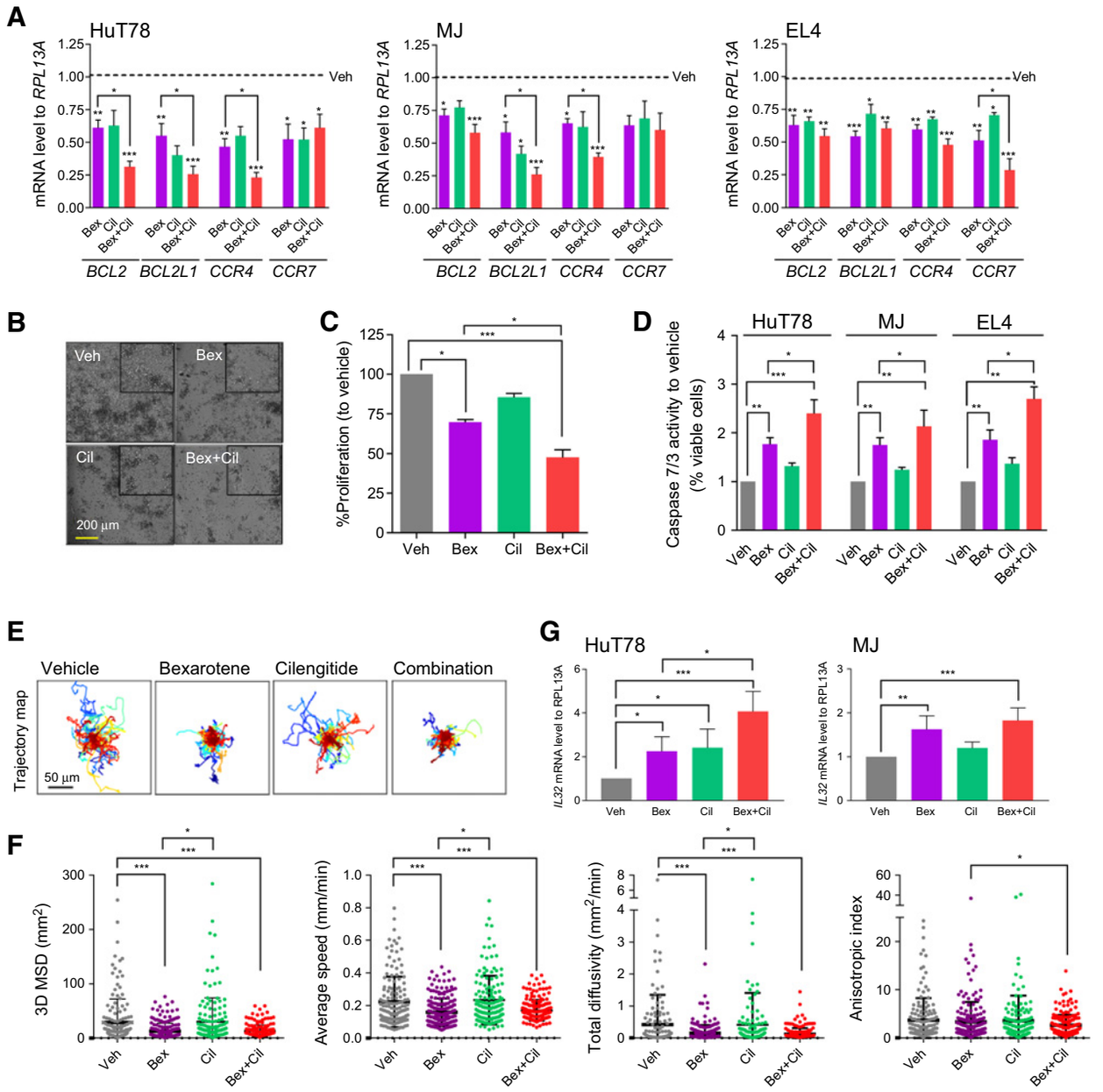

Figure 5.

Integrin αVβ3 inhibition increases the *in vitro* activity of bexarotene in CTCL. **A,** BCL2, BCL2L1, CCR4, and CRR7 gene expression in HUT78, MJ, and EL4 cells upon 6 hours of treatment with vehicle, 1.5 µmol/L bexarotene (Bex), 1.5 µmol/L cilengitide (Cil), or the combination of both drugs (Bex+Cil) in the presence of physiologic concentration of TH. Data from four independent experiments. **B,** Proliferation of HuT78 cells in a 3D culture system treated for 96 hours with the treatments as in **A**. Representative photographs of four independent experiments are shown. **C,** Quantification of cell proliferation of the experiments shown in **B**. **D,** Apoptosis induction (caspase 7/3 activity) at 72 hours in HuT78, MJ, and EL4 cells. **E,** Origin-centered trajectories of spontaneous motility of HuT78 cells embedded in collagen-1 gels as a function of treatment conditions. **F,** Quantification of cell motility parameters from the experiments shown in **E**. **G,** IL32 expression in HuT78 and MJ cells upon the different treatment conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

levothyroxine (**Fig. 6C**; Supplementary Figs. S2A and S2B). Moreover, the Th1-based cytotoxic activity measured by the production of IFNγ in lymphoma explants, grown as organotypic cultures, significantly increased in mice receiving the combination compared with those receiving bexarotene supplemented with levothyroxine (**Fig. 6D**, $P = 0.006$). These effects were translated in significantly improved lymphoma control in mice receiving the combination versus each drug alone (**Fig. 6E**, $P = 0.004$ and 0.006, vs. bexarotene or cilengitide, respectively). Taken together, our data indicate that inhibition of the integrin αVβ3 is an effective strategy to improve bexarotene-based treatments in CTCL by increasing its antiproliferative effect while maintaining lymphoma immunity.

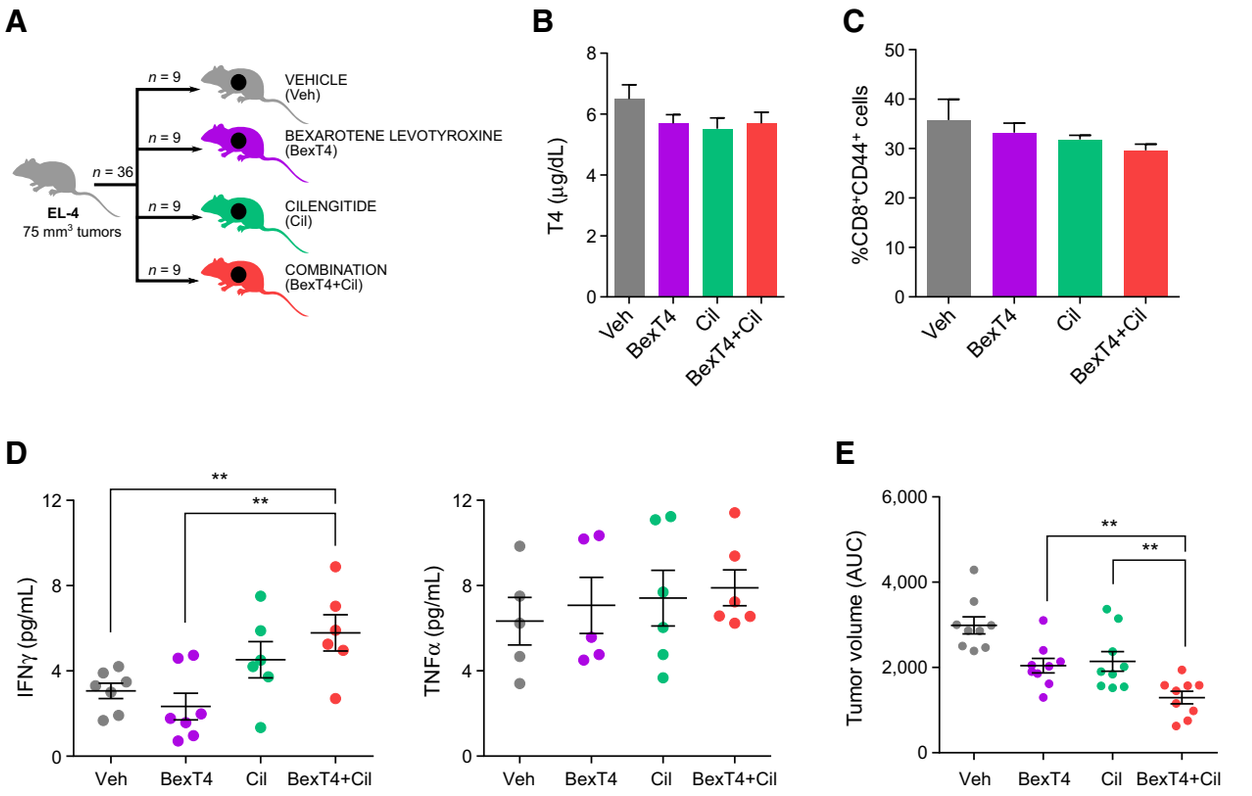

Figure 6.
Pharmacologic inhibition of integrin αVβ3 improves bexarotene effect. **A,** Graphical representation of the subcutaneous TCL model and treatment schedule, including vehicle (Veh), bexarotene with levothyroxine replacement (BexT4), cilengitide alone (Cil), or bexarotene with levothyroxine and cilengitide (BexT4+Cil). **B,** Plasma levels of levothyroxine (by radioimmunoassay) on the four treatment groups. **C,** Activated $CD8^+CD44^+$ T cells in TDLN cell suspensions from the four treatment groups. **D,** IFNγ and TNFα concentration (both in pg/mL) after 24 hours in cell suspensions from lymphoma explants grown as organotypic cultures. **E,** Tumor volume (measured by AUC from day 1 to 12 of treatment) of the EL4 model according to the four treatment groups.

Discussion

Our work addresses a frequently neglected topic in the treatment of cancer patients represented by the therapeutic consequences of supportive medications that patients with cancer receive to mitigate antineoplastic drug's side effects. The RXR agonist bexarotene is commonly used for the treatment of CTCL when systemic therapy is recommended such as in the refractory and advance-stage diseases (1, 26, 27). The antilymphoma activity of bexarotene derives from the activation of RXR homo- and heterodimers that regulate the transcription of retinoid-regulated genes ultimately inducing cell-cycle arrest and apoptosis of cancer cells (4). However, in the clinic as well as in animal models, its administration is associated with the development of hypothyroidism that requires levothyroxine replacement therapy concomitant to initiating bexarotene treatment (7, 28). Here, we described the effects of levothyroxine replacement in the antineoplastic activity of bexarotene as well as in the lymphoma immunity; and provided a rationale to improve its antilymphoma effect by combining with an inhibitor of integrin αVβ3.

In a CTCL cell line, we characterized the spectrum of the rexinoid-regulated genes to expand their involvement in motility and immune response from their previously described roles in cell proliferation and apoptosis. In accordance, we found that bexarotene decreases CTCL cell proliferation, viability, and motility, supporting their antineoplastic activity. Using cell lines and a mice model of TCL, we found that although levothyroxine may decrease the antiproliferative and apoptotic effects of bexarotene it also maintains a favorable immune microenvironment. TH can activate the ubiquitously expressed nuclear receptor TRA and the restricted membrane receptor, the integrin αVβ3, that we found overexpressed in T-cell lymphomas including CTCL (8). We described here that selective inhibition of the integrin αVβ3 with genomic and pharmacologic approaches blunts the proliferative and anti-apoptotic effects of levothyroxine while preserving the lymphoma immune effects. This is likely due to the undisturbed activation of the nuclear receptor TRA in lymphoma cells as well as in healthy cells by levothyroxine supplementation.

Bexarotene and cilengitide affect CTCL motility in different yet complimentary ways as we demonstrated by using 3D culture assays. This is likely to the decrease in *CCR7* expression induced by bexarotene with the blocking of the integrin αVβ3 by cilengitide. This is in agreement with recent data demonstrating that contributions of integrin LFA-1 and CCR7 to T-cell extravasation are complementary rather than positioned in a linear pathway (29). Although CCR7 regulates cortical actin flows, integrins mediate substrate friction during intranodal migration of T cells (29). Bexarotene modifies the expression of other chemokine receptors, including CCR4 that likely participates in the skin homing of malignant lymphocytes (30, 31). We

confirmed the previously reported bexarotene-dependent decrease in CCR4 expression (5) and found that is further downregulated in the presence of cilengitide, thus providing an additional therapeutic advantage to this combination. In fact, a recent trial in CTCL patients with the CCR4 inhibitor mogamulizumab has shown objective responses, demonstrating that CCR4 is a *bona fide* therapeutic target in this disease (32).

Our results demonstrated that the combination of bexarotene and cilengitide promoted a favorable lymphoma immune microenvironment by increasing IL32, TBX21, and IFNG expression. In early disease stages, a significant proportion of CTCL immune infiltration is represented by $CD8^+$ and Th1 cells (14, 33), which likely suppress the expansion of malignant cells (34, 35). Disease progression is, however, associated with increased secretion of Th2 cytokines from malignant and infiltrating T cells (16, 36, 37). In fact, the downregulation of the critical Th1-polarizing factor TBX21 (38) has been associated with CTCL pathogenesis and progression (36). TBX21 regulates IFNγ expression, which have been shown to be absent from the clonal malignant T cells in CTCL samples (39). It is also part of a positive feedback loop promoting Th1 development (40). Restoring the Th1 microenvironment contributes to disease control (16), as demonstrated by clinical responses in patients with CTCL receiving intralesional viral-mediated administration of the Th1 cytokine IFNγ (41). Because bexarotene-induced IL32 may activate the integrin αVβ3 (25), it could potentially decrease bexarotene's antineoplastic effect. This could further support the rationale for combining bexarotene and cilengitide in patients with CTCL.

In addition to the lymphoma microenvironment, the systemic impairment of cellular immunity contributes to CTCL morbidity and mortality (42). Lymphopenia and loss of T-cell repertoire have been observed in patients with limited and advanced stage disease (43). Both quantitative and qualitative defects in dendritic cell-, natural killer cell-, and T-cell-mediated immunity are reported in patients with CTCL (37, 44–46). This immune dysfunction provides an additional justification for levothyroxine supplementation during bexarotene treatment and strengthens our rationale to selectively inhibit the integrin αVβ3, which expression is mostly restricted to the malignant T cells. Our work also provides a potential therapeutic strategy to counteract immune deleterious effects of hypothyroidism induced by other antineoplastic drugs such as tyrosine kinase inhibitors (47) and immune checkpoint inhibitors (48) in patients with cancer.

Authors' Disclosures

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Authors' Contributions

F. Cayrol: Formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **M.V. Revuelta:** Data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **M. Debernardi:** Investigation. **A. Paulazo:** Investigation. **J.M. Phillip:** Investigation, visualization. **N. Zamponi:** Investigation, visualization. **H.A. Sterle:** Investigation. **M.C. Díaz Flaqué:** Investigation. **C.M. Magro:** Investigation. **R. Marullo:** Investigation. **E. Mulvey:** Resources, investigation. **J. Ruan:** Resources. **G.A. Cremaschi:** Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing. **L. Cerchietti:** Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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