Bortezomib enhances cytotoxicity of ex vivo-expanded gamma delta T cells against acute myeloid leukemia and T-cell acute lymphoblastic leukemia

Christopher Doering, Emory University
HT Trent Spencer, Emory University
Sunil Raikar, Emory University
Jamie Story, Emory University
JT Zoine, Emory University
RE Burnham, Emory University
JAG Hamilton, Emory University

Journal Title: CYTOTHERAPY
Volume: Volume 23, Number 1
Publisher: ELSEVIER SCI LTD | 2021-01-01, Pages 12-24
Type of Work: Article
Publisher DOI: 10.1016/j.jcyt.2020.09.010
Permanent URL: https://pid.emory.edu/ark:/25593/vxkqc

Final published version: http://dx.doi.org/10.1016/j.jcyt.2020.09.010
Accessed November 4, 2022 4:04 PM EDT
Bortezomib enhances cytotoxicity of ex vivo expanded gamma delta (γδ) T cells against AML and T-ALL

Jamie Y. Story1,4, Jaquelyn T. Zoine2,4, Becca E. Ryan1,4, Jamie A. Hamilton2,4, H. Trent Spencer1,2,3,4, Christopher B. Doering1,3,4, Sunil S. Raikar3,4

1Molecular and Systems Pharmacology Graduate Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University School of Medicine, Atlanta, GA, USA
2Cancer Biology Graduate Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University School of Medicine, Atlanta, GA, USA
3Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA
4Aflac Cancer and Blood Disorders Center, Children’s Healthcare of Atlanta, Atlanta, GA, USA

Abstract

Engagement between the natural killer group 2, member D (NKG2D) receptor and its ligands is one of the main mechanisms used by immune cells to target stressed cells for cell death. NKG2D ligands are known markers of cellular stress and are often upregulated on tumor cells. Certain drugs can further increase NKG2D ligand levels, thereby making tumor cells more susceptible to immune cell detection and destruction. However, the effectiveness of this approach appears to be limited with drug treatment alone, possibly due to immune dysregulation in the setting of malignancies. We hypothesized that a more effective approach would be the combination of NKG2D ligand inducing drugs, such as the proteasome inhibitor bortezomib, with ex vivo expanded peripheral blood γδ T cells (Vγ9Vδ2 T cells). Acute myeloid leukemia (AML) is a high-risk hematologic malignancy, and treatment has shown limited benefit from the addition of bortezomib to standard chemotherapy regimens. Two AML cell lines, Nomo-1 and Kasumi-1, were treated with increasing concentrations of bortezomib and changes in NKG2D ligand expression were measured. Bortezomib treatment significantly increased expression of the
NKG2D ligand UL16 binding protein (ULBP) 2/5/6 in both cell lines. Vγ9Vδ2 T cells were expanded and isolated from peripheral blood of healthy donors to generate a final cellular product with a mean of 96% CD3⁺/γδ TCR⁺ cells. Combination treatment of the AML cell lines with γδ T cells and bortezomib resulted in significantly greater cytotoxicity than γδ T cells alone, even at lower effector to target ratios. Based on the positive results against AML and the generalizable mechanism of this combination approach, it was also tested against T-cell acute lymphoblastic leukemia (T-ALL), another high-risk leukemia. Similarly, bortezomib increased ULBP2/5/6 expression in T-ALL cell lines, Jurkat and MOLT-4, and improved the cytotoxicity of γδ T cells against each line. Collectively, these results show that bortezomib enhances γδ T cell-mediated killing of both AML and T-ALL cells in part through increased NKG2D ligand-receptor interaction. Furthermore, proof-of-concept for the combination of ex vivo expanded γδ T cells with stress ligand inducing drugs as a therapeutic platform for high-risk leukemias is demonstrated.

**Keywords**
gamma delta T cells; bortezomib; NKG2D ligands; AML; T-ALL

**INTRODUCTION**

Gamma delta (γδ) T cells are a unique class of immune cells, which play important roles in both innate and adaptive cellular immunity [1, 2]. Unlike the more abundant αβ T cells, they do not require antigen presentation and can identify their targets in a major histocompatibility complex (MHC) independent manner [1–6]. This distinctive property suggests they could be developed into allogeneic ‘off-the-shelf’ therapeutics without expectation of graft versus host disease (GvHD) and utilization of their inherent anti-tumor properties. A large tumor molecular profiling study showing that infiltrating γδ T cells were the strongest leukocyte predictor of favorable outcome [3–7]. In humans, γδ T cells constitute about 1–5% of peripheral blood T cells, and are primarily composed of the Vδ2Vγ9 T-cell subset [1–6]. We previously developed a good manufacturing practice (cGMP) compliant method to expand Vδ2Vγ9 T cells from peripheral blood mononuclear cells (PBMCs) by using serum-free media in combination with cytokines and bisphosphonates [8, 9]. This method is highly safe as it relies on recombinant protein reagents and not on the addition of bovine or human serum, thereby significantly reducing the risk of pathogen contamination.

γδ T cells exhibit anti-tumor properties through several different mechanisms [1–6]. The γδ T-cell receptor (TCR) is specifically reactive to butyrophilin molecules whose surface expression are driven by intracellular phosphoantigens, such as isopentenyl pyrophosphate. These phosphoantigens are upregulated in certain stressed, infected and tumor cells. γδ T cells also have upregulated CD16 expression, and thus play an important role in antibody-dependent cellular cytotoxicity (ADCC). In addition, γδ T cells also target cancer cells through expression of the natural killer group 2, member D (NKG2D) receptor that can recognize stress-induced ligands on tumor cells [1–6]. These NKG2D ligands include the UL16 binding proteins (ULBP) 1–6 and the MHC class I chain-related protein A and B.
Past clinical trials have used γδ T cells to target different malignancies either through autologous γδ T-cell infusions post expansion with zoledronic acid, or by activating circulating γδ T cells by directing administering zoledronic acid and interleukin-2 (IL-2) to patients [10–18]. These studies were primarily designed to activate γδ T cells through stimulation of their TCR with bisphosphonates. While the therapy was safely tolerated, mixed results were seen in terms of efficacy. In order to maximize the inherent anti-tumor properties of γδ T cells, non-γδ TCR-mediated cytotoxic pathways must also be explored. One potential method to enhance γδ T cell-mediated lysis is through upregulation of NKG2D ligands on tumor cells, which can be achieved through different mechanisms such as proteasome inhibition, epigenetic modification, and use of traditional chemotherapy [19–27]. Thus, we hypothesize that using a combination of a stress ligand-inducing drug with an ‘off-the-shelf’ allogeneic γδ T-cell product from a healthy donor would be an effective strategy to target different cancers.

In this study, the combination of the proteasome inhibitor bortezomib and γδ T cells is tested as a novel therapy for acute myeloid leukemia (AML), a hematologic malignancy in which chimeric antigen receptor (CAR)-based adoptive T cell immunotherapy strategies has been challenging to develop due to on-target off-tumor effects [28]. Relapsed AML continues to have extremely poor outcomes, with only allogeneic hematopoietic stem cell transplantation (HSCT) offering a realistic chance at cure [29]. Newer therapies are being developed to induce remission in these patients, which is a prerequisite for allogeneic HSCT [30]. A combinatory strategy utilizing a stress ligand-inducing drug with an ‘off-the-shelf’ γδ T-cell product could potentially induce remission in these patients, thus providing a bridge to allogeneic HSCT. As proof-of-concept, bortezomib was chosen to act as an immunomodulatory agent to sensitize AML cells to γδ T cell-mediated lysis, given its known efficacy in AML. Bortezomib inhibits the 26S subunit of the proteasome, causing build-up of ubiquitinated proteins in cancer cells, which results in cell death [31, 32]. While the exact mechanism of cell death is unknown given the multiple pathways affected, several studies have shown that bortezomib can upregulate the expression of NKG2D ligands in tumor cells due to the resulting cellular stress from proteasome inhibition [19–22]. Here, the use of bortezomib as an agent to sensitize AML to γδ T cell-mediated cytotoxicity was further explored. Subsequently, this combination therapy was tested in a different cancer, T-cell acute lymphoblastic leukemia (T-ALL), another hematologic malignancy in which there are significant barriers to CAR-based therapies [33]. These findings show that utilizing bortezomib to increase NKG2D ligand surface expression enhances γδ T cell cytotoxicity against AML and T-ALL, allowing for the development of an adoptive cellular immunotherapy platform to treat these aggressive blood cancers.

**MATERIALS AND METHODS**

**Expansion of γδ T cells in serum free media**

Peripheral blood mononuclear cells (PBMCs, 50–100 million per donor) from six different healthy donors were obtained through AllCells (Alameda, CA) and cultured in OpTmizer serum free media (Thermo Fisher Scientific, Waltham, MA) supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin. Eight total expansions from PBMCs from
AllCells were performed, with two separate expansions from two donors occurring from two separate vials obtained from the same blood donation. Alternatively, peripheral blood of two different healthy donors (30–40 mL) was obtained through the Emory Children’s Clinical and Translational Discovery Core (IRB00101797) (Atlanta, GA) and PBMCs were isolated using Ficoll-Paque Plus density gradient media (Sigma Aldrich, St. Louis, MO). Media changes were made every 3 days and cultures were stimulated with 500 IU/mL of IL-2 (Peprotech, Rocky Hill, NJ) and 5 μM zoledronic acid (Sigma-Aldrich) on days 0 and 3, and with 1000 IU/mL of IL-2 on days 6, 9, and 12. On day 6 of expansion, αβ T cells were removed from the culture with positive selection using anti-αβ TCR antibodies and magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Total cell numbers were obtained every 3 days over the course of expansion via Cellometer (Nexcelom, Lawrence, MA). γδ T cell percentage and receptors/ligands associated with cytotoxicity were monitored via flow cytometry on days 0, 6, 9, and 12. Downstream experiments were performed with γδ T cells at day 10 to 12 of their expansion.

**Monitoring cell culture composition during γδ T-cell expansions**

Live cell counts were determined using Trypan blue dye (Thermo Fisher, Waltham, MA) after thoroughly pipetting cell clumps to achieve a uniform cell culture, and 3–5 × 10⁵ cells of each sample were washed with phosphate-buffered saline (PBS) and spun at 300 × g in flow tubes. Supernatant was decanted and cells were incubated at room temperature with eBioscience eFluor780 Viability Dye (Invitrogen, San Diego, CA) for 30 minutes, shaking. The cells were washed with 2 mL PBS and spun at 300 × g. Supernatant was decanted and replaced with the appropriate antibody cocktail in PBS. The antibodies used for flow cytometry are listed in Table S1. Cells were incubated with antibody cocktails for at least 15 minutes, then the cells were washed with 2 mL PBS and spun at 300 × g. Supernatant was decanted and cells were resuspended in 100 μL PBS prior to data collection using an Aurora flow cytometer (Cytek, Fremont, CA). Data was analyzed using FlowJo (BD, Franklin Lakes, NJ).

**Cell lines**

The Nomo-1, Kasumi-1 and MOLT-4 cell lines were kindly provided to us by the laboratory of Dr. Douglas Graham (Emory University, Atlanta, GA). The Jurkat cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The primary culture media for the Nomo-1, Jurkat, and MOLT-4 cell lines was RPMI (Corning, Manassas, VA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), while the culture media for Kasumi-1 cells was RPMI with 20% FBS and 1% P/S. Green fluorescent protein (GFP)-expressing Nomo-1 and Jurkat cells were generated by transducing 500,000 cells at a multiplicity of infection (MOI) of 10 with a lentivirus (p-HIV-EGFP, Addgene, Watertown, MA). Cells were incubated with virus in complete media supplemented with 8 μg/mL polybrene (EMD Millipore, Billerica, MA) for 24 hours, then media was replaced. Five days later, transduced cells were assessed for GFP expression via flow cytometry.

**Dose-response curves for bortezomib**

Bortezomib was purchased from Sigma-Aldrich (St. Louis, MO), reconstituted in 100% DMSO at a stock concentration of 1 mM, and further diluted to 100 μM with DMSO before
being stored at −20°C. 100 μM aliquots were thawed just before experiments and diluted to 10 μM with DMSO. The 10 μM solution was then diluted in the culture media for each cell type to reach a working concentration of 1 μM (10% DMSO). Dose-response curves for bortezomib (treated cells were generated by plating 3 × 10^5 cells in 1 mL in 12 well plates for 48 hours and treating with increasing concentrations of bortezomib ranging from 0.1 nM to 50 nM. Cell growth percentages for each bortezomib dose were determined by normalizing live cell counts at 48 hours post-treatment to the vehicle control treated group. Cell viability was determined by gating on live cells (7-AAD−/Annexin V−) via flow cytometry and normalizing live cell percentage at 48 hours to the untreated group. Dose-response curves were plotted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and the half-maximal inhibitory concentration (IC_{50}) and lethal concentration (LC_{50}) were determined by interpolating the drug concentration at x = 50% cell growth or viability on these curves.

**Stress antigen surface expression**

Baseline surface expression of NKG2D ligands for three healthy donor PBMCs, three healthy donor bone marrow samples and each cancer cell line was determined via flow cytometry by gating on live cells (7-AAD−/Annexin V−). Relative median fluorescence intensity (MFI) for each ligand was calculated by dividing MFI of the cell line by MFI of healthy donor PBMCs. Time course experiments were performed to determine the optimal dose and treatment time of bortezomib for increasing stress antigen expression on leukemia cell lines. 3 × 10^5 cells were seeded in 1 mL in 12 well plates and treated with 2.5, 5, or 10 nM bortezomib or vehicle control (0.1% DMSO) over 48 hours, with NKG2D ligand expression being measured at several time points. Median fluorescence intensity (MFI) was determined based on live cells (7-AAD−/Annexin V−) using either an Aurora flow cytometer (Cytek, Fremont, CA) or BD LSRII (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo (BD, Franklin Lakes, NJ). Relative MFI was calculated dividing MFI of bortezomib-treated cells by MFI of vehicle control group. Similar effects for NKG2D ligand upregulation with bortezomib were tested in healthy donor PBMC and bone marrow samples.

**NKG2D ligand gene expression data in AML and T-ALL patient samples**

The R2 Genomics Analysis and Visualization Platform (https://r2.amc.nl/) is a publicly accessible genomics analysis and visualization platform allowing researchers to query cellular, clinical and genomic data. In this study, the Megasampler R2 module was utilized to query multiple datasets for the expression of ULBP1, ULBP2, ULBP3, ULBP4, and MICA/B. Exclusively human databases queried, and the u133p2 chip with MAS5.0 normalization were used throughout the study. Databases queried were as follows include: PBMC (Tangye, 14 samples), AML (Delwel, 460 samples and Verhaak, 525 samples) and T-ALL (Peiters, 92 samples, and Meijerink, 124 samples).

**Effect of bortezomib on γδ T cells**

γδ T cells were plated in 500 μL at 1.5 × 10^6 cells/mL in a 12 well plate and treated with vehicle control (0.1% DMSO), 1 nM, 2.5 nM or 5 nM bortezomib. After 24 hours of treatment, cell viability and surface expression of NKG2D receptor and CD107a were
assessed via flow cytometry. Cell growth was also determined by total live cell counts via Cellometer (Nexcelom, Lawrence, MA). To determine whether bortezomib treatment has any delayed effects on γδ T cells, bortezomib was removed from the cultures after 24 hours of treatment and γδ T cells were cultured for another 24 hours. γδ T cell growth, cell viability, and cytotoxicity capability were assessed after this time point.

Cytotoxicity assays

Cytotoxic capabilities of γδ T cells against leukemia cells or non-malignant hematopoietic cells was assessed in a flow cytometry-based assay. Target cells included acute myeloid leukemia (AML) cell lines Nomo-1 and Kausmi-1, T-cell acute lymphoblastic leukemia (T-ALL) cell lines Jurkat clone E6-1 and MOLT-4 (ATCC, Manassas, VA), and healthy donor PBMCs and bone marrow cells from AllCells. Target cells were stained with Violet Proliferation Dye 450 (BD Biosciences, San Jose, CA) and treated with vehicle control, 5 nM (Kasumi-1, Jurkat, and MOLT-4 cells) or 10 nM bortezomib (Nomo-1 cells) for 24 hours prior to the cytotoxicity assay. Fifty-thousand target cells were then incubated with day 12 ex vivo expanded γδ T cells at various effector to target (E:T) ratios: 0:1, 1:4, 1:2, 1:1, and 2.5:1 for 4 hours at 37°C. Target cell death was assessed by flow cytometry via 7-AAD (7-aminoactinomycin D) and Annexin V (Biolegend, San Diego, CA) staining of necrotic and apoptotic cells. Samples were washed with 2 mL PBS, spun down at 300 × g, then incubated with Annexin V in Annexin V binding buffer for 15 minutes at room temperature. 7-AAD was added immediately prior to data acquisition on the cytometer. For the time course cytotoxicity assays, 200,000 GFP+ Nomo-1 cells or GFP+ Jurkat cells were incubated with day 11 ex vivo expanded γδ T cells at 0:1 and 1:2 (Nomo-1) or 1:4 and 1:20 (Jurkat) E:T ratios for 4, 24, and 48 hours. Target cell disappearance was assessed by flow cytometry via GFP and 7-AAD detection. 7-AAD+ cells were gated out to determine decrease in the percentage of live GFP+ cells over time.

NKG2D receptor blocking cytotoxicity assays and bortezomib washout experiments

The importance of the NKG2D receptor and its interaction with NKG2D ligands in bortezomib treated AML and T-ALL cells for γδ T cell-mediated cell death was assessed. To determine the optimal concentration for blocking the NKG2D receptor on γδ T cells, γδ T cells were seeded in a 96 well plate in 100 μL at a density of 2 × 10^6 cells/mL and incubated with 0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, or 50 μg/mL of an unconjugated NKG2D receptor antibody (Biolegend, San Diego, CA) at room temperature. After 30 minutes, the cells were washed with PBS and spun at 300 × g, then incubated with FITC conjugated NKG2D receptor antibody (Biolegend, San Diego, CA) for 15 minutes at room temperature. MFI was assessed for each concentration treatment via flow cytometry to determine the concentration at which yielded the lowest binding of the FITC NKG2D receptor antibody. For cytotoxicity assays, 2 × 10^5 γδ T cells were incubated with 50 μg/mL of the unconjugated NKG2D receptor antibody for 30 minutes, then spun down at 300 × g and resuspended in fresh media prior to incubation with VPD450 stained target cells at a 1:2 E:T ratio. Bortezomib washout experiments were performed to test the efficacy of γδ T-cell killing once the NKG2D ligand expression had returned to baseline. Briefly, all cell lines were treated with bortezomib for 24 hours, then a media change was performed to remove any residual drug and NKG2D ligand expression was followed over the next several days.
Cytotoxicity assays at an E:T ratio of 1:2 were set up immediately after bortezomib treatment and at the time point post-washout when NKG2D ligand expression returned back to baseline.

**Statistics**

All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA). Results were presented as mean ± standard deviation of the mean and were considered significant at p-value < 0.05. Unpaired 2-tailed Student t test and one-way or two-way ANOVA were used to determine statistical significance. The specific statistical test used for analysis of each experiment is detailed in the figure legends.

**RESULTS**

**Robust expansion of healthy donor-derived \( \gamma \delta \) T cells in serum free media**

Our laboratory has previously developed a GMP compliant method to expand \( \gamma \delta \) T cells from peripheral blood using serum-free media in combination with bisphosphonates and cytokines, depicted in the schematic (Figure 1A) [8]. The percentage of \( \gamma \delta \) T cells was measured via flow cytometry on days 0, 6, 9, and 12 of expansion (Figure 1B). Gating strategy for flow cytometry experiments is shown in Figure S1. To enrich for \( \gamma \delta \) T cells only, an \( \alpha \beta \) T-cell depletion was performed on day 6 of our *ex vivo* expansion. This step resulted in a cellular product with an mean of 95.4 ± 3.5% CD3+/\( \gamma \delta \) TCR+ cells by day 9 and 95.5% ± 4% CD3+/\( \gamma \delta \) TCR+ cells by day 12 (Figure 1B and 1C). Downstream experiments were done with \( \gamma \delta \) T cells between Day 10 and 12 of the expansion depending on the experimental setup.

**Baseline surface expression of NKG2D ligands in AML cell lines and gene expression in primary AML patient samples compared to non-malignant hematopoietic cells**

ULBP1, ULBP3, and MICA/B were significantly higher in Nomo-1 cells compared to healthy donor PBMCs (Figure 2A, \( p < 0.01 \), one-way ANOVA with Dunnett’s multiple comparisons test). ULBP1, ULBP3, and MICA/B were also elevated in Kasumi-1 cells compared to PBMCs (Figure 2A, \( p < 0.0001 \), one-way ANOVA with Dunnett’s multiple comparisons test). ULBP2/5/6 was also elevated in both Nomo-1 and Kasumi-1 cells (Figure 2A, \( p = 0.01 \) and 0.03, respectively, one-way ANOVA with Dunnett’s multiple comparisons test). Bone marrow cells from healthy donors showed no significant difference in any NKG2D ligand surface expression compared to PBMCs (Figure 2A, \( p > 0.05 \), one-way ANOVA with Dunnett’s multiple comparisons test). Primary samples obtained at time of diagnosis from two separate databases showed elevated RNA expression of all NKG2D ligands in AML patients, compared to healthy PBMC controls (Figure 2B, \( p < 0.001 \), one-way ANOVA with Dunnett’s multiple comparisons test). To assess whether \( \gamma \delta \) T cells mediated cell death of non-malignant hematopoietic cells, a cytotoxicity assay was performed on healthy donor PBMCs and bone marrow cells at 1:2 and 1:1 effector to target (E:T) ratios. Cell death of healthy donor PBMCs was minimal (mean at 1:2 E:T ratio was 4.5 ± 1.6% and 4 ± 1.2% at 1:1 E:T ratio), as was cell death of healthy donor bone marrow cells (mean 2.8 ± 2.9% at 1:2 E:T ratio and 2.9 ± 3.5% at 1:1 E:T ratio). There was no
significant difference in cell death between PBMCs and bone marrow cells (Figure 2C, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test).

**Effects of bortezomib on NKG2D ligand expression in AML cell lines, PBMCs and bone marrow cells**

Bortezomib decreased cell growth and viability in a dose-dependent manner in Nomo-1 and Kasumi-1 cell lines (Figure S2). The effect of bortezomib on cell growth varied between the cell lines, with Kasumi-1 cells having the most sensitivity to growth inhibition (IC$_{50}$ = 4.2 nM), while Nomo-1 cells had the least sensitivity to growth inhibition (IC$_{50}$ = 9.6 nM). The effect of bortezomib on cell viability also varied, with Kasumi-1 cells being most sensitive to bortezomib-induced cell death (LC$_{50}$ = 4.3 nM) and Nomo-1 cells being the least sensitive (LC$_{50}$ > 50 nM). To determine the optimal treatment dose and time for increasing NKG2D ligand expression, the AML cell lines were treated at 2.5, 5, and 10 nM bortezomib for 48 hours and NKG2D ligand expression (ULBP1, ULBP2/5/6, ULBP3, ULBP4, MICA/B) was measured at several time points by flow cytometry using MFI (Figure 3A, 3B and S4). Gating strategy for these experiments is shown in Figure S3. Relative MFI for each drug concentration was determined by comparing to a vehicle treated control group. Peak expression for ULBP2/5/6 was seen between hours 18 and 24 for both AML cell lines (Figure 3A and 3B). Treating Kasumi-1 cells with 5 nM bortezomib for 24 hours significantly increased expression of ULBP2/5/6 by 1.9-fold, respectively (Figure 3C, p <0.0001, n = 3, one-way ANOVA with Dunnett’s multiple comparisons test). Significant increase was also seen in ULBP1 and ULBP3 expression at 18 and 24 hours in Kasumi-1 cells treated with 5 nM bortezomib (Figure S4). Twenty-four hour treatment with 10 nM bortezomib significantly increased expression of ULBP2/5/6 in Nomo-1 cells by 2.0-fold (Figure 3D, p = 0.0124, n = 3, one-way ANOVA with Dunnett’s multiple comparisons test). Significant elevation was also seen in MICA/B at 24 hours (Figure S3). Treatment of healthy donor PBMCs and bone marrow samples with 5 nM bortezomib for 24 hours did not show any significant increase in expression NKG2D ligands, demonstrating ligand expression remains low in healthy non-malignant hematopoietic cells even with bortezomib treatment (Figure 3E and 3F, n = 3, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test).

**Effects of bortezomib on γδ T cell growth and functionality**

To determine whether bortezomib has any negative effects on γδ T cells, γδ T cells at day 10 of expansion were treated for 24 hours at 1 nM, 2.5 nM, or 5 nM bortezomib and assessed for decreases in cell viability, growth, and surface expression of NKG2D receptor and CD107a, a marker of immune cell activation and function. The dose range was chosen since the expected concentration of bortezomib 24 hours after intravenous or subcutaneous administration (1.3 mg/m$^2$) in adults is between 1 and 5 nM [34–36]. No changes in NKG2D receptor or CD107a expression were observed at any bortezomib treatment (Figure 4A and 4B, n = 3, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test). Though there was no significant decrease in cell growth at any dose (Figure 4C, n = 3, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test), there was a significant decrease in cell viability from 98.9% to 74.8% at the 5 nM dose (Figure 4D, p = 0.004, n = 3, one-way ANOVA with Dunnett’s multiple comparisons test). To determine whether
bortezomib treatment had an delayed effects on γδ T cells, γδ T cells were treated with vehicle, 1 nM, 2.5 nM, or 5 nM bortezomib for 24 hours, then bortezomib was removed with a media change and γδ T cells were cultured for an additional 24 hours. γδ T-cell growth, viability, and cytotoxicity capabilities were then assessed. There was no significant decrease in γδ T-cell growth or viability 24 hours after bortezomib was removed (Figure 4E and 4F, n = 3, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test). No change in cytotoxicity was seen when the bortezomib treated γδ T cells were tested against the Nomo-1 AML cell line at a 1:2 effector to target (E:T) ratio (Figure S5, n = 3, p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test). These findings suggest that administration of γδ T cells 24 hours after the bortezomib injection would not adversely affect their in vivo cytotoxicity against cancer cells. Bortezomib is typically given twice a week over the course of 14 days, and the highest plasma concentration of residual bortezomib was reported in adults after their fourth dose (~5 nM). Therefore, the optimal dosing time for γδ T cells would be 24 hours after bortezomib dosing to eliminate any risk of negatively affecting the γδ T cells.

Bortezomib treatment enhances the cytotoxicity of healthy donor-derived γδ T cells against AML cells

Bortezomib treatment increased surface expression of NKG2D ligands in AML cell lines. To determine whether this increased AML cells sensitivity to γδ T cell-mediated cell death, the effect of treating leukemia cells with bortezomib prior to an in vitro cytotoxicity assay with γδ T cells was assessed. Results from these cytotoxicity assays showed a much lower percentage of live cells (7-AAD⁻/Annexin V⁻) with the bortezomib and γδ T cell combination than with either single treatment (Figure 5A). Gating strategy for these cytotoxicity experiments is shown in Figure S6. Compared to vehicle control treated controls, bortezomib treated AML cell lines had higher total cell death at all effector to target (E:T) ratios (Figure 5B and 5C). Cytotoxicity of Nomo-1 cells was significantly increased with bortezomib and γδ T cell combination treatment, compared to vehicle control and γδ T cell treatment- from 8.8% to 33.7%, 13.9% to 43.6%, 23.1% to 56.5%, and 45.8% to 71.3% at the 1:4, 1:2, 1:1, and 2.5:1 E:T ratios, respectively (Figure 5B, p < 0.002, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test). Total cell death of Kasumi-1 cells was significantly increased with bortezomib treatment compared to vehicle control from 16.8% to 52.1% at the 1:4 E:T ratio, 30.0 % to 64.0% at the 1:2 ratio, and 48.0% to 77.8% at the 1:1 ratio (Figure 5C, p < 0.02, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test). Variability in cytotoxicity between the individual donors is shown in Figure S7.

To determine if increased target cell killing in the cytotoxicity assays was dependent on NKG2D receptor-ligand interactions, we sought to block the NKG2D receptor on γδ T cells prior to incubation with target cells. We performed a titration assay and determined 50 μg/mL of an unconjugated NKG2D receptor antibody was optimal for NKG2D receptor blocking (Figure S8). A cytotoxicity assay was then performed in which the NKG2D receptor on γδ T cells was blocked prior to incubation with bortezomib treated AML cell lines. γδ T cell-specific cytotoxicity was normalized to target cell death mediated by unblocked γδ T cells and the relative γδ T cell-specific cytotoxicity with the NKG2D
receptor blocked was compared to with the NKG2D receptor unblocked. γδ T cell-specific cytotoxicity against bortezomib treated Nomo-1 cells was significantly decreased by 53.7% when the NKG2D receptor on γδ T cells was blocked (Figure 5D, p = 0.004, n = 3 separate donors, unpaired t-test). γδ T cell-specific cytotoxicity against bortezomib treated Kasumi-1 cells was also significantly decreased when the NKG2D receptor was blocked (Figure S9A, p = 0.04, n = 3 separate donors, unpaired t-test). This confirmed that the increased killing in bortezomib treated cells was in part through NKG2D receptor-ligand interaction. As an alternative method to show that increased NKG2D ligand expression was involved in γδ T-cell killing of AML cell lines, we pulsed the tumor cells with bortezomib and then performed cytotoxicity assays after drug washout and recovery once NKG2D ligand expression decreased. We treated the AML cells with bortezomib for 24 hours, then changed media to remove any residual drug. NKG2D ligand expression was then followed over the next few days and was shown to decrease back to baseline level in both cell lines by 48 hours (Figures S9B and S9C). A cytotoxicity assay was set up immediately after bortezomib treatment and 48 hours after bortezomib was washed out at a 1:2 E:T ratio. Both cell lines showed a significant decrease in γδ T cell-specific cytotoxicity after bortezomib was removed and cells were allowed to recover for 48 hours (Figures 5E and S9D, n = 3 separate donors, p = 0.003 for Nomo-1, p = 0.02 for Kasumi-1, unpaired t-test). To assess whether bortezomib treatment accelerated γδ T cell-mediated cell death of AML cells over a longer incubation period, vehicle and bortezomib treated GFP+ Nomo-1 cells were co-cultured with γδ T cells at a 1:2 E:T ratio and analyzed the culture at 4, 24, and 48 hours. The combination of bortezomib and γδ T cells eliminated 39.2% of target cells at 24 hours and 77.2% of the target cells after 48 hours, whereas γδ T cells alone only eliminated 15.1% at 24 hours and 45.3% at 48 hours (Figure 5F, p = 0.003 and 0.0002, respectively, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test).

Validation of bortezomib and γδ T cells as a combination therapy for T-ALL

To determine whether this combination therapy could be applied to other cancer types, the use of bortezomib and γδ T cells was also investigated in the T-ALL setting. Baseline surface expression of NKG2D ligands on two T-ALL cell lines, Jurkat and MOLT-4, showed elevated expression of ULBP2/5/6 (Figure 6A, n = 3, p < 0.0001 and p = 0.01, respectively, one-way ANOVA with Dunnett’s multiple comparisons test). This correlated with gene expression data that showed higher expression of ULBP2 in primary T-ALL patient samples compared to normal PBMCs (Figure 6B, n = 3, p < 0.0001, one-way ANOVA with Dunnett’s multiple comparisons test). Expression of ULBP1, ULBP3, ULBP4, and MICA/B was also elevated in T-ALL patients and T-ALL cell lines when compared to healthy donor PBMCs (Figure S10, p < 0.001, one-way ANOVA with Dunnett’s multiple comparisons test). Bortezomib treatment in T-ALL cell lines decreased cell growth and viability in a dose-dependent manner (Figure S11). Jurkat cells and MOLT-4 cells had intermediate sensitivity to cell growth inhibition, with IC$_{50}$ of 5.4 nM and 7.2 nM, respectively. The T-ALL cell lines had similar sensitivity to bortezomib-induced cell death, with Jurkat cells having an LC$_{50}$ of 7.1 nM and MOLT-4 cells having LC$_{50}$ of 6.6 nM. To determine the optimal treatment dose and time for increasing NKG2D ligand expression in T-ALL cell lines, Jurkat and MOLT-4 cells were treated at 2.5, 5, and 10 nM bortezomib for 48 hours and measured NKG2D ligand expression (ULBP1, ULBP2/5/6, ULBP3, ULBP4, MICA/B).
at several time points by flow cytometry (Figure 6 and S12). Expression was compared to a vehicle treated control group. Peak expression for ULBP2/5/6 was seen between hours 18 and 24 for both cell lines (Figure 6C and 6E). Treatment with 5 nM bortezomib for 24 hours significantly increased expression of ULBP2/5/6 in Jurkats and MOLT-4 cells by 2.0- and 2.1-fold, respectively (Figure 6D and 6F, p = 0.0007 and 0.0037, respectively, n = 3, one-way ANOVA with Dunnett’s multiple comparisons test).

In a four-hour cytotoxicity assay with \(\gamma\delta\) T cells, there was a much lower percentage of live cells (7-AAD\(^-\)/Annexin V\(^-\)) with the bortezomib and \(\gamma\delta\) T cell combination than with either single treatment (Figure 7A). The total cytotoxicity against bortezomib treated Jurkat cells significantly increased from 39.9% to 64.0% at the 1:4 E:T ratio and from to 58.1% to 76.4% 1:2 ratio compared to vehicle control treated cells (Figure 7B, p = 0.005 and 0.045, respectively, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test). The total cytotoxicity of MOLT-4 cells was also significantly increased with bortezomib treatment compared to vehicle treated cells- from 19.6% to 35.5% at the 1:4 E:T ratio, 36.7% to 48.4% at the 1:2 E:T ratio, and 57.4% to 68.3% at the 1:1 E:T ratio (Figure 7C, p = 0.001, 0.014, and 0.023, respectively, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test). Variability in cytotoxicity among the different donors is shown in Figure S13. The NKG2D receptor blocking cytotoxicity assay previously described was then performed with T-ALL cell lines. \(\gamma\delta\) T cell-specific cytotoxicity against bortezomib treated Jurkat cells was significantly decreased by 46.6% when the NKG2D receptor on \(\gamma\delta\) T cells was blocked (Figure 7D, p = 0.004, n = 3 separate donors, unpaired t-test). Similarly, \(\gamma\delta\) T cell-specific cytotoxicity against bortezomib treated MOLT-4 cells was significantly decreased by 64.8% with blockage of the NKG2D receptor (Figure 7E, p < 0.0001, n = 3 separate donors, unpaired t-test). We repeated the bortezomib washout experiments with both T-ALL cell lines, and saw a similar significant decrease in \(\gamma\delta\) T cell-specific cytotoxicity at 48 hours after washout once the NKG2D ligand expression had decreased back to baseline (Figure S14). To determine whether the combination treatment of bortezomib and \(\gamma\delta\) T cells accelerated Jurkat cell death over a longer incubation time, GFP\(^+\) Jurkat cells were co-cultured with \(\gamma\delta\) T cells at a 1:4 and 1:20 E:T ratio and measured the percentage of live target cells after 4 and 24 hours. These results showed a more rapid decrease in the percentage of live GFP\(^+\) Jurkat cells at 24 hours compared to vehicle treated Jurkat cells at both the 1:4 and 1:20 E:T ratios (Figure 7F, p = 0.011 and 0.003, respectively, n = 3 separate donors, two-way ANOVA with Bonferroni’s multiple comparisons test).

**DISCUSSION**

Outcomes for patients with acute leukemias have greatly improved in the past several decades with the intensification of chemotherapy, especially in children [37, 38]. However, resistance or recurrence in AML and T-ALL patients remain a critical barrier in the field [39, 40]. Cancer immunotherapies offer a promising strategy to improve clinical outcomes in patients not responsive to current first-line therapies. Although CAR T-cell therapies have proven promising in B-cell malignancies, primarily by targeting the B-cell antigen CD19, identifying cancer-specific target antigens in both AML and T-ALL that have limited on-target off-tumor effects has been difficult [28, 33]. One way to overcome this challenge is to
utilize an alternative adoptive cell therapy strategy with ex vivo expanded allogeneic \( \gamma \delta \) T cells (\( V\gamma 9\delta 2 \) T cells).

\( \gamma \delta \) T cells are a unique immune cell that offer the potential for an allogeneic cellular product. Unlike \( \alpha \beta \) T cells, \( \gamma \delta \) T cells possess anti-tumor effects without the risk of inducing graft vs. host disease (GvHD). Additionally, our laboratory has developed a serum-free ex vivo expansion of \( V\gamma 9\delta 2 \) T cells, which, along with the incorporation of an \( \alpha \beta \) T-cell depletion step, ensures a safe ‘off-the-shelf’ cellular product. Currently, there are two clinical trials utilizing an allogeneic \( \gamma \delta \) T-cell product in the post-transplantation setting and relapsed AML patient population, which highlights the potential of \( \gamma \delta \) T cells as a single agent therapy (NCT03533816, NCT03790072). It is anticipated that allogeneic \( \gamma \delta \) T-cell therapy would be well tolerated, have a minimal risk of causing GvHD, and result in minimal off-target side effects; thus potentially providing patients with relapsed AML and T-ALL an effective bridge to allogeneic HSCT. We have previously shown that our expansion protocol consistently yields a final \( \gamma \delta \) T-cell product of 85.5% \( V\gamma 9\delta 2+ \) and only 7% \( V\delta 1 \) \( \gamma \delta \) T cells [9]. Thus, the vast majority of our cellular product consisted of anti-tumor \( V\gamma 9\delta 2 \) T cells. While it is known that \( V\gamma 9\delta 2 \) T cells can potentially polarize towards pro-tumorigenic \( \gamma \delta \) T cell subsets such as \( \gamma \delta \) Treg and \( \gamma \delta \) T17, this transformation requires stimulation with specific cytokines such as TGF-\( \beta \) and IL-15 for FoxP3+ \( \gamma \delta \) Treg and IL-6, IL-1\( \beta \), and TGF-\( \beta \) for \( \gamma \delta \) T17 [41–43]. Since our expansion protocol only consists of stimulation with IL-2 and zoledronate, we do not expect a significant portion of \( \gamma \delta \) T cells to transform into protumorigenic subsets. However, we plan to evaluate this further while optimizing the development of our clinical cellular product. Our expanded \( \gamma \delta \) T-cell product showed minimal toxicity against normal PBMCs and bone marrow cells, which had low baseline NKG2D ligand expression. Previous clinical trials using ex vivo expanded autologous \( \gamma \delta \) T cells or attempted in vivo activation of endogenous \( \gamma \delta \) T cells have been safely tolerated with minimal reported toxicity [10–18].

However, only limited success was seen in terms of efficacy in these trials, possibly due to insufficient recognition of tumor cells by \( \gamma \delta \) T cells [10–18]. This suggests \( \gamma \delta \) T cells may function best in combination with drugs that induce expression of stress antigens. In the current study, we show that the combination of \( \gamma \delta \) T cells and bortezomib may be an effective strategy to target two cancer types, AML and T-ALL, with increased cytotoxicity seen even at low effector to target ratios. Currently, ~1–100 million \( \gamma \delta \) T cells per kg of body weight are used for each injection in clinical trials (NCT03790072 and NCT03533816). These results suggest sufficient anti-tumor effects may be achieved with a lower dose of \( \gamma \delta \) T cells when used in combination with bortezomib, making it a more practical immunotherapy. Additionally, previous in vitro cytotoxicity studies with \( \gamma \delta \) T cells required E:T ratios from 5:1 to 40:1 in order to achieve >30% killing of tumor cells, which would not likely be achievable in a clinical setting [44, 45]. Target cell death >30% was seen at a 1:4 E:T ratio with combinations of \( \gamma \delta \) T cells and bortezomib treatment in all the cell lines tested. Treating AML and T-ALL cells with bortezomib also led to faster killing. Blocking of the NKG2D receptor showed that there was a significant decrease in \( \gamma \delta \) T-cell killing in all four cell lines suggesting that the NKG2D ligand-receptor interaction was critical in triggering \( \gamma \delta \) T cell-mediated killing. However, blocking did not completely eliminate \( \gamma \delta \) T-cell killing, thereby suggesting that the increased killing could have been...
mediated by other γδ T cell receptor/ligand interactions through the γδ TCR, DNAX Accessory Molecule-1 (DNAM-1), Fas Ligand (FasL) or Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Further investigations are needed to study the effect of bortezomib on expression of these ligands/receptors on AML and T-ALL cells. The washout experiment demonstrated a significant decrease in γδ T cell-specific cytotoxicity after bortezomib was removed and the NKG2D ligand expression had decreased back to baseline, further confirming the role of the NKG2D axis in mediating γδ T-cell killing. There was also likely to have been a partial additive effect on killing from the bortezomib treatment. However, we only consider this to be beneficial as it increased the overall killing. These findings highlight the feasibility of γδ T-cell immunotherapy in the setting of AML and T-ALL, where multiple and more frequent doses of γδ T cells may be required to treat such aggressive malignancies.

The current dosing of bortezomib in adult cancer patients typically consists of bi-weekly intravenous or subcutaneous injections at 1.0 or 1.3 mg/m² for two weeks [34–36]. Peak bortezomib levels shortly after injection of drug have been reported to be between 20.4–22.5 ng/mL vs. 162–223 ng/mL for subcutaneous vs. IV administration respectively; however, previous pharmacokinetic studies have shown that the plasma concentration of bortezomib at 24 hours after injection ranges from 1–5 nM, depending on the number of previous doses a subject had received [34–36]. Here, we have shown there is minimal negative effects on γδ T cell growth and viability at these low doses, and no significant decrease in γδ T cell-mediated killing following exposure to bortezomib. A repeat assessment post-drug removal showed there was no significant delayed effect from the bortezomib treatment on γδ T-cell functionality. This is important as we would expect to dose bortezomib repeatedly as discussed above. Additionally, a previous study showed 10 nM bortezomib had no significant effects on γδ T-cell viability or expression of the degranulation marker CD107a [22]. Our data shows that bortezomib is effective at increasing ULBP2/5/6 surface expression on AML and T-ALL cells with peak expression around 18–24 hours. Thus, an intravenous infusion of ex vivo expanded γδ T cells after 24 hours of bortezomib treatment, when AML and T-ALL cells are still sensitive to enhanced γδ T cell-mediated cytotoxicity via increased NKG2D ligand expression, may offer an effective treatment strategy.

One limitation of single antigen-targeting immunotherapy, such as CAR T cells, is tumor heterogeneity. Targeting one antigen on tumor cells allows potential for tumor cell escape by ‘selecting’ for tumor cells which do not express the target antigen or adapt by downregulating the antigen, making the cells resistant to CAR T cells [46–48]. Our approach does not rely on a single antigen targeting system, but rather utilizing γδ T cells intrinsic cytotoxic mechanisms, thereby decreasing the chances of tumor escape. γδ T cells also mediate tumor cell identification and lysis through their TCR, DNAM-1, FasL and TRAIL interactions, mechanisms which are independent of the NKG2D ligand/receptor mechanism enhanced with bortezomib treatment. The ability of γδ T cells to kill cancer cells through multiple mechanisms, as well as leukemia cells’ susceptibility to cell death from bortezomib treatment alone, makes this combination therapy attractive, as there is less risk of tumor cells surviving treatment. Collectively, data from the current study provide a solid rationale for developing a platform of combination therapy with γδ T cells and stress ligand inducing drugs, such as bortezomib, to a variety of cancers.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (K12HD072245, K08CA248962), Hyundai Hope on Wheels, Curing Kids Cancer, and Children’s Healthcare of Atlanta. Additionally, research reported in this publication was supported in part by the Pediatrics/Winship Flow Cytometry Core of Winship Cancer Institute of Emory University, Children’s Healthcare of Atlanta and NIH/NCI under award number P30CA138292 and by the National Center for Advancing Translational Sciences of the NIH under award number UL1TR002378. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

GLOSSARY

MHC     major histocompatibility complex; linked polymorphic genes which code for cell surface proteins involved in adaptive immune system

GvHD    graft versus host disease; condition where immune cells from a donor attack receipt (host) tissue after a transplant

PBMCs   peripheral blood mononuclear cells; cellular components of blood, consisting of T cells, B cells, NK cells, monocytes, and granulocytes

TCR     T-cell receptor; complex of proteins on T cell surface which recognizes pieces of antigens as peptides bound to MHC molecules

ADCC    antibody-dependent cellular cytotoxicity; immune cell-mediated killing of target cells through antibodies present on surface of the target cells

NKG2D   natural killer group 2, member D; proteins in the CD94/NKG2 family of receptor-ligands

ULBP    UL16 binding proteins; cell surface proteins related to MHC class I molecules which are ligands for NKG2D receptor

MICA/B  MHC class I chain-related proteins A and B; stress induced ligand for NGK2D receptor

IL-2    interleukin-2; cytokine signaling molecule which regulates activation of lymphocytes

AML     acute myeloid leukemia; cancer which arises from rapid expansion of abnormal myeloblasts, leading to bone marrow crowding and decrease of normal blood cells

CAR     chimeric antigen receptor; an artificial T cell receptor engineered to target a specific antigen on target cells for immunotherapy

T-ALL   T-cell acute lymphoblastic leukemia; cancer characterized by rapid expansion of lymphoblasts
**NK cells**

Natural killer cells; lymphocyte involved in the innate immune system which rapidly targets and kills infected or cancer cells

**DNAM-1**

DNAX-accessory molecule-1 or CD226, surface protein expressed on NK cells, monocytes, and subset of T cells which binds CD112 and CD155 to drive cellular activation

**FasL**

Fas ligand, a transmembrane protein in the tumor necrosis factor (TNF) family which induces apoptosis when bound to the Fas receptor; one mechanism T cells induce cell death in infected or tumor cells

**TRAIL**

TNF-related apoptosis-inducing ligand which triggers caspase-8-dependent apoptosis in cells when it binds death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2)

**REFERENCES**

15. Bennouna J, et al., Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with


Figure 1. Expansion of γδ T cells from healthy donor PBMCs.

(A) Healthy donor PBMCs were cultured in serum free media over a day 12 period. Cultures were supplemented with 500 IU IL-2 and 5 μM zoledronic acid on days 0 and 3, and 1000 IU IL-2 on days 6, 9, and 12. On day 6, cultures were depleted of αβ T cells using positive αβ T-cell selection (n = 10 separate expansions, 8 different donors). (B) Flow cytometry was performed every 3 days to monitor the percentage of γδ T cells over the expansion. Live cells were gated on and γδ T-cell percentage was determined by CD3+/γδ TCR+. Post-αβ depleted cultures were 80% γδ T cells, compared to 49% prior to depletion on day 6 (n = 10 separate expansions, 8 different donors). By day 9 and 12 of expansion, all cultures were...
>92% γδ T cells (n = 10 separate expansions, 8 different donors day 9 mean = 95.4% ± 3.5%, day 12 mean = 95.5% ± 4%). (C) Representative flow of the percentage of γδ T cells over the course of expansion for one donor. Depleting cultures of αβ T cells yielded a highly pure γδ T-cell product by day 9 of expansion.
Figure 2. Expression of NKG2D ligands in AML cell lines and primary AML patient samples.
(A) Baseline protein expression of NKG2D ligands on AML cell lines was assessed by gating on Annexin V and 7-AAD double negative cells and analyzing surface marker expression (median fluorescent intensity) via flow cytometry. ULBP1 and ULBP3 were significantly higher in both Nomo-1 and Kasumi-1 cells compared to healthy donor PBMCs (n = 3, p < 0.0001). Surface expression of MICA/B was also significantly higher in Kasumi-1 cells compared to healthy donor PBMCs (n = 3, p < 0.0001). There was no significant difference in any NKG2D ligand surface expression on healthy donor bone...
marrow cells compared to PBMCs (n = 3, p > 0.05). (B) RNA sequencing data from primary AML patient samples was obtained from the Delwel (n=460) and Verhaak (n=525) data sets in the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) and showed elevated gene expression in all NKG2D ligands compared to gene expression in normal PBMCs (p < 0.0001). (C) Healthy donor PBMCs and bone marrow cells (n = 3 different donors) were stained with VPD450 and then incubated with day 12 γδ T cells (n = 3 different donors) at 1:2 and 1:1 E:T ratios for 4 hours at 37°C. Cell death of healthy donor PBMCs was minimal (mean at 1:2 E:T ratio was 4.5 ± 1.6% and 4 ± 1.2% at 1:1 E:T ratio), as was cell death of healthy donor bone marrow cells (mean 2.8 ± 2.9% at 1:2 E:T ratio and 2.9 ± 3.5% at 1:1 E:T ratio). There was no significant difference between PBMCs or bone marrow cell death at either E:T ratio (n = 3, p > 0.05). Significance was determined using two-way ANOVA with Bonferroni’s multiple comparisons test.
Figure 3. Bortezomib significantly increases expression of ULBP2/5/6 in AML cell lines after 24 hours of treatment.

(A) and (B) AML cell lines Kasumi-1 and Nomo-1 were treated with vehicle control or increasing doses of bortezomib for 48 hours. Live cells were gated on (7-AAD⁻/Annexin V⁻) and analyzed for changes in NKG2D ligand surface expression at the various time points and concentrations (n = 3, mean relative MFI to vehicle control). Peak expression of ULBP 2/5/6 was seen between hour 18 and 24 in both cell lines. (C) Surface expression of ULBP2/5/6 on Kasumi-1 cells significantly increased with 5 nM and 10 nM bortezomib treatment at 24 hours (n = 3, p < 0.0001 for both groups). (D) Nomo-1 cells had a significant increase in ULBP2/5/6 at the 10 nM dose (n = 3, p = 0.012) at 24 hours. Data are displayed as mean ± standard deviation of relative MFI of bortezomib treated cells compared to vehicle control treated cells. (E) No increase in NKG2D ligand expression was seen in healthy donor PBMCs and bone marrow cells after 24 hour treatment with 5 nM bortezomib. (E) and (F) No increase in NKG2D ligand expression was seen in PBMCs and bone marrow cells from healthy donors treated with 5 nM bortezomib for 24 hours. Significance was determined using one-way ANOVA with Dunnett’s multiple comparisons test.
Figure 4. Bortezomib has no major negative effects on γδ T cells at clinically relevant dose levels. (A) and (B) γδ T cells were treated with 1, 2.5, or 5 nM bortezomib for 24 hours at day 10 of the expansion. There was no significant decrease in surface expression of NKG2D receptor and CD107a on live γδ T cells (7-AAD−/Annexin V−) at any bortezomib dose (n = 3 separate donors, p > 0.05). (C) Bortezomib treatment had no negative effect on cell growth (n = 3 separate donors, p > 0.05). (D) There was a significant decrease in cell viability at 5 nM, from 98.9% to 74.8% (p = 0.004, n = 3 separate donors). (E) and (F) γδ T cells were treated with 1, 2.5, or 5 nM bortezomib for 24 hours at day 10 of the expansion, then bortezomib was removed and cells were cultured for an additional 24 hours. There was no significant decrease in cell growth or cell viability. Data are displayed as mean ± standard deviation of relative MFI, cell growth, and cell viability of bortezomib treated cells compared to vehicle control treated cells. Significance was determined using one-way ANOVA with Dunnett’s multiple comparisons test.
Figure 5. Bortezomib enhances cytotoxicity of γδ T cells against AML cell lines. 
(A) Representative flow plots from a 4 hour cytotoxicity assay with γδ T cells and Nomo-1 cells at a 1:1 effector to target (E:T) ratio. After 24 hour treatment with bortezomib or vehicle, AML cells were incubated with day 12 ex vivo expanded γδ T cells at 1:4, 1:2, 1:1, and 2.5:1 E:T ratios for 4 hours. The difference in cytotoxicity of γδ T cells against bortezomib-treated target cells and vehicle-treated target cells was analyzed at each E:T ratio. Replicates represent γδ T cells expanded from 3 different donors. (B) Cytotoxicity of Nomo-1 cells significantly increased in bortezomib-treated cells compared to vehicle-treated cells at all E:T ratios. (C) Cytotoxicity of Kasumi-1 cells was also increased in bortezomib-treated cells compared to vehicle-treated cells. (D) Inhibition of NKG2D-mediated cytotoxicity by 2.5 mM bortezomib in NKG2D-R blocked γδ T cells. (E) Cytotoxicity against Nomo-1 cells was not blocked completely by 2.5 mM bortezomib. (F) Bortezomib treatment resulted in a significant decrease in live GFP+ cells over time compared to vehicle-treated control.
cells at all E:T ratios (n= 3, p = 0.0025, 0.0005, 0.0001, and 0.0021, respectively). (C) Treating Kasumi-1 cells with bortezomib significantly increased target cell death at the 1:4, 1:2, and 1:1 E:T ratios (n = 3, p = 0.0044, 0.0058, and 0.016, respectively). (D) Blocking the NKG2D receptor on γδ T cells significantly decreased γδ T cell-specific cytotoxicity against bortezomib treated Nomo-1 cells and Kasumi-1 cells when tested at a 1:2 E:T ratio (n = 3 separate donors, p = 0.004 and p = 0.04, respectively). (E) Bortezomib washout experiment at a 1:2 E:T ratio showed a significant decrease in γδ T cell-specific cytotoxicity against Nomo-1 48 hours after washout, once the NKG2D ligand expression had decreased back to baseline (n = 3 separate donors, p = 0.003, unpaired t-test). (F) Representative flow plots from a longer time course cytotoxicity assay with γδ T cells and Nomo-1 cells at a 1:2 E:T ratio. After 24 hours of treatment with bortezomib or vehicle, GFP+ Nomo-1 cells were incubated with day 11 ex vivo expanded γδ T cells for 4, 24, and 48 hours. The percentage of remaining live GFP+ cells (GFP+/7-AAD−) was determined at each time point to determine whether bortezomib treatment increased the cytotoxicity of GFP+ Nomo-1 cells compared to vehicle-treated cells. Bortezomib-treated Nomo-1 cells incubated with γδ T cells had a significantly lower percentage of live cells after 24 hours and 48 hours compared with vehicle-treated cells (n = 3, p = 0.003 and 0.0002, respectively). Significance was determined using two-way ANOVA with Bonferroni’s multiple comparisons test or unpaired t-test.
Figure 6. Bortezomib significantly increases expression of ULBP2/5/6 in T-ALL cell lines after 24 hours of treatment.

(A) Baseline protein expression of NKG2D ligands on T-ALL was assessed by gating on Annexin V and 7-AAD double negative cells and analyzing surface marker expression via flow cytometry. ULBP2/5/6 was significantly higher in both Jurkat and MOLT-4 cells compared to healthy donor PBMCs (n = 3, p < 0.0001 and p = 0.01, respectively, one-way ANOVA with Dunnett’s multiple comparisons test). There was no significant difference in any NKG2D ligand surface expression on healthy donor bone marrow cells compared to PBMCs (n = 3, p > 0.05). (B) RNA sequencing data from primary T-ALL patient samples was obtained from the Peiters (n= 92) and Meijerink (n=124) data sets in the R2 Genomics Analysis and Visualization Platform ([http://r2.amc.nl](http://r2.amc.nl)) and showed elevated gene expression in ULBP2 compared to gene expression in normal PBMCs (p < 0.0001). (C) and (D) T-ALL cell lines Jurkat and MOLT-4 were treated with vehicle control or increasing doses of bortezomib for 48 hours. Live cells were gated on (7-AAD-/Annexin V-) and analyzed for changes in NKG2D ligand surface expression at the various time points and concentrations (mean relative MFI, n= 3). Surface expression of ULBP2/5/6 in both cell lines peaked between 24 and 48 hours at all doses. (E) There was a significant increase in ULBP2/5/6 with 5 nM and 10 nM bortezomib treatment of Jurkat cells at the 24 hour time point (n = 3, p = 0.007, one-way ANOVA with Dunnett’s multiple comparisons test). (F) Surface expression of ULBP2/5/6 in MOLT-4 cells peaked between 12 and 24 hours at all doses. (F) ULBP2/5/6 expression was also significantly increased on MOLT-4 cells at 5 nM and 10 nM bortezomib (n = 3, p = 0.0037 and p < 0.0001, respectively, one-way ANOVA with Dunnett’s multiple comparisons test).
Figure 7. Bortezomib increases cytotoxicity of γδ T cells against T-ALL cell lines.

(A) Representative flow plots from a 4 hour cytotoxicity assay with γδ T cells and Jurkat cells at a 1:1 effector to target (E:T) ratio. (B) Vehicle or bortezomib treated cells Jurkat and MOLT-4 cells were incubated for 4 hours with day 12 ex vivo expanded γδ T cells at 1:4, 1:2, 1:1, and 2.5:1 E:T ratios. The difference in target cell death between bortezomib-treated cells and vehicle-treated cells was compared at each E:T ratio. Replicates represent γδ T cells expanded from 3 different donors. Bortezomib-treated Jurkat cells had a significantly higher cytotoxicity (% dead target cells) compared to vehicle-treated Jurkat cells at the 1:4 E:T ratio.
and 1:2 E:T ratios (n = 3, p = 0.0054 and 0.0448, respectively). (C) MOLT-4 cells treated with bortezomib also had a significantly higher percentage of dead target cells at the 1:4, 1:2, and 1:1 E:T ratios (n = 3, p = 0.001, 0.014, and 0.023, respectively). (D) & (E) Blocking the NKG2D receptor on γδ T cells significantly decreased γδ T cell-specific cytotoxicity against bortezomib treated Jurkat and MOLT-4 cells when tested at 1:2 E:T ratio (n = 3 separate donors, p < 0.0001, respectively). (F) After 24 hours of treatment with bortezomib or vehicle, GFP+ Jurkat cells were incubated with day 11 ex vivo expanded γδ T cells for 4 and 24 hours at a 1:4 or 1:20 E:T ratio. The percentage of remaining live GFP+ cells (GFP+/7-AAD-) was determined at each time point to determine whether bortezomib treatment increased the cytotoxicity of GFP+ Jurkat cells compared to vehicle-treated cells. Bortezomib-treated Jurkat cells had a significantly lower percentage of live cells after 24 hours compared with vehicle-treated cells at both E:T ratios (n = 3, p = 0.011 and p = 0.003, respectively). Significance was determined using two-way ANOVA with Bonferroni’s multiple comparisons test or unpaired t-test.