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Basic Research

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

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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 a combination of NKG2D ligand-inducing drugs, such as the proteasome inhibitor bortezomib, and *ex vivo*-expanded peripheral blood $\gamma\delta$ T cells (i.e., V γ 9V δ 2 T cells). Acute myeloid leukemia (AML) is a high-risk hematologic malignancy, and treatment has shown limited benefit with 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. V γ 9V δ 2 T cells were expanded and isolated from peripheral blood of healthy donors to generate a final cellular product with a mean of 96% CD3⁺/ $\gamma\delta$ T-cell receptor-positive cells. Combination treatment of the AML cell lines with $\gamma\delta$ T cells and bortezomib resulted in significantly greater cytotoxicity than $\gamma\delta$ 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 ULBP 2/5/6 expression in T-ALL cell lines, Jurkat and MOLT-4 and improved the cytotoxicity of $\gamma\delta$ T cells against each line. Collectively, these results show that bortezomib enhances $\gamma\delta$ 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 $\gamma\delta$ T cells with stress ligand-inducing drugs as a therapeutic platform for high-risk leukemias is demonstrated.

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Introduction

The $\gamma\delta$ T cells are a unique class of immune cells that play important roles in both innate and adaptive cellular immunity [1,2]. Unlike

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the more abundant $\alpha\beta$ 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 for utilization of their inherent anti-tumor properties without expectation of graft-versus-host disease (GVHD). A large tumor molecular profiling study showed that infiltrating $\gamma\delta$ T cells were the strongest leukocyte predictor of favorable outcome [7]. In humans, $\gamma\delta$ T cells constitute about 1–5% of peripheral blood T cells and are

primarily composed of the V δ 2V γ 9 T-cell subset [1–6]. The authors previously developed a Good Manufacturing Practice-compliant method to expand V δ 2V γ 9 T cells from peripheral blood mononuclear cells (PBMCs) using serum-free media in combination with cytokines and bisphosphonates [8,9]. This method is quite 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.

The $\gamma\delta$ T cells exhibit anti-tumor properties through several different mechanisms [1–6]. The $\gamma\delta$ T-cell receptor (TCR) is specifically reactive to butyrophilin molecules, whose surface expression is driven by intracellular phosphoantigens, such as isopentenyl pyrophosphate. These phosphoantigens are upregulated in certain stressed, infected and tumor cells. The $\gamma\delta$ T cells also have upregulated CD16 expression and thus play an important role in antibody-dependent cellular cytotoxicity. In addition, $\gamma\delta$ T cells target cancer cells through expression of the natural killer group 2, member D (NKG2D) receptor, which can recognize stress-induced ligands on tumor cells [1–6]. These NKG2D ligands include UL16 binding proteins (ULBP) 1–6 and the MHC class I chain-related proteins A and B (MICA/B). Past clinical trials have used $\gamma\delta$ T cells to target different malignancies either through autologous $\gamma\delta$ T-cell infusions post expansion with zoledronic acid or by activating circulating $\gamma\delta$ T cells by directly administering zoledronic acid and IL-2 to patients [10–18]. These studies were primarily designed to activate $\gamma\delta$ T cells through stimulation of their TCR with bisphosphonates. Although the therapy was safely tolerated, mixed results were seen in terms of efficacy. To maximize the inherent anti-tumor properties of $\gamma\delta$ T cells, non- $\gamma\delta$ TCR-mediated cytotoxic pathways must also be explored. One potential method to enhance $\gamma\delta$ 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, the authors hypothesize that using a combination of a stress ligand-inducing drug with an off-the-shelf allogeneic $\gamma\delta$ 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 $\gamma\delta$ 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 have been challenging to develop because of 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 $\gamma\delta$ 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 $\gamma\delta$ 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]. Although 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 because of the resulting cellular stress from proteasome inhibition [19–22]. Here the use of bortezomib as an agent to sensitize AML to $\gamma\delta$ 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]. The findings show that utilizing bortezomib to increase NKG2D ligand surface expression enhances $\gamma\delta$ T-cell cytotoxicity against AML and T-ALL, allowing

for the development of an adoptive cellular immunotherapy platform to treat these aggressive blood cancers.

Methods

Expansion of $\gamma\delta$ T cells in serum-free media

PBMCs (50–100 million per donor) from six different healthy donors were obtained through AllCells (Alameda, CA, USA) and cultured in OpTmizer serum-free media (Thermo Fisher Scientific, Waltham, MA, USA) 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, USA), and PBMCs were isolated using Ficoll-Paque Plus density gradient media (Sigma-Aldrich, St Louis, MO, USA). Media changes were made every 3 days, and cultures were stimulated with 500 IU/mL of IL-2 (PeproTech, Rocky Hill, NJ, USA) and 5 μ M zoledronic acid (Sigma-Aldrich) on day 0 and day 3 and with 1000 IU/mL of IL-2 on day 6, day 9 and day 12. On day 6 of expansion, $\alpha\beta$ T cells were removed from the culture with positive selection using anti- $\alpha\beta$ 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, USA). The $\gamma\delta$ T-cell percentage and receptors/ligands associated with cytotoxicity were monitored via flow cytometry on day 0, day 6, day 9 and day 12. Downstream experiments were performed with $\gamma\delta$ T cells at day 10 to day 12 of their expansion.

Monitoring cell culture composition during $\gamma\delta$ T-cell expansion

Live cell counts were determined using Trypan Blue dye (Thermo Fisher Scientific) after thoroughly pipetting cell clumps to achieve a uniform cell culture, and $3\text{--}5 \times 10^5$ cells of each sample were washed with phosphate-buffered saline (PBS) and spun at $300 \times g$ in flow tubes. Supernatant was decanted, and cells were incubated at room temperature with eBioscience eFluor 780 viability dye (Invitrogen, San Diego, CA, USA) for 30 min while shaking. The cells were washed with 2 mL PBS and spun at $300 \times g$. Supernatant was decanted and replaced with the appropriate antibody cocktail in PBS. The antibodies used for flow cytometry are listed in supplementary Table 1. Cells were incubated with antibody cocktails for at least 15 min, and then the cells were washed with 2 mL PBS and spun at $300 \times g$. Supernatant was decanted, and cells were resuspended in 100 μ L PBS prior to data collection using an Aurora flow cytometer (Cytek Biosciences, Fremont, CA, USA). Data were analyzed using FlowJo (BD Biosciences, Franklin Lakes, NJ, USA).

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, USA). The Jurkat cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The primary culture medium for the Nomo-1, Jurkat and MOLT-4 cell lines was RPMI (Corning, Manassas, VA, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin, whereas the culture medium for the Kasumi-1 cells was RPMI with 20% fetal bovine serum and 1% penicillin/streptomycin. Green fluorescent protein (GFP)-expressing Nomo-1 and Jurkat cells were generated by transducing 500 000 cells at a multiplicity of infection of 10 with a lentivirus (p-HIV-EGFP; Addgene, Watertown, MA, USA). Cells were incubated with virus in complete media supplemented with 8 μ g/mL Polybrene (EMD Millipore,

Billerica, MA, USA) for 24 h, then media were 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, reconstituted in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 1 mM and further diluted to 100 μ M with DMSO before being stored at -20°C . Next, 100- μ M aliquots were thawed just before experiments and diluted to 10 μ M with DMSO. The 10- μ M solution was then diluted in the culture medium for each cell type to reach a working concentration of 1 μ M (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 h 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 h post-treatment to the vehicle control-treated group. Cell viability was determined by gating on live cells (7-aminocaproic acid [7-AAD]⁻/Annexin V⁻) via flow cytometry and normalizing live cell percentage at 48 h to the untreated group. Dose-response curves were plotted using Prism 8 (GraphPad Software Inc, San Diego, CA, USA), and the half-maximal inhibitory concentration (IC₅₀) and lethal concentration (LC₅₀) were determined by interpolating the drug concentration at $\times = 50\%$ 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. Next, 3×10^5 cells were seeded in 1 mL in 12-well plates and treated with 2.5 nM, 5 nM or 10 nM bortezomib or vehicle control (0.1% DMSO) over 48 h, with NKG2D ligand expression being measured at several time points. MFI was determined based on live cells (7-AAD⁻/Annexin V⁻) using either an Aurora flow cytometer (Cytex Biosciences) or BD LSRII (BD Biosciences). Data were analyzed using FlowJo (BD Biosciences). Relative MFI was calculated by 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 were queried, and the u133p2 chip with MAS5.0 normalization was used throughout the study. Databases queried were as follows: PBMC (Tangye, 14 samples), AML (Delwel, 460 samples, and Verhaak, 525 samples) and T-ALL (Pieters, 92 samples, and Meijerink, 124 samples).

Effect of bortezomib on $\gamma\delta$ T cells

The $\gamma\delta$ T cells were plated in 500 μ L 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 h 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). To determine whether bortezomib treatment had any delayed effects on $\gamma\delta$ T cells, bortezomib was removed from the cultures after 24 h of treatment and $\gamma\delta$ T cells were cultured for another 24 h. The $\gamma\delta$ T-cell growth, cell viability and cytotoxicity capability were assessed after this time point.

Cytotoxicity assays

Cytotoxic capabilities of $\gamma\delta$ T cells against leukemia cells or non-malignant hematopoietic cells were assessed in a flow cytometry-based assay. Target cells included AML cell lines Nomo-1 and Kausmi-1, T-ALL cell lines Jurkat clone E6-1 and MOLT-4 (American Type Culture Collection) and healthy donor PBMCs and bone marrow cells from AllCells. Target cells were stained with Violet proliferation dye 450 (BD Biosciences) and treated with vehicle control, 5 nM (Kasumi-1, Jurkat and MOLT-4 cells) or 10 nM bortezomib (Nomo-1 cells) for 24 h prior to the cytotoxicity assay. Next, 50 000 target cells were incubated with day 12 *ex vivo*-expanded $\gamma\delta$ T cells at various effector-to-target (E:T) ratios—0:1, 1:4, 1:2, 1:1 and 2.5:1—for 4 h at 37°C . Target cell death was assessed by flow cytometry via 7-AAD and Annexin V (BioLegend, San Diego, CA, USA) staining of necrotic and apoptotic cells. Samples were washed with 2 mL PBS, spun down at $300 \times g$ and then incubated with Annexin V in Annexin V binding buffer for 15 min at room temperature. Next, 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 $\gamma\delta$ T cells at E:T ratios of 0:1 and 1:2 (Nomo-1) or 1:4 and 1:20 (Jurkat) for 4 h, 24 h and 48 h. Target cell disappearance was assessed by flow cytometry via GFP and 7-AAD detection. Next, 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 $\gamma\delta$ T-cell-mediated cell death was assessed. To determine the optimal concentration for blocking the NKG2D receptor on $\gamma\delta$ T cells, $\gamma\delta$ T cells were seeded in a 96-well plate in 100 μ L at a density of 2×10^6 cells/mL and incubated with 0 μ g/mL, 5 μ g/mL, 10 μ g/mL, 20 μ g/mL or 50 μ g/mL of an unconjugated NKG2D receptor antibody (BioLegend) at room temperature. After 30 min, the cells were washed with PBS and spun at $300 \times g$, then incubated with fluorescein isothiocyanate-conjugated NKG2D receptor antibody (BioLegend) for 15 min at room temperature. MFI was assessed for each concentration treatment via flow cytometry to determine the concentration that yielded the lowest binding of the fluorescein isothiocyanate NKG2D receptor antibody. For cytotoxicity assays, 2×10^5 $\gamma\delta$ T cells were incubated with 50 μ g/mL of the unconjugated NKG2D receptor antibody for 30 min, then spun down at $300 \times g$ and resuspended in fresh media prior to incubation with Violet proliferation dye 450-stained target cells at a 1:2 E:T ratio. Bortezomib washout experiments were performed to test the efficacy of $\gamma\delta$ T-cell killing once the NKG2D ligand expression had returned to baseline. Briefly, all cell lines were treated with bortezomib for 24 h, 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 Prism 8 software (GraphPad Software Inc). Results were presented as mean $\pm$ standard deviation of the mean and were considered significant at $P < 0.05$. Unpaired two-tailed Student's *t*-test and one-way or two-way analysis of variance (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

The authors' laboratory has previously developed a Good Manufacturing Practice-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 day 0, day 6, day 9 and day 12 of expansion (Figure 1B). Gating strategy for flow cytometry experiments is shown in supplementary Figure 1. To enrich for $\gamma\delta$ T cells only, an $\alpha\beta$ T-cell depletion was performed on day 6 of the *ex vivo* expansion. This step resulted

in a cellular product with a mean of $95.4 \pm 3.5\%$ CD3⁺/ $\gamma\delta$ TCR⁺ cells by day 9 and $95.5 \pm 4\%$ CD3⁺/ $\gamma\delta$ TCR⁺ cells by day 12 (Figure 1B,C). Downstream experiments were done with $\gamma\delta$ T cells between day 10 and day 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 with non-malignant hematopoietic cells

ULBP1, ULBP3 and MICA/B were significantly higher in Nomo-1 cells compared with healthy donor PBMCs ($P < 0.01$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 2A). ULBP1, ULBP3 and MICA/B were also elevated in Kasumi-1 cells compared with PBMCs ($P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 2A). Moreover, ULBP2/5/6 was elevated in both Nomo-1 and Kasumi-1 cells ($P = 0.01$ and 0.03 , respectively, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 2A). Bone marrow cells from healthy donors showed no significant difference in any NKG2D ligand surface expression compared with PBMCs ($P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 2A). Primary samples obtained at time of diagnosis from two separate databases showed elevated RNA expression of all NKG2D ligands in AML patients compared with healthy PBMC controls ($P < 0.001$, one-way ANOVA with Dunnett's multiple comparisons test)

Figure 1. Expansion of $\gamma\delta$ T cells from healthy donor PBMCs. (A) Healthy donor PBMCs were cultured in serum-free media over a 12-day period. Cultures were supplemented with 500 IU IL-2 and 5 μ M zoledronic acid on day 0 and day 3 and 1000 IU IL-2 on day 6, day 9 and day 12. On day 6, cultures were depleted of $\alpha\beta$ T cells using positive $\alpha\beta$ T-cell selection ($n = 10$ separate expansions, 8 different donors). (B) Flow cytometry was performed every 3 days to monitor the percentage of $\gamma\delta$ T cells over the expansion. Live cells were gated on, and $\gamma\delta$ T-cell percentage was determined by selecting CD3⁺/ $\gamma\delta$ TCR⁺ cells. Post- $\alpha\beta$ -depleted cultures were 80% $\gamma\delta$ T cells compared with 49% prior to depletion on day 6 ($n = 10$ separate expansions, 8 different donors). By day 9 and day 12 of expansion, all cultures were $>92\%$ $\gamma\delta$ T cells ($n = 10$ separate expansions, 8 different donors, day 9 mean = $95.4 \pm 3.5\%$, day 12 mean = $95.5 \pm 4\%$). (C) Representative flow of the percentage of $\gamma\delta$ T cells over the course of expansion for one donor. Depleting cultures of $\alpha\beta$ T cells yielded a highly pure $\gamma\delta$ 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 (MFI) via flow cytometry. ULBP1 and ULBP3 were significantly higher in both Nomo-1 and Kasumi-1 cells compared with healthy donor PBMCs ($n = 3$, $P < 0.0001$). Surface expression of MICA/B was also significantly higher in Kasumi-1 cells compared with 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 with PBMCs ($n = 3$, $P > 0.05$). (B) RNA sequencing data from primary AML patient samples were obtained from the Delwel ($n = 460$) and Verhaak ($n = 525$) datasets in the R2 Genomics Analysis and Visualization Platform and showed elevated gene expression in all NKG2D ligands compared with 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 $\gamma\delta$ T cells ($n = 3$ different donors) at 1:2 and 1:1 E:T ratios for 4 h at 37°C. Cell death of healthy donor PBMCs was minimal (mean at 1:2 E:T ratio, $4.5 \pm 1.6\%$, mean at 1:1 E:T ratio, $4 \pm 1.2\%$), as was cell death of healthy donor bone marrow cells (mean at 1:2 E:T ratio, $2.8 \pm 2.9\%$, mean at 1:1 E:T ratio, $2.9 \pm 3.5\%$). 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. mRNA, messenger RNA; VPD450, Violet proliferation dye 450; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

(Figure 2B). 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 E:T ratios of 1:2 and 1:1. Cell death of healthy donor PBMCs was minimal (mean at 1:2 E:T ratio, $4.5 \pm 1.6\%$, mean at 1:1 E:T ratio, $4 \pm 1.2\%$), as was cell death of healthy donor bone marrow cells (mean at 1:2 E:T ratio, $2.8 \pm 2.9\%$, mean at 1:1 E:T ratio, $2.9 \pm 3.5\%$). There was no significant difference in cell death between PBMCs and bone marrow cells

($P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 2C).

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 (see supplementary

Figure 2). 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), whereas 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 the 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 with 2.5 nM, 5 nM and 10 nM bortezomib for 48 h, 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,B; also see supplementary Figure 4). Gating strategy for these experiments is shown in supplementary Figure 3. Relative MFI for each drug concentration was determined by comparing with a vehicle control-treated group. Peak expression for ULBP2/5/6 was seen between 18 h and 24 h for both AML cell lines (Figure 3A,B). Treating Kasumi-1 cells with 5 nM bortezomib for 24 h significantly increased expression of ULBP2/5/6 by 1.9-fold ($n = 3$, $P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 3C). A significant increase was also seen in ULBP1 and ULBP3 expression at 18 h and 24 h in Kasumi-1 cells treated with 5 nM bortezomib (see supplementary Figure 4). The 24-h treatment with 10 nM bortezomib significantly increased expression of ULBP2/5/6 in Nomo-1 cells by 2.0-fold ($n = 3$, $P = 0.0124$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 3D). Significant elevation was also seen in MICA/B at 24 h (see supplementary Figure 3). Treatment of healthy donor PBMC and bone marrow samples with 5 nM bortezomib for 24 h did not show any significant increase in expression of NKG2D ligands, demonstrating that ligand expression remains low in healthy, non-malignant hematopoietic cells even with bortezomib treatment ($n = 3$, $P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 3E,F).

Effects of bortezomib on $\gamma\delta$ T-cell growth and functionality

To determine whether bortezomib had any negative effects on $\gamma\delta$ T cells, $\gamma\delta$ T cells at day 10 of expansion were treated for 24 h 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. This dose range was chosen since the expected concentration of bortezomib 24 h after intravenous or subcutaneous administration (1.3 mg/ m^2) in adults is between 1 nM and 5 nM [34–36]. No changes in NKG2D receptor or CD107a expression were observed with any bortezomib treatment ($n = 3$, $P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 4A,B). Though there was no significant decrease in cell growth at any dose ($n = 3$, $P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 4C), there was a significant decrease in cell viability, from 98.9% to 74.8%, at the 5-nM dose ($n = 3$, $P = 0.004$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 4D).

To determine whether bortezomib treatment had any delayed effects on $\gamma\delta$ T cells, $\gamma\delta$ T cells were treated with vehicle control, 1 nM, 2.5 nM or 5 nM bortezomib for 24 h, then bortezomib was removed with a media change and $\gamma\delta$ T cells were cultured for an additional 24 h. The $\gamma\delta$ T-cell growth, viability and cytotoxicity capabilities were then assessed. There was no significant decrease in $\gamma\delta$ T-cell growth or viability 24 h after bortezomib was removed ($n = 3$, $P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 4E,F). No change in cytotoxicity was seen when the bortezomib-treated $\gamma\delta$ T cells were tested against the Nomo-1 AML cell line at a 1:2 E:T ratio ($n = 3$, $P > 0.05$, one-way ANOVA with Dunnett's multiple comparisons test) (see supplementary Figure 5). These findings suggest that administration of $\gamma\delta$ T cells 24 h after 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

Figure 3. Bortezomib significantly increases expression of ULBP2/5/6 in AML cell lines after 24 h of treatment. (A,B) AML cell lines Kasumi-1 and Nomo-1 were treated with vehicle control or increasing doses of bortezomib for 48 h. Live cells were gated on 7-AAD⁺/Annexin V⁺ and analyzed for changes in NKG2D ligand surface expression at various time points and concentrations (mean relative MFI to vehicle control, $n = 3$). Peak expression of ULBP 2/5/6 was seen between 18 h and 24 h 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 h ($n = 3$, $P < 0.0001$ for both groups). (D) Nomo-1 cells had a significant increase in ULBP2/5/6 at the 10-nM dose ($n = 3$, $P = 0.0124$) at 24 h. Data are displayed as mean $\pm$ standard deviation of relative MFI of bortezomib-treated cells compared with vehicle control-treated cells. (E) No increase in NKG2D ligand expression was seen in healthy donor PBMCs and bone marrow cells after 24 h treatment with 5 nM bortezomib. (E,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 h. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

Figure 4. Bortezomib has no major negative effects on $\gamma\delta$ T cells at clinically relevant dose levels. (A,B) The $\gamma\delta$ T cells were treated with 1 nM, 2.5 nM or 5 nM bortezomib for 24 h at day 10 of the expansion. There was no significant decrease in surface expression of NKG2D receptor and CD107a on live $\gamma\delta$ 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% ($n = 3$ separate donors, $P = 0.004$). (E,F) The $\gamma\delta$ T cells were treated with 1 nM, 2.5 nM or 5 nM bortezomib for 24 h at day 10 of the expansion, then bortezomib was removed and cells were cultured for an additional 24 h. There was no significant decrease in cell growth or cell viability. Data are displayed as mean $\pm$ standard deviation of relative MFI, cell growth and cell viability of bortezomib-treated cells compared with vehicle control-treated cells. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

bortezomib is reported in adults after their fourth dose (~5 nM) [34–36]. Therefore, the optimal dosing time for $\gamma\delta$ T cells, to eliminate any risk of negatively affecting the $\gamma\delta$ T cells, would be 24 h after bortezomib dosing.

Bortezomib treatment enhances the cytotoxicity of healthy donor-derived $\gamma\delta$ T cells against AML cells

Bortezomib treatment increased surface expression of NKG2D ligands in AML cell lines. To determine whether this increased AML cell sensitivity to $\gamma\delta$ T-cell-mediated cell death, the effect of treating leukemia cells with bortezomib prior to an *in vitro* cytotoxicity assay with $\gamma\delta$ 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 $\gamma\delta$ T-cell combination than with either treatment alone (Figure 5A). Gating strategy for these cytotoxicity experiments is shown in supplementary Figure 6. Compared with vehicle control-treated cells, bortezomib-treated AML cell lines had higher total cell death at all E:T ratios (Figure 5B,C). Cytotoxicity of Nomo-1 cells was significantly increased with bortezomib and $\gamma\delta$ T-cell combination treatment compared with vehicle control and $\gamma\delta$ 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 E:T ratios of 1:4, 1:2, 1:1 and 2.5:1, respectively ($n = 3$ separate donors, $P < 0.002$, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 5B). Total cell death of Kasumi-1 cells was significantly increased with bortezomib treatment compared with vehicle control, from 16.8% to 52.1% at the 1:4 E:T ratio, 30.0% to 64.0% at the 1:2 E:T ratio and 48.0% to 77.8% at the 1:1 E:T ratio ($n = 3$ separate donors, $P < 0.02$, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 5C). Variability in cytotoxicity between individual donors is shown in supplementary Figure 7.

To determine whether increased target cell killing in the cytotoxicity assays was dependent on NKG2D receptor–ligand interactions,

the authors sought to block the NKG2D receptor on $\gamma\delta$ T cells prior to incubation with target cells. The authors performed a titration assay and determined that 50 $\mu\text{g}/\text{mL}$ of an unconjugated NKG2D receptor antibody was optimal for NKG2D receptor blocking (see supplementary Figure 8). A cytotoxicity assay was then performed in which the NKG2D receptor on $\gamma\delta$ T cells was blocked prior to incubation with bortezomib-treated AML cell lines. The $\gamma\delta$ T-cell-specific cytotoxicity was normalized to target cell death mediated by unblocked $\gamma\delta$ T cells, and the relative $\gamma\delta$ T-cell-specific cytotoxicity with the NKG2D receptor blocked was compared to that with the NKG2D receptor unblocked. The $\gamma\delta$ T-cell-specific cytotoxicity against bortezomib-treated Nomo-1 cells was significantly decreased by 53.7% when the NKG2D receptor on $\gamma\delta$ T cells was blocked ($n = 3$ separate donors, $P = 0.004$, unpaired *t*-test) (Figure 5D). The $\gamma\delta$ T-cell-specific cytotoxicity against bortezomib-treated Kasumi-1 cells was also significantly decreased when the NKG2D receptor was blocked ($n = 3$ separate donors, $P = 0.04$, unpaired *t*-test) (see supplementary Figure 9A). 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 $\gamma\delta$ T-cell killing of AML cell lines, the authors pulsed the tumor cells with bortezomib and then performed cytotoxicity assays after drug washout and recovery once NKG2D ligand expression decreased. The authors treated the AML cells with bortezomib for 24 h, 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 h (see supplementary Figure 9B,C). A cytotoxicity assay was set up immediately after bortezomib treatment and 48 h after bortezomib was washed out at a 1:2 E:T ratio. Both cell lines showed a significant decrease in $\gamma\delta$ T-cell-specific cytotoxicity after bortezomib was removed and cells were allowed to recover for 48 h ($n = 3$ separate donors, $P = 0.003$ for Nomo-1, $P = 0.02$ for Kasumi-1,

Figure 5. Bortezomib enhances cytotoxicity of $\gamma\delta$ T cells against AML cell lines. (A) Representative flow plots from a 4-h cytotoxicity assay with $\gamma\delta$ T cells and Nomo-1 cells at a 1:1 E:T ratio. After 24-h treatment with bortezomib or vehicle control, AML cells were incubated with day 12 *ex vivo*-expanded $\gamma\delta$ T cells at E:T ratios of 1:4, 1:2, 1:1 and 2.5:1 for 4 h. The difference in cytotoxicity of $\gamma\delta$ T cells against bortezomib-treated target cells and vehicle control-treated target cells was analyzed at each E:T ratio. Replicates represent $\gamma\delta$ T cells expanded from three different donors. (B) Cytotoxicity of Nomo-1 cells significantly increased in bortezomib-treated cells compared with vehicle control-treated 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 E:T ratios of 1:4, 1:2 and 1:1 ($n = 3$, $P = 0.0044$, 0.0058 and 0.016 , respectively). (D) Blocking the NKG2D receptor on $\gamma\delta$ T cells significantly decreased $\gamma\delta$ 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 0.04 , respectively). (E) Bortezomib washout experiment at a 1:2 E:T ratio showed a significant decrease in $\gamma\delta$ T-cell-specific cytotoxicity against Nomo-1 at 48 h after washout once NKG2D ligand expression had decreased back to baseline ($n = 3$ separate

unpaired *t*-test) (Figure 5E; also see supplementary Figure 9D). To assess whether bortezomib treatment accelerated $\gamma\delta$ T-cell-mediated cell death of AML cells over a longer incubation period, vehicle control- and bortezomib-treated GFP⁺ Nomo-1 cells were co-cultured with $\gamma\delta$ T cells at a 1:2 E:T ratio, and the culture was analyzed at 4 h, 24 h and 48 h. The combination of bortezomib and $\gamma\delta$ T cells eliminated 39.2% of target cells at 24 h and 77.2% of target cells after 48 h, whereas $\gamma\delta$ T cells alone eliminated only 15.1% of target cells at 24 h and 45.3% of target cells at 48 h ($n = 3$ separate donors, $P = 0.003$ and 0.0002 , respectively, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 5F).

Validation of bortezomib and $\gamma\delta$ 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 $\gamma\delta$ 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 ($n = 3$, $P < 0.0001$ and $P = 0.01$, respectively, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 6A). This correlated with gene expression data that showed higher expression of ULBP2 in primary T-ALL patient samples compared with normal PBMCs ($n = 3$, $P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 6B). Expression of ULBP1, ULBP3, ULBP4 and MICA/B was also elevated in T-ALL patients and T-ALL cell lines compared with healthy donor PBMCs ($P < 0.001$, one-way ANOVA with Dunnett's multiple comparisons test) (see supplementary Figure 10). Bortezomib treatment in T-ALL cell lines decreased cell growth and viability in a dose-dependent manner (see supplementary Figure 11). Jurkat cells and MOLT-4 cells had intermediate sensitivity to cell growth inhibition, with an 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 an 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 with 2.5 nM, 5 nM and 10 nM bortezomib for 48 h, and NKG2D ligand expression (ULBP1, ULBP2/5/6, ULBP3, ULBP4, MICA/B) was measured at several time points by flow cytometry (Figure 6; also see supplementary Figure 12). Expression was compared with a vehicle control-treated group. Peak expression for ULBP2/5/6 was seen between 18 h and 24 h for both cell lines (Figure 6C,E). Treatment with 5 nM bortezomib for 24 h significantly increased expression of ULBP2/5/6 in Jurkat and MOLT-4 cells by 2.0- and 2.1-fold, respectively ($n = 3$, $P = 0.0007$ and 0.0037 , respectively, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 6D,F).

In a 4-h 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 treatment alone (Figure 7A). The total cytotoxicity against bortezomib-treated Jurkat cells increased significantly, from 39.9% to 64.0% at the 1:4 E:T ratio and from 58.1% to 76.4% at the 1:2 E:T ratio, compared with vehicle control-treated cells ($n = 3$ separate donors, $P = 0.005$ and 0.045 , respectively, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 7B). The total cytotoxicity of MOLT-4 cells was also significantly increased with bortezomib treatment compared with vehicle control-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 ($n = 3$ separate donors, $P = 0.001$, 0.014 and 0.023 , respectively, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 7C). Variability in cytotoxicity among the different donors is shown in supplementary Figure 13.

The NKG2D receptor blocking cytotoxicity assay previously described was then performed with T-ALL cell lines. The $\gamma\delta$ 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 ($n = 3$ separate donors, $P = 0.004$, unpaired *t*-test) (Figure 7D). 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 ($n = 3$ separate donors, $P < 0.0001$, unpaired *t*-test) (Figure 7E). The authors 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 h after washout once the NKG2D ligand expression had decreased back to baseline (see supplementary Figure 14). 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 E:T ratios of 1:4 and 1:20, and the percentage of live target cells was measured after 4 h and 24 h. These results showed a more rapid decrease in the percentage of live GFP⁺ Jurkat cells at 24 h compared with vehicle control-treated Jurkat cells at E:T ratios of both 1:4 and 1:20 ($n = 3$ separate donors, $P = 0.011$ and 0.003 , respectively, two-way ANOVA with Bonferroni's multiple comparisons test) (Figure 7F).

Discussion

Outcomes for patients with acute leukemias have greatly improved over the past several decades with the intensification of chemotherapy, especially in children [37,38]. However, resistance and recurrence in AML and T-ALL patients remain a critical barrier in the field [39,40]. Cancer immunotherapies offer a promising strategy for improving 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 by utilizing an alternative adoptive cell therapy strategy with *ex vivo*-expanded allogeneic $\gamma\delta$ T cells (V γ 9V δ 2 T cells).

The $\gamma\delta$ T cells are unique types of immune cells 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 GVHD. Additionally, the authors' laboratory has developed a serum-free *ex vivo* expansion of V γ 9V δ 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 (NCT03533816, NCT03790072) 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. 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. The authors have previously shown that our expansion protocol consistently yields a final $\gamma\delta$ T-cell product of 85.5% V γ 9V δ 2+ and only 7% V δ 1 $\gamma\delta$ T cells [9]. Thus, the vast majority of the authors' cellular product consists of

donors, $P = 0.003$, unpaired *t*-test). (F) Representative flow plots from a longer time course cytotoxicity assay with $\gamma\delta$ T cells and Nomo-1 cells at a 1:2 E:T ratio. After 24 h of treatment with bortezomib or vehicle control, GFP⁺ Nomo-1 cells were incubated with day 11 *ex vivo*-expanded $\gamma\delta$ T cells for 4 h, 24 h and 48 h. The percentage of remaining live GFP⁺ cells (GFP⁺/7-AAD⁻) was determined at each time point to establish whether bortezomib treatment increased the cytotoxicity of GFP⁺ Nomo-1 cells compared with vehicle control-treated cells. Bortezomib-treated Nomo-1 cells incubated with $\gamma\delta$ T cells had a significantly lower percentage of live cells after 24 h and 48 h compared with vehicle control-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. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

Figure 6. Bortezomib significantly increases expression of ULBP2/5/6 in T-ALL cell lines after 24 h 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 with 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 with PBMCs ($n = 3$, $P > 0.05$). (B) RNA sequencing data from primary T-ALL patient samples were obtained from the Pieters ($n = 92$) and Meijerink ($n = 124$) datasets in the R2 Genomics Analysis and Visualization Platform and showed elevated gene expression in ULBP2 compared with gene expression in normal PBMCs ($P < 0.0001$). (C,D) T-ALL Jurkat and MOLT4 cell lines were treated with vehicle control or increasing doses of bortezomib for 48 h. Live cells were gated on 7-AAD⁻/Annexin V⁻ and analyzed for changes in NKG2D ligand surface expression at various time points and concentrations (mean relative MFI, $n = 3$). Surface expression of ULBP2/5/6 in both cell lines peaked between 24 h and 48 h 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-h 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 h and 24 h at all doses. 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). mRNA, messenger RNA; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

anti-tumor $\gamma\delta$ T cells. Although it is known that $\gamma\delta$ T cells can potentially polarize toward 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- β and IL-15 for FoxP3⁺ $\gamma\delta$ Treg and IL-6, IL-1 β and TGF- β for $\gamma\delta$ T17 [41–43]. Since the authors' expansion protocol consists only of stimulation with IL-2 and zoledronate, we do not expect a significant portion of $\gamma\delta$ T cells to transform into pro-tumorigenic subsets. However, the authors plan to evaluate this further while optimizing the development of our clinical cellular product. The authors' 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 that $\gamma\delta$ T cells may function best in combination with drugs that induce expression of stress antigens. In the current study, the authors show that the combination of $\gamma\delta$ T cells and bortezomib may be an effective strategy for targeting two cancer types, AML and T-ALL, with increased cytotoxicity seen even at low E:T ratios. Currently, ~1–100 million $\gamma\delta$ T cells per kg of body weight are used for each injection in clinical trials (NCT03790072, 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 this 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 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 $\gamma\delta$ TCR–ligand interactions through the $\gamma\delta$ TCR, DNAX accessory molecule-1, Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand. 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 $\gamma\delta$ 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 $\gamma\delta$ T-cell killing. There was also likely to have been a partial additive effect on killing from the bortezomib treatment. However, the authors only consider this to be beneficial as it increased the overall killing. These findings highlight the feasibility of $\gamma\delta$ T-cell immunotherapy in the setting of AML and T-ALL, where multiple and more frequent doses of $\gamma\delta$ T cells may be required to treat such aggressive malignancies.

The current dosing of bortezomib in adult cancer patients typically consists of biweekly intravenous or subcutaneous injections at 1.0 or 1.3 mg/m² for 2 weeks [34–36]. Peak bortezomib levels shortly after injection of the drug have been reported to be between 20.4 and 22.5 ng/mL versus 162 and 223 ng/mL for subcutaneous versus

Figure 7. Bortezomib increases cytotoxicity of $\gamma\delta$ T cells against T-ALL cell lines. (A) Representative flow plots from a 4-h cytotoxicity assay with $\gamma\delta$ T cells and Jurkat cells at a 1:1 E:T ratio. (B) Vehicle control- or bortezomib-treated Jurkat and MOLT-4 cells were incubated for 4 h with day 12 *ex vivo*-expanded $\gamma\delta$ T cells at E:T ratios of 1:4, 1:2, 1:1 and 2.5:1. The difference in target cell death between bortezomib-treated cells and vehicle control-treated cells was compared at each E:T ratio. Replicates represent $\gamma\delta$ T cells expanded from three different donors. Bortezomib-treated Jurkat cells had a significantly higher cytotoxicity (% dead target cells) compared with vehicle control-treated Jurkat cells at E:T ratios of 1:4 and 1:2 ($n = 3$, $P = 0.0054$ and 0.0448 , respectively). (C) MOLT-4 cells treated with bortezomib had a significantly higher percentage of dead target cells at E:T ratios of 1:4, 1:2 and 1:1 ($n = 3$, $P = 0.001$, 0.014 and 0.023 , respectively). (D,E) Blocking the NKG2D receptor on $\gamma\delta$ T cells significantly decreased $\gamma\delta$ T-cell-specific cytotoxicity against bortezomib-treated Jurkat and MOLT-4 cells when tested at an E:T ratio of 1:2 ($n = 3$ separate donors, $P < 0.0001$). (F) After 24 h of treatment with bortezomib or vehicle control, GFP+ Jurkat cells were incubated with day 11 *ex vivo*-expanded $\gamma\delta$ T cells for 4 h and 24 h at an E:T ratio of 1:4 or 1:20. The percentage of remaining live GFP+ cells (GFP+/7-AAD⁻) was determined at each time point to establish whether bortezomib treatment increased the cytotoxicity of GFP+ Jurkat cells compared with vehicle control-treated cells. Bortezomib-treated Jurkat cells had a significantly lower percentage of live cells after 24 h compared with vehicle control-treated cells at both E:T ratios ($n = 3$, $P = 0.011$ and 0.003 , respectively). Significance was determined using two-way ANOVA with Bonferroni's multiple comparisons test or unpaired *t*-test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns, not significant.

intravenous administration, respectively; however, previous pharmacokinetic studies have shown that the plasma concentration of bortezomib at 24 h after injection ranges from 1 nM to 5 nM, depending on the number of previous doses a subject has received [34–36]. Here the authors have shown that there are minimal negative effects on $\gamma\delta$ T-cell growth and viability at these low doses and no significant decrease in $\gamma\delta$ T-cell-mediated killing following exposure to bortezomib. A repeat assessment post-drug removal showed that there was no significant delayed effect from the bortezomib treatment on $\gamma\delta$ T-cell functionality. This is important, as the authors would expect to dose bortezomib repeatedly, as discussed earlier. Additionally, a previous study showed that 10 nM bortezomib had no significant effects on $\gamma\delta$ T-cell viability or expression of the degranulation marker CD107a [22]. The authors' data show that bortezomib is effective at increasing ULBP2/5/6 surface expression on AML and T-ALL cells, with peak expression around 18–24 h. Thus, an intravenous infusion of *ex vivo*-expanded $\gamma\delta$ T cells after 24 h of bortezomib treatment, when AML and T-ALL cells are still sensitive to enhanced $\gamma\delta$ 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 the potential for tumor cell escape by “selecting” for tumor cells that do not express the target antigen or adapt by down-regulating the antigen, making the cells resistant to CAR T cells [46–48]. The authors' approach does not rely on a single-antigen targeting system, but rather utilizes the intrinsic cytotoxic mechanisms of $\gamma\delta$ T cells, thereby decreasing the chances of tumor escape. The $\gamma\delta$ T cells also mediate tumor cell identification and lysis through their TCR, DNAX accessory molecule-1, Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand interactions, mechanisms that are independent of the NKG2D ligand/receptor mechanism enhanced with bortezomib treatment. The ability of $\gamma\delta$ 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 $\gamma\delta$ T cells and stress ligand-inducing drugs, such as bortezomib, to treat a variety of cancers.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: JYS, HTS, CBD and SSR. Acquisition of data: JYS, JTZ, REB, JAGH and SSR. Analysis and interpretation of data: JYS, JTZ, REB, JAGH, HTS, CBD and SSR. Drafting or revising the

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Supplementary materials

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