



A Bispecific Single-Domain Antibody Boosts Autologous V γ 9V δ 2-T Cell Responses Toward CD1d in Chronic Lymphocytic Leukemia

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ABSTRACT

Purpose: Although considerable progress has been made with autologous T cell–based therapy in B-cell malignancies, application in chronic lymphocytic leukemia (CLL) lags behind due to disappointing response rates as well as substantial toxicity that is of particular concern in the elderly CLL population. V γ 9V δ 2-T cells form a conserved T-cell subset with strong intrinsic immunotherapeutic potential, largely because of their capacity to be triggered by phosphoantigens that can be overproduced by CLL and other malignant cells. Specific activation of V γ 9V δ 2-T cells by a bispecific antibody may improve the efficacy and toxicity of autologous T-cell–based therapy in CLL.

Experimental Design: We evaluated CD1d expression in a cohort of 78 untreated patients with CLL and generated and functionally characterized a CD1d-specific V γ 9V δ 2-T cell engager based on single-domain antibodies (VHH).

Results: CD1d was expressed by CLL in the majority of patients, particularly in patients with advanced disease. The CD1d-specific V γ 9V δ 2-T cell engager induced robust activation and degranulation of V γ 9V δ 2-T cells, enabling V γ 9V δ 2-T cells from patients with CLL to lyse autologous leukemic cells at low effector-to-target ratios. Expression of CD1d on CLL cells is upregulated by *all-trans* retinoic acid, and sensitizes the malignant cells to bispecific VHH-induced lysis. Furthermore, we provide evidence that the V γ 9V δ 2-T cell receptor retains responsiveness to phosphoantigens when the bispecific VHH is bound, and aminobisphosphonates can therefore enhance bispecific V γ 9V δ 2-T cell engager–mediated tumor-specific killing.

Conclusions: Collectively, our data demonstrate the immunotherapeutic potential of this novel CD1d-specific V γ 9V δ 2-T cell engager in CLL.

Introduction

In recent years, the therapeutic arsenal for chronic lymphocytic leukemia (CLL) has substantially increased with the introduction of tyrosine kinase inhibitors, Bcl-2 inhibitors, and mAbs. However, the need for long-term or continuous treatment, resistance, and toxicity concerns highlight the need for novel treatment options (1, 2).

The curative responses observed after allogeneic stem cell transplantation in CLL indicate the therapeutic potential of T cells (3).

Recent attempts to exploit T cell–based therapies in CLL have focused on the development of chimeric antigen receptor (CAR) T cells, in which a diverse, non-antigen-specific pool of T cells is endowed with a CAR that recognizes a tumor-associated surface antigen. In contrast to the promising clinical responses observed with CAR T-cell therapy in acute lymphoblastic leukemia and diffuse large B-cell lymphomas, the response rate and response duration in CLL has been comparatively disappointing (4–6). Toxicity is another important concern with CAR T-cell therapy, particularly in the elderly CLL population (6, 7).

Selective activation of intrinsically tumor-responsive cytotoxic T lymphocytes represents an alternative approach that could generate a potent and focused antitumor response. V γ 9V δ 2-T cells form a conserved T-cell subset that can induce apoptosis in a wide range of malignant cells in a human leukocyte antigen–independent fashion (8–10). The V γ 9V δ 2-T cell receptor (TCR) senses butyrophilin (BTN) 2A1 in complex with BTN3A1 upon binding of phosphoantigens to the intracellular B30.2 domain of BTN3A1 (11, 12). Phosphoantigens are metabolites that accumulate upon dysregulation of the mevalonate pathway and are therefore upregulated during cellular stress, such as infection or malignant transformation, or through pharmacologic manipulation with aminobisphosphonates (ABP). In addition, natural killer (NK) receptors allow V γ 9V δ 2-T cells to recognize malignant cells via stress ligands, such as the NKG2D ligands MICA/B and ULBP4 (13–15). Following activation, V γ 9V δ 2-T cell functions include cytotoxicity, secretion of chemokines and proinflammatory cytokines, and antigen presentation (13, 16, 17).

These characteristics have led to clinical trials that aimed to exploit the antitumor potential of V γ 9V δ 2-T cells, including ABP-based approaches to induce *in vivo* V γ 9V δ 2-T cell proliferation (18–20). Alternative strategies employed adoptive transfer of *ex vivo* expanded

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Translational Relevance

This study describes the generation of a CD1d-specific T-cell engager that specifically triggers V γ 9V δ 2-T cells, a subset with potent antitumor properties. The identification of CD1d expression in the majority of patients with chronic lymphocytic leukemia (CLL) indicates the capacity of the bispecific antibody to leverage the immunotherapeutic potential of V γ 9V δ 2-T cells in CLL.

V γ 9V δ 2-T cells (21–23). With both strategies, objective responses were observed in hematologic malignancies, along with a limited toxicity profile, thus establishing the feasibility of V γ 9V δ 2-T cell therapy (19–22). However, these pilot studies demonstrated substantial variability in responses, indicating that novel strategies that more consistently trigger clinically relevant V γ 9V δ 2-T cell–mediated anti-tumor activity are needed.

We previously reported that V γ 9V δ 2-T cells are capable of recognizing and lysing patient CLL cells, although the function of V γ 9V δ 2-T cells from patients with CLL is suppressed, presumably by altered synapse formation between CLL cells and V γ 9V δ 2-T cells (9). Defective synapse formation also underlies the diminished cytotoxicity of CD8⁺ T cells of patients with CLL (24). Bispecific T-cell engagers represent a promising strategy for T cell–based therapy, and bispecific antibodies have recently been shown to overcome synapse formation impairments in patients with CLL (25–27). To selectively activate V γ 9V δ 2-T cells and direct them toward CLL cells, we aimed to generate a V γ 9V δ 2-T cell engager based on variable domains of camelid-derived heavy chain–only antibodies, also termed VHH. We have previously shown that a similar bispecific VHH directed against EGFR can induce efficient tumor lysis in a solid tumor setting *in vitro* and *in vivo* (28).

CD1d is an MHC class I-like molecule that endows antigen-presenting cells, including B cells, with the capacity to present endogenous or exogenous glycolipid antigens to type I NK T (NKT) cells and subsets of V δ 1-T cells (29, 30). Because of the surface expression of CD1d by several hematologic malignancies, including CLL, acute myeloid leukemia, marginal zone lymphoma, Hodgkin disease, and multiple myeloma, development of anti-CD1d antibody-based treatment has been suggested (31–34). Although reported data on the level of CD1d expression on CLL cells are inconsistent, higher CD1d expression levels were observed in patients with progressive or clinically advanced disease (35–40). Therefore, we aimed to characterize the expression of CD1d in patients with CLL with variable disease characteristics to evaluate its use as a target for antibody-based treatment.

Here, we analyzed CD1d expression in a cohort of 78 untreated patients with CLL, indicating that CD1d is a suitable target for bispecific V γ 9V δ 2-T cell engager–based treatment in CLL, specifically in patients with advanced disease. We describe the generation and functional evaluation of a CD1d-specific V γ 9V δ 2-T cell engaging VHH. Our data demonstrate that the bispecific V γ 9V δ 2-T cell engager can activate V γ 9V δ 2-T cells from healthy controls and patients with CLL, leading to CD1d-specific lysis. We provide evidence that V γ 9V δ 2-T cells remain phosphoantigen responsive in the presence of the bispecific V γ 9V δ 2-T cell engager, and that ABP accordingly enhances the antileukemic activity mediated by the bispecific V γ 9V δ 2-T cell engager. Together, our data indicate the capacity of this CD1d-specific bispecific antibody to leverage the immunotherapeutic potential of V γ 9V δ 2-T cells in CLL.

Materials and Methods

Patient and healthy donor material

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood (PB) samples and fine-needle lymph node biopsies from untreated patients with CLL or age-matched healthy control (HC) buffy coats from Sanquin Blood Supply and cryopreserved as described previously (Table 1; ref. 41). The presence of monoclonal B-cell lymphocytosis was excluded in HCs by CD5, CD19, κ , and λ immunophenotyping. Healthy B cells were obtained by CD19 selection (130-050-301) and monocytes were obtained by CD14 selection from HC PBMCs (130-050-201, both magnetic microbeads, Miltenyi Biotec).

The study was approved by the medical ethics committee at the Amsterdam UMC. Written informed consent from all subjects was obtained in accordance with the Declaration of Helsinki.

Cell lines

Culture medium was supplemented with 10% FCS (F7524), 0.05 mmol/L β -mercapto-ethanol (M6250, both Merck), 200 mmol/L L-glutamine (25030-123), and 10,000 U/mL penicillin/streptomycin (15140-122, both Thermo Fisher Scientific). The multiple myeloma cell line MM.1s [either wildtype (WT) or stably transduced with CD1d (kind gift from R. Groen, Amsterdam UMC, Vrije Universiteit, Amsterdam, the Netherlands)] mantle cell lymphoma cell line Jeko-1 (DSMZ) and T cell acute lymphoblastic leukemia cell line CCRF-CEM (ATCC) were cultured in RPMI1640 medium (52400-025, Thermo Fisher Scientific). NIH/3T3 fibroblasts (ATCC) stably transfected with CD40L were cultured in supplemented Iscove's Modified Dulbecco's Medium (12440-053, Thermo Fisher Scientific). Authentication of human cell lines was performed using short tandem repeat analysis (DC6531, Promega). All cell lines were used within 3 months of thawing and checked for *Mycoplasma* using PCR at least every 3 months.

V γ 9V δ 2-T cell lines were generated as described previously (28). In short, V δ 2⁺-T cells were isolated from HC PBMCs using FITC-conjugated anti-V δ 2 TCR (Supplementary Table) in combination with anti-mouse IgG microbeads (130-048-401, Miltenyi Biotec) and cultured weekly with irradiated feeder mix consisting of PBMCs from two HCs, JY cells, IL7 (10 U/mL, 207-IL025), IL15 (10 ng/mL, 247-ILB-25, R&D Systems), and PHA (50 ng/mL, R30852801, Thermo Fisher Scientific). Purity of V γ 9V δ 2-T cell lines was maintained at >90%. M1 and M2 macrophages were generated by isolation of CD14⁺ cells from HC PBMCs, followed by a 7-day culture period in the presence of GM-CSF (1,000 U/mL, M1) or M-CSF (50 ng/mL, M2). IFN γ (20 ng/mL) and LPS (100 ng/mL, M1) or IL4 (20 ng/mL, M2) was added during the last 2 days of culture.

Table 1. Patient characteristics.

N	78
Age in years	63 (39–89)
Sex, % female	37.2
IGHV, % M-CLL	54.3 (for 46 known)
Rai, % stage 0	47.2 (for 53 known)
ALC $\times 10^9/L$	67.5 (15.3–422)

Note: Data presented as percentage or median (range). Abbreviations: ALC, absolute leukocyte count; M-CLL, CLL with mutated immunoglobulin heavy chains.

Flow cytometry

Cells were stained with antibodies and viability dyes (Supplementary Table) and measured on an LSR Fortessa or FACS Canto cytometer (BD Biosciences). Samples were analyzed with Flowjo Mac v10. Cytofix/Cytoperm reagent was used for detection of intracellular cytokines (BD Biosciences). Relative CD1d expression is defined as geometric mean fluorescence intensity (MFI; CD1d stained)—geometric MFI (fluorescence minus one).

Generation, production, and purification of bispecific V γ 9V δ 2-T cell engager

The CD1d-specific VHH 1D7 (42) and the V γ 9V δ 2-TCR-specific VHH 5C8 (43), binding to the V δ 2 chain of the TCR, were previously generated. In short, VHHs were generated by llama immunization and identified by subsequent phage display and screening. To create the CD1d-V δ 2 bispecific VHH, VHH 5C8 (C-terminal) was joined to VHH 1D7 (N-terminal) with a Gly₄Ser linker. Bispecific VHH protein from this gene sequence was produced in mammalian HEK293E-253 cells by UPE (Utrecht, The Netherlands) and purified from the supernatant by sequential protein A-based selection and size exclusion using fast protein liquid chromatography (ÅKTAexplorer, GE Healthcare).

Binding of the bispecific V γ 9V δ 2-T cell engager

To assess binding, CD1d-transduced MM.1s or V γ 9V δ 2-T cell lines were incubated with the bispecific V γ 9V δ 2-T cell engager for 30 minutes at 37°C. Bound bispecific V γ 9V δ 2-T cell engager was detected by sequential incubation with rabbit-anti-llama and PE-conjugated goat-anti-rabbit antibodies for 20 minutes at 4°C (Supplementary Table).

Cytokine and degranulation assays

V γ 9V δ 2-T cell lines were incubated with the bispecific V γ 9V δ 2-T cell engager or medium control for 30 minutes at 37°C. Subsequently, V γ 9V δ 2-T cells were cocultured with Jeko-1 cells for 4 hours in a 1:1 ratio in the presence of Brefeldin A (10 μ g/mL; B7651, Sigma-Aldrich), GolgiStop (554724, BD Biosciences) and anti-CD107a (Supplementary Table).

In the assays with autologous V γ 9V δ 2-T cells, CLL PBMCs were partially depleted of CD19⁺ cells using magnetic beads (Miltenyi Biotec; after depletion \pm 50% of cells were CD19⁺) and then cultured overnight with the bispecific V γ 9V δ 2-T cell engager or medium control in the presence of Brefeldin A, GolgiStop, and anti-CD107a.

Cytotoxicity assays

For cytotoxicity assays, target cells were labeled with carboxy-fluorescein succinimidyl ester (CFSE; C1157, Thermo Fisher Scientific) or Cell Trace Violet (CTV; C34557, Thermo Fisher Scientific) and incubated with the bispecific V γ 9V δ 2-T cell engager or medium control for 30 minutes at 37°C. Target cells were then cocultured overnight with V γ 9V δ 2-T cell lines in a 1:1 ratio unless otherwise indicated. For monocyte cytotoxicity assays only, $\gamma\delta$ -T cells obtained by negative $\gamma\delta$ -T cell selection from CD14-depleted HC PBMCs were used as effector cells (130-092-892, Miltenyi Biotec). Viability was measured using 7-AAD (10-minute incubation at room temperature, 559925, BD Biosciences) for monocyte and macrophage assays or Mitotracker Orange (25-minute incubation at 37°C, M7510) and To-pro-3 (10-minute incubation at room temperature; T3605, both Thermo Fisher Scientific) for all other cytotoxicity assays.

If indicated, target cells were pretreated with 25 μ mol/L mevastatin (M2537, Sigma-Aldrich), 50 μ mol/L aminobisphosphonates (pamidronate, #12J08RD, TEVA Pharmachemie) or medium control for 2 hours.

If indicated, cells were cultured with the indicated concentrations of *all-trans* retinoic acid (ATRA; R2625, Sigma-Aldrich) for 48 hours unless otherwise indicated. ATRA was washed away prior to cytotoxicity assays.

If indicated, target cells were preincubated with 5 μ g/mL anti-CD1d mAb (clone 51.1, 350304, BioLegend) and/or 10 μ g/mL anti-BTN3A1 mAb (clone 103.2, Creative Biolabs) for 10 minutes.

For cytotoxicity assays performed with patient-derived V γ 9V δ 2-T cells, CD3⁺ cells were enriched from patient PBMC by magnetic bead selection (130-050-101, Miltenyi Biotec; \geq 90% purity). Total PBMCs were preincubated with the bispecific V γ 9V δ 2-T cell engager or medium control for 30 minutes at 37°C and cultured overnight with purified CD3⁺ cells from the same patient in a 5:1 or 20:1 (CD3⁺:PBMC) ratio. Viable cells were then quantified by fluorescently-labeled antibodies and viability dyes in combination with counting beads (01-1234-42, Thermo Fisher Scientific).

Expansion assays

For expansion assays, PBMCs from patients with CLL were enriched for T cells by depletion of CD19⁺ CLL cells. After attachment of irradiated (30 Gy) CD40L-expressing fibroblasts to culture plates, T cell-enriched PBMC (\leq 10% CD19⁺) and purified CD19⁺ (\geq 90% CD19⁺) PBMC fractions were added in a 2:1 (CD19⁻:CD19⁺) ratio. Cells were cultured in the presence of 50 IU/mL IL2 (200-02, Peprotech) or 50 IU/mL IL2 and 50 nmol/L bispecific V γ 9V δ 2-T cell engager for 1 week.

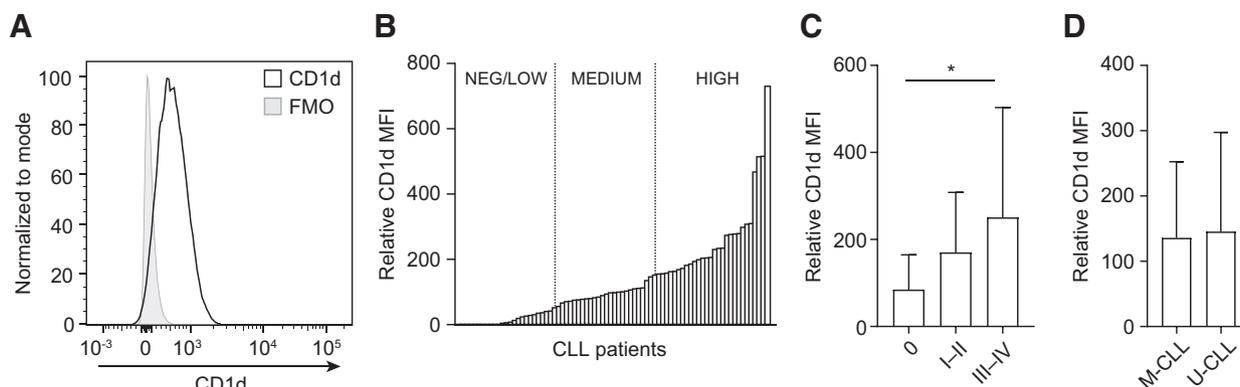
Statistical analyses

Specific lysis was calculated as: (% cell death in treated cells) – (% cell death in untreated cells)/(% viable cells in untreated cells) \times 100. Data were analyzed using paired *t* test, unpaired *t* test, one-way ANOVA (followed by Dunnett *post hoc* test or Holm-Šidák's *post hoc* test), two-way ANOVA (followed by Tukey, Šidák, or Dunnett *post hoc* test as appropriate), Friedman test, Pearson correlation analysis, linear regression, or nonlinear regression analysis as appropriate and as indicated with significance set at *P* < 0.05 using GraphPad Prism v7.

Results

The majority of patients with CLL express CD1d on the leukemic cell surface

To assess the suitability of CD1d as a target for bispecific V γ 9V δ 2-T cell engager-based treatment in CLL, we analyzed CD1d surface expression on CLL cells in a cohort of 78 untreated patients. Within each patient sample, CD1d was expressed homogeneously (Fig. 1A). However, the expression of CD1d was highly variable across the cohort (median relative MFI 131.2 \pm 135.1; Fig. 1B). We categorized the cohort into three groups, that each contained approximately one-third of the cohort, based on negative or low (relative MFI <50), medium (relative MFI >50 and <150), or high (relative MFI >150) CD1d levels. Among eight healthy donors tested, CD1d expression on CD5⁻CD19⁺ B cells was 58.6 \pm 21.7 (mean \pm SD). Within the PB and lymph nodes of patients with CLL, CD1d expression was also lower on nonleukemic B cells versus CLL cells (Supplementary Fig. S1A).

**Figure 1.**

CD1d expression on CLL cells. CD1d expression was measured on CD5⁺CD19⁺ lymphocytes from untreated patients with CLL and corrected for background fluorescence using fluorescence minus one (FMO) samples of the same patient material. **A**, Representative example histogram of CD1d expression on CLL cells. **B**, CD1d expression in the total cohort; each bar represents an individual patient ($n = 78$). The dotted lines indicate the boundaries between CLL with CD1d^{NEG/LOW} (relative MFI < 50), CD1d^{MEDIUM} (relative MFI > 50 and < 150), and CD1d^{HIGH} (relative MFI > 150) expression. CD1d expression according to Rai stage (Rai 0: $n = 25$, Rai I-II: $n = 22$, Rai III-IV: $n = 6$; **C**) and IGHV status (M-CLL: $n = 26$, U-CLL: $n = 21$; **D**) for those patients with data available. Data are presented as mean (bars) and SD (error bars). *, $P < 0.05$. (**C**: one-way ANOVA followed by Holm-Šidák's *post hoc* test; **D**: unpaired *t* test).

CD1d expression was higher on CLL cells from patients with advanced stage disease according to Rai classification (**Fig. 1C**). There was no difference in CD1d levels between patients with mutated (M-CLL) or unmutated (U-CLL) immunoglobulin heavy chain (IGHV) genes (**Fig. 1D**).

Type I NKT cells as well as subsets of $\gamma\delta$ -T cells recognize antigens in a CD1d-dependent manner (44). The frequency of type I NKT cells in patients with CLL was low (<0.1% of T cells; Supplementary Fig. S1B). We could not confirm previously reported data that suggested a correlation between CD1d expression and the percentage of type I NKT cells in patients with CLL (35, 40), nor did the expression of CD1d correlate with the percentage of V γ 9V δ 2-T cells in these patients (Supplementary Fig. S1B and S1C).

In summary, CD1d is expressed on the surface of leukemic cells from approximately two-thirds of patients with CLL, particularly in patients with advanced disease.

Bispecific anti-CD1d-V δ 2 VHH induces effector responses of V γ 9V δ 2-T cells

Next, we constructed a bispecific anti-CD1d V γ 9V δ 2-T cell engager by joining the previously generated CD1d-specific VHH 1D7 and the V δ 2-specific VHH 5C8 with a Gly₄Ser linker (43).

We first confirmed that binding to CD1d and V γ 9V δ 2-T cells is retained in the bispecific format. The bispecific V γ 9V δ 2-T cell engager bound with high apparent affinity to cultured V γ 9V δ 2-T cells (apparent K_D 0.36 nmol/L, 95% confidence interval 0.18–0.71 nmol/L; **Fig. 2A** and **B**) and to CD1d-transfected MM.1s cells (apparent K_D 0.40 nmol/L, 95% confidence interval 0.27–0.60 nmol/L; **Fig. 2C**).

Subsequently, we tested whether the bispecific V γ 9V δ 2-T cell engager could induce V γ 9V δ 2-T cell activation. For this purpose, V γ 9V δ 2-T cells were cocultured with Jeko-1 cells, a malignant B-cell line that naturally expresses CD1d at moderate levels (Supplementary Fig. S2). V γ 9V δ 2-T cells did not appreciably produce IFN γ or degranulate in response to exposure to target cells or the bispecific V γ 9V δ 2-T cell engager alone, but this was dose dependently enhanced in cultures containing both Jeko-1 cells and the

bispecific V γ 9V δ 2-T cell engager (**Fig. 2D**). The half-maximum activating effect on V γ 9V δ 2-T cells was reached around 3 pmol/L [EC₅₀ and 95% confidence interval IFN γ : 2.6 pmol/L (1.2–10.0), TNF: 3.5 pmol/L (1.4–7.0), CD107a: 3.1 pmol/L (1.3–5.2); **Fig. 2E**].

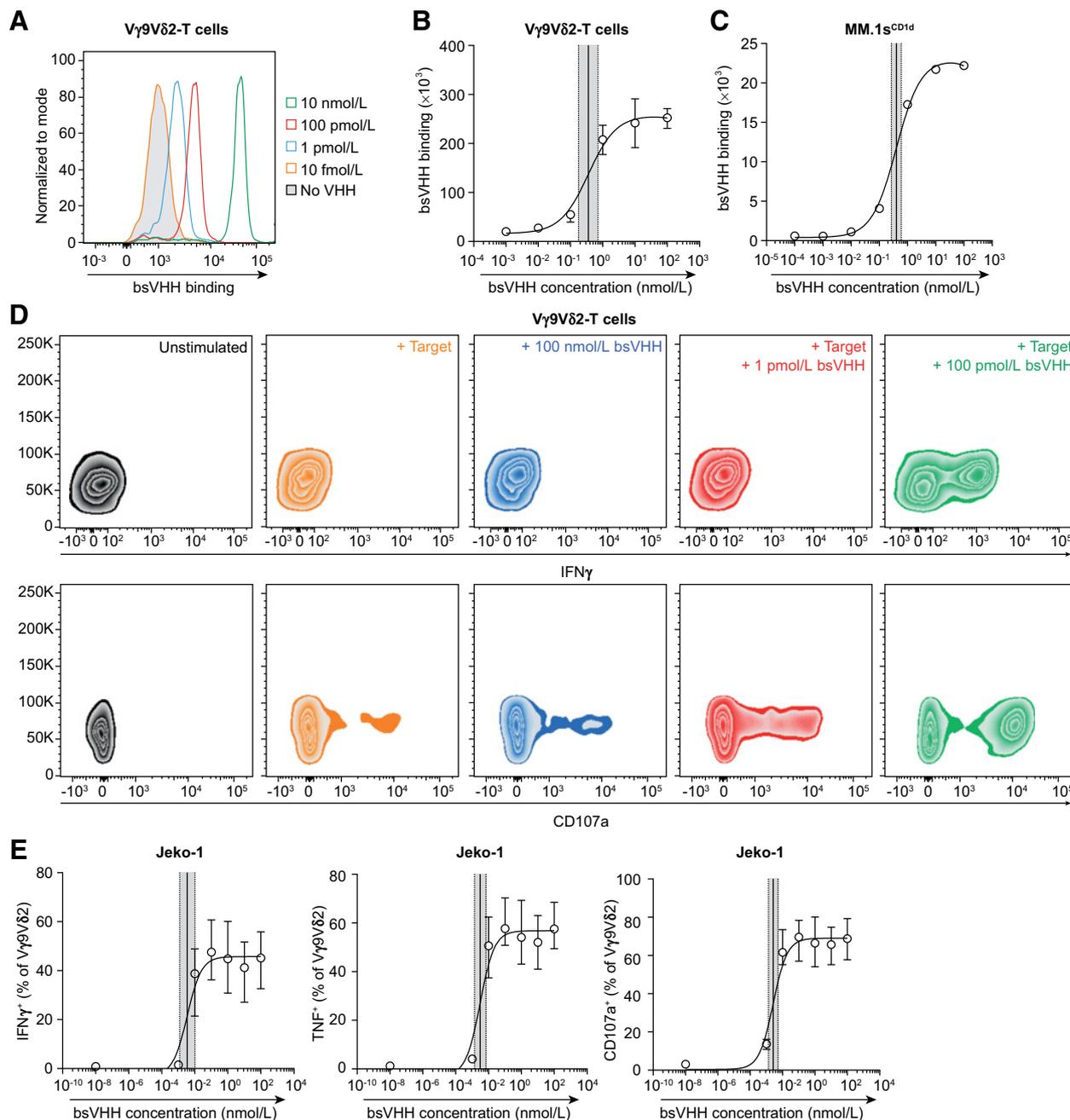
In conclusion, we generated a bispecific anti-CD1d-V δ 2 VHH that elicits target-dependent V γ 9V δ 2-T cell activation at low picomolar concentrations.

The CD1d-specific V γ 9V δ 2-T cell engager promotes CD1d-dependent cytotoxicity

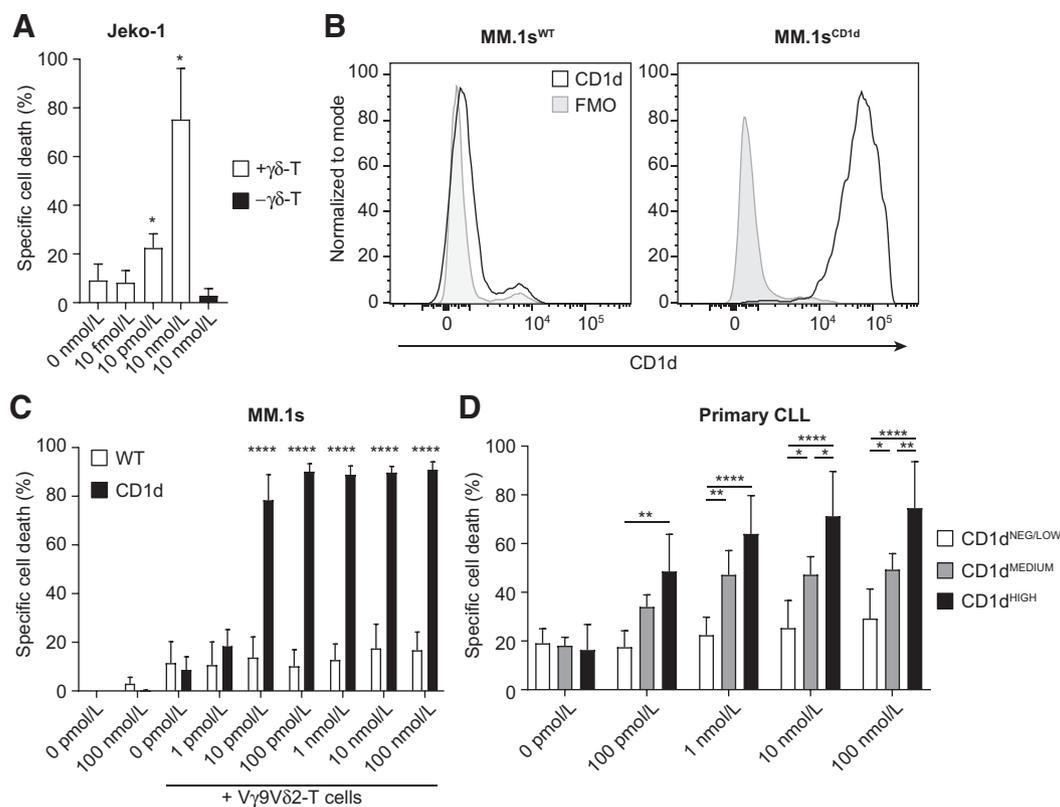
We then evaluated whether the activation of V γ 9V δ 2-T cells also led to lysis of CD1d⁺ tumor cells. Less than 10% of Jeko-1 cells were lysed during overnight coculture with V γ 9V δ 2-T cells alone. The bispecific V γ 9V δ 2-T cell engager enhanced target lysis in a dose-dependent manner, lysing 75.2% \pm 21.0 of the Jeko-1 cells in the presence of 10 nmol/L bispecific V γ 9V δ 2-T cell engager (**Fig. 3A**). The bispecific V γ 9V δ 2-T cell engager did not induce target cell lysis in the absence of V γ 9V δ 2-T cells.

Next, we analyzed the specificity of bispecific V γ 9V δ 2-T cell engager-induced cytotoxicity using CD1d-transduced and WT CD1d-negative MM.1s cells (**Fig. 3B**). When the MM.1s cells were exposed to V γ 9V δ 2-T cells alone, less than 20% lysis of both the WT and CD1d⁺ cells was observed during overnight coculture (**Fig. 3C**). The bispecific V γ 9V δ 2-T cell engager strongly increased lysis of CD1d⁺ cells but not of CD1d⁻ cells, demonstrating that the V γ 9V δ 2-T cell-mediated cytotoxicity triggered by the bispecific V γ 9V δ 2-T cell engager is CD1d specific.

We then tested the efficacy of the bispecific V γ 9V δ 2-T cell engager against primary CLL cells in samples with variable CD1d expression levels. Again, a minority of CLL target cells was killed when exposed to V γ 9V δ 2-T cells alone after overnight coculture (**Fig. 3D**). The bispecific V γ 9V δ 2-T cell engager significantly enhanced cytotoxicity against CLL cells with medium or high CD1d expression, but not with negative/low CD1d expression. Furthermore, CD1d^{HIGH} CLL cells were more susceptible to bispecific V γ 9V δ 2-T cell engager-induced cell death than CD1d^{MEDIUM} CLL cells, as 100 nmol/L bispecific V γ 9V δ 2-T cell engager-induced lysis

**Figure 2.**

The bispecific anti-CD1d-V δ 2 VHH activates V γ 9V δ 2-T cells. **A–C**, Binding of the bispecific VHH to healthy donor-derived V γ 9V δ 2-T cells and CD1d-transfected MM.1s cells by flow cytometric detection of anti-llama antibodies. **A**, Representative histograms of binding to V γ 9V δ 2-T cells. Summary of binding to V γ 9V δ 2-T cells (**B**) and CD1d-transfected MM.1s cells (**C**) for three experiments. **D** and **E**, Cytokine production and degranulation of V γ 9V δ 2-T cells in the presence of the bispecific VHH, brefeldin, monensin, and anti-CD107a as measured by flow cytometry. **D**, Healthy donor-derived V γ 9V δ 2-T cells were cultured with medium control, Jeko-1 cells (1:1 ratio), bispecific VHH (100 nmol/L), Jeko-1 cells and bispecific VHH (1:1 ratio, 10 pmol/L), or Jeko-1 cells and bispecific VHH (1:1 ratio, 100 pmol/L). Representative plots for three experiments. **E**, Healthy donor-derived V γ 9V δ 2-T cells were cultured with Jeko-1 cells (1:1 ratio) and the indicated concentrations of bispecific VHH ($n = 3$). Symbols and error bars represent mean and range; vertical line and shaded area represent half-maximal binding capacity (apparent K_D ; **B** and **C**) or EC_{50} and 95% confidence interval (**E**). (**B**, **C**, **E**: nonlinear regression analysis).

**Figure 3.**

The bispecific anti-CD1d-V δ 2 VHH induces lysis of malignant B cells. Lysis of target cells after overnight culture with healthy donor-derived V γ 9V δ 2-T cells in a 1:1 E:T ratio in the presence of the indicated concentrations of the bispecific VHH. **A**, Specific lysis of Jeko-1 cell line ($n = 3$). **B**, CD1d expression on WT or CD1d-transfected MM.1s cell line [black histogram: CD1d-stained, gray-filled histogram: fluorescence minus one (FMO) control]. **C**, Specific lysis of WT or CD1d-transfected MM.1s cell line ($n = 4$). **D**, Specific lysis of primary CLL cells with negative/low, medium, or high CD1d expression ($n = 4$ /group). Specific lysis is calculated by correcting for background cell death in condition without V γ 9V δ 2-T cells. Data represent mean and SD. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. (**A**: one-way ANOVA followed by Dunnett *post hoc* test compared with untreated condition, **C**: two-way ANOVA followed by Šidák's *post hoc* test comparing WT vs. CD1d for each concentration, **D**: two-way ANOVA followed by Tukey *post hoc* test comparing each CD1d group for each concentration).

in $49.3\% \pm 6.6$ of CD1d^{MEDIUM} cells versus $74.5\% \pm 19.1$ of CD1d^{HIGH} cells ($P = 0.0083$).

Taken together, the bispecific V γ 9V δ 2-T cell engager potently enhances V γ 9V δ 2-T cell-mediated cytotoxicity against tumor cells in a CD1d-dependent manner.

The bispecific V γ 9V δ 2-T cell engager induces lysis of leukemic cells by autologous V γ 9V δ 2-T cells

Because V γ 9V δ 2-T cells from patients with CLL are functionally suppressed (9, 45), we assessed whether CLL patient-derived V γ 9V δ 2-T cells could be activated by the bispecific V γ 9V δ 2-T cell engager. V γ 9V δ 2-T cells present in the PBMC fraction of patients with CLL upregulated the activation marker CD25 on average 20.8-fold upon overnight culture with the bispecific V γ 9V δ 2-T cell engager, to a level that was comparable with and not significantly different from that observed when PBMC were cultured with ABP (20.5-fold; Fig. 4A).

The bispecific V γ 9V δ 2-T cell engager also induced production of IFN γ and TNF by the patient-derived V γ 9V δ 2-T cells (Fig. 4B) and in addition triggered V γ 9V δ 2-T cell degranulation, comparable with ABP (Fig. 4C).

Next, we evaluated whether the induction of V γ 9V δ 2-T cell effector responses by the bispecific V γ 9V δ 2-T cell engager would

also enable lysis of autologous leukemic cells. For this purpose, we enriched for V γ 9V δ 2-T cells by culturing magnetically isolated CD3⁺ cells ($\geq 90\%$ purity) together with full PBMCs from the same donor in a CD3:PBMC ratio of 5:1 ($\pm 1:20$ V δ 2:CLL) or 20:1 ($\pm 1:5$ V δ 2:CLL) in the presence or absence of the bispecific V γ 9V δ 2-T cell engager. Patient-derived V γ 9V δ 2-T cells were capable of inducing cell death in autologous CLL cells, with a higher V γ 9V δ 2-T cell:tumor ratio leading to more leukemic cell death (Fig. 4D).

We then determined whether the bispecific V γ 9V δ 2-T cell engager could also promote enrichment of V γ 9V δ 2-T cells when added to PBMCs from patients with CLL enriched for T cells by CD19 magnetic isolation (2:1 CD19⁻:CD19⁺ ratio). After 1 week of culture in the presence of 50 IU/mL IL2, the percentage of V γ 9V δ 2-T cells within the T-cell fraction (baseline $1.2\% \pm 1.0$) increased in all eight samples tested and was 3.1-fold higher with the bispecific VHH ($5.6\% \pm 4.7$) than without ($1.8\% \pm 1.5$, $P = 0.0353$; Supplementary Fig. S3).

Together, these data indicate that the bispecific V γ 9V δ 2-T cell engager is capable of activating CLL patient-derived V γ 9V δ 2-T cells and thereby enables autologous tumor lysis at relatively low effector to target (E:T) ratios.

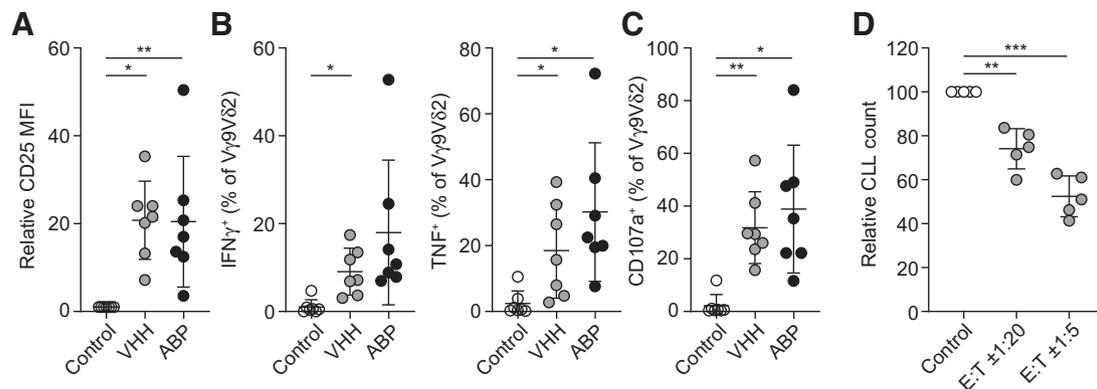


Figure 4.

The bispecific anti-CD1d-V δ 2 VHH activates V γ 9V δ 2-T cells from patients with CLL and induces autologous tumor lysis. **A-C**, Activation of and cytokine production and degranulation by patient-derived V γ 9V δ 2-T cells. PBMCs from patients with CLL were enriched for T cells by depletion of CD19⁺ CLL cells and then cultured overnight with CD19⁺ CLL cells in a 1:1 ratio with bispecific VHH (50 nmol/L) or medium control in the presence of brefeldin, monensin, and anti-CD107a to measure CD25 expression (**A**), cytokine production (**B**), and degranulation (**C**) by flow cytometry ($n = 7$). **D**, Lysis of primary CLL cells by autologous V γ 9V δ 2-T cells. CD3⁺ cells were isolated from CLL PBMCs and cultured with total CLL PBMCs in a low (5:1 CD3:PBMC, which equals \pm 1:20 effector V δ 2:target CLL cells) or higher (20:1 CD3:PBMC, which equals \pm 1:5 effector V δ 2:target CLL cells) ratio with bispecific VHH (10 nmol/L) or medium control. Living CLL cells were quantified by flow cytometry using counting beads ($n = 5$). Data are presented as mean and SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (**A-D**): one-way ANOVA followed by Dunnett *post hoc* test compared with medium control).

Phosphoantigen recognition by V γ 9V δ 2-T cells alters bispecific V γ 9V δ 2-T cell engager-induced target lysis

Next, we set out to assess whether residual recognition of the phosphoantigen-BTN3A1 complex by the V γ 9V δ 2-TCR contributes to the level of V γ 9V δ 2-T cell activation in the presence of the bispecific V γ 9V δ 2-T cell engager. The mevalonate metabolic pathway, of which the endogenous phosphoantigen isopentenyl pyrophosphate is an intermediate, is often overactive in malignant cells. The mevalonate pathway can be blocked by ABP resulting in intracellular isopentenyl pyrophosphate accumulation and subsequently in increased recognition by V γ 9V δ 2-T cells. We therefore tested whether ABP pretreatment of target cells could enhance bispecific V γ 9V δ 2-T cell engager-induced cytotoxicity against tumor cells.

Jeko-1 cells were more sensitive to V γ 9V δ 2-T cell-mediated cytotoxicity following pretreatment with the ABP pamidronate (**Fig. 5A**). In contrast, pretreatment with mevastatin, which reduces intracellular phosphoantigen levels, led to a reduction in lysis. A similar pattern was observed in the presence of the bispecific V γ 9V δ 2-T cell engager, where pamidronate pretreatment led to an increase in lysis of Jeko-1 cells, whilst mevastatin decreased bispecific V γ 9V δ 2-T cell engager-induced lysis. Together, this indicates that the V γ 9V δ 2-TCR can still detect phosphoantigens through sensing changes in the conformation of BTN3A1 when the bispecific V γ 9V δ 2-T cell engager is bound to the TCR.

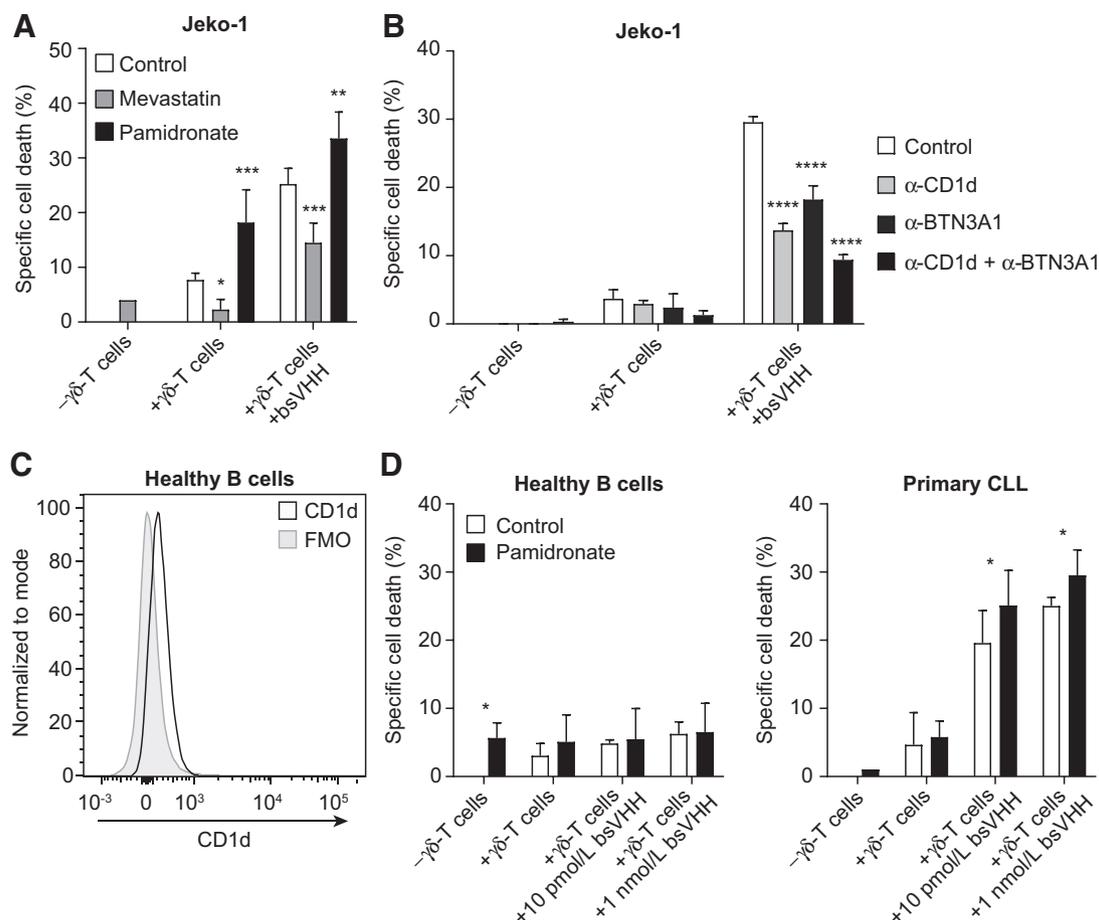
To further confirm the ability to recognize BTN3A1 in the presence of the bispecific V γ 9V δ 2-T cell engager and to assess the relative contribution of bispecific V γ 9V δ 2-T cell engager-induced versus phosphoantigen/BTN3A1-V γ 9V δ 2 TCR-dependent target cell recognition, we evaluated the effect of blocking BTN3A1 and CD1d. The bispecific V γ 9V δ 2-T cell engager-induced cytotoxicity of Jeko-1 cells ($29.6\% \pm 0.9$, lysis without bispecific V γ 9V δ 2-T cell engager $3.7\% \pm 1.3$) was reduced 11.4% on average by a BTN3A1-blocking antibody [clone 103.2 (11), $18.2\% \pm 2.0$; 29.7% relative reduction in bispecific V γ 9V δ 2-T cell engager-induced cytotoxicity], confirming a remaining role for ligand recognition by the V γ 9V δ 2-TCR (**Fig. 5B**). Bispecific V γ 9V δ 2-T cell engager-induced lysis also decreased on average

15.9% with a CD1d-blocking antibody (to $13.7\% \pm 1.0$; relative reduction 47.1%), and declined by 20.2% when BTN3A1 was simultaneously blocked (to $9.4\% \pm 0.8$; relative reduction 63.7%).

Healthy B cells express low levels of CD1d (**Fig. 5C**). We therefore assessed the bispecific V γ 9V δ 2-T cell engager-induced lysis of healthy B cells, and evaluated whether ABP had differential effects on healthy versus leukemic B cells. For this purpose, we performed mixed coculture experiments, in which B cells from healthy donors and CLL cells were pretreated with pamidronate for 2 hours and subsequently cultured for 6 hours with V γ 9V δ 2-T cells from a third donor in a 1:1:1 ratio in the presence of the bispecific V γ 9V δ 2-T cell engager. Lysis of healthy B cells by V γ 9V δ 2-T cells was minimal, irrespective of ABP pretreatment and the presence of the V γ 9V δ 2-T cell engager (10 pmol/L bispecific VHH; no ABP: $4.8\% \pm 0.6$, with ABP: $5.4\% \pm 4.5$; **Fig. 5D**). In contrast, while V γ 9V δ 2-T cell lysis of CLL cells was triggered by the bispecific V γ 9V δ 2-T cell engager, CLL cells were also more prone to V γ 9V δ 2-T cell lysis in the presence of the bispecific V γ 9V δ 2-T cell engager following pamidronate pretreatment (10 pmol/L bispecific V γ 9V δ 2-T cell engager; no ABP: $19.6\% \pm 4.8$, with ABP: $25.1\% \pm 5.2$), indicating that ABP treatment can enhance tumor specificity.

In addition to B cells, several other cell types including monocytes and macrophages have also been reported to express CD1d (46, 47). Similar to healthy B cells, lysis of monocytes from healthy individuals was negligible, and contrasted with lysis of the positive control CD1d⁺ T-ALL CCRF-CEM cell line (Supplementary Fig. S4A). CD1d expression was confirmed on M1 and M2 macrophages present in lymph nodes of patients with CLL and M1 and M2 macrophages generated *in vitro*, with somewhat lower CD1d expression on M2 macrophages (Supplementary Fig. S4B and S4C). Of interest, while overall the bispecific V γ 9V δ 2-T cell engager induced limited lysis of *in vitro* differentiated M1 and M2 macrophages (Supplementary Fig. S4D), lysis of (tumor-promoting) M2 macrophages was more pronounced.

Taken together, these data indicate that the V γ 9V δ 2-TCR of V γ 9V δ 2-T cells bound to bispecific V γ 9V δ 2-T cell engager can still recognize phosphoantigen-induced conformational changes in

**Figure 5.**

V γ 9V δ 2-T cell activation by the bispecific VHH can be modified through modulation of phosphoantigen recognition. **A**, Specific lysis of Jeko-1 cells after pretreatment with mevastatin or pamidronate. Jeko-1 cells were treated with 25 μ mol/L mevastatin, 50 μ mol/L pamidronate, or medium control for 2 hours and cultured for 6 hours with healthy donor-derived V γ 9V δ 2-T cells in a 1:2 ratio with 100 nmol/L bispecific VHH ($n = 3$). **B**, Specific lysis of Jeko-1 cells after blockade of CD1d or BTN3A1. Jeko-1 cells were incubated with an anti-CD1d antibody (5 μ g/mL), anti-BTN3A1 antibody (10 μ g/mL), anti-CD1d and anti-BTN3A1 antibodies, or medium control for 10 minutes. Target cells were then cultured overnight with healthy donor-derived V γ 9V δ 2-T cells in a 1:3 ratio with 10 pmol/L bispecific VHH ($n = 3$). **C**, CD1d expression on B cells from healthy donor PBMCs. **D**, Specific lysis of healthy B cells and CLL cells after pretreatment with ABP. Healthy B cells (isolated from healthy donor PBMCs, CFSE-labeled) and CLL cells (isolated from CLL PBMCs, CTV-labeled) were mixed in 1:1 ratio and pretreated with 50 μ mol/L pamidronate or medium control for 2 hours. Target cells were then cultured for 6 hours with healthy donor-derived V γ 9V δ 2-T cells in a 1:1 (V γ 9V δ 2-T cell: B cell: CLL cell) ratio with the indicated concentrations of bispecific VHH ($n = 4$). Specific lysis is calculated by correcting for background cell death in condition without V γ 9V δ 2-T cells. Data represent mean and SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**A**): two-way ANOVA followed by Dunnett *post hoc* test comparing control vs. each pretreatment; **B**: one-way ANOVA followed by Dunnett *post hoc* test compared with medium control; **D**: two-way ANOVA followed by Šidák *post hoc* test comparing untreated vs. ABP pretreated).

BTN3A1 on target cells, allowing for the potential of a further increase in bispecific V γ 9V δ 2-T cell engager-induced tumor cell lysis in the presence of ABP.

ATRA upregulates CD1d expression and enhances bispecific V γ 9V δ 2-T cell engager-induced cytotoxicity

ATRA can increase the expression of CD1d on B cells (48). Since the capacity of the bispecific V γ 9V δ 2-T cell engager to induce target lysis was dependent on CD1d expression levels, we hypothesized that ATRA-induced upregulation of CD1d could increase cytotoxicity. ATRA caused upregulation of CD1d expression on Jeko-1 cells, which was detectable with 10 pmol/L of ATRA and further increased with higher doses (Fig. 6A). Peak CD1d expression occurred after 2 days of culture (Fig. 6B).

ATRA did not induce cell death directly, nor did ATRA pretreatment increase V γ 9V δ 2-T cell-mediated cytotoxicity in the absence of the bispecific V γ 9V δ 2-T cell engager (Fig. 6C and D). However, Jeko-1 cells that were pretreated with ATRA were more sensitive to bispecific V γ 9V δ 2-T cell engager-induced cell death than control Jeko-1 cells (Fig. 6D).

Next, we tested whether ATRA would also increase CD1d expression on primary CLL cells. CD1d levels increased 2.6-fold with 1 nmol/L of ATRA and the highest expression occurred with 100 nmol/L ATRA (average 3.7-fold increase; Fig. 6E). There was considerable variation in the observed increase in CD1d expression upon exposure to ATRA (relative MFI after 100 nmol/L ATRA: 504.6 ± 366.0), which could partially be explained by baseline CD1d expression ($r^2 = 0.4025$, $P = 0.0062$; Supplementary Fig. S5).

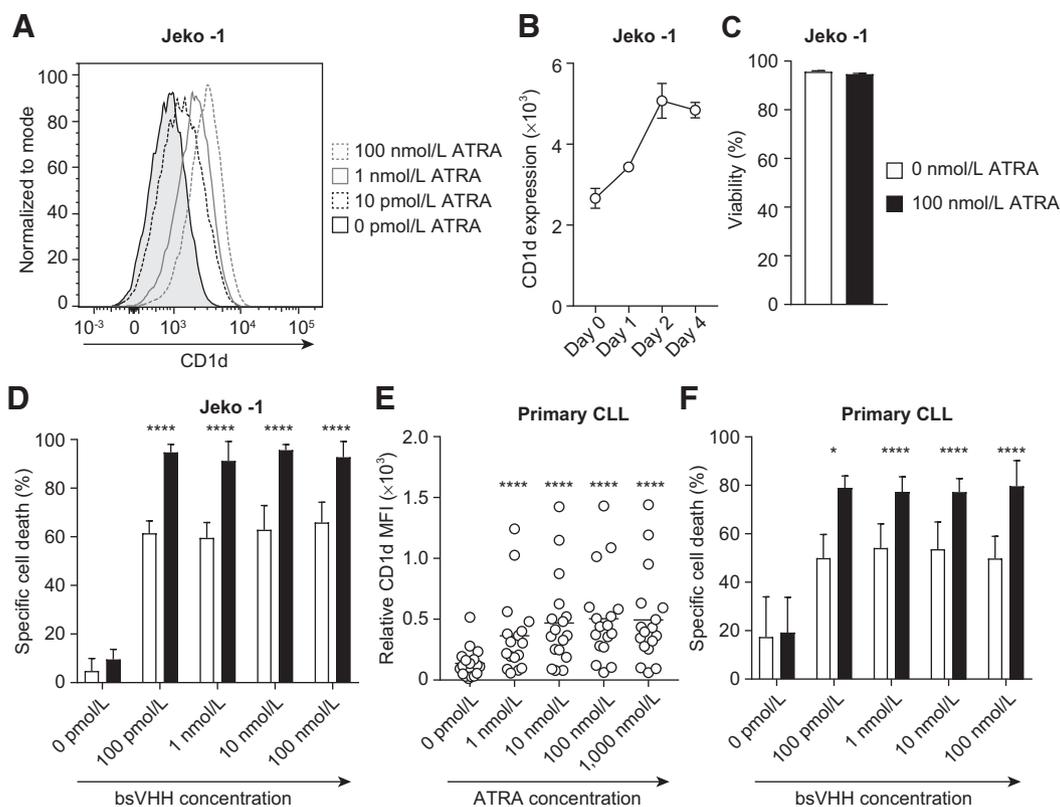


Figure 6.

ATRA enhances bispecific VHH-induced killing by upregulation of CD1d expression. **A**, CD1d expression on Jeko-1 cells after 48 hours of ATRA treatment. **B**, Time course of CD1d expression upon ATRA treatment ($n = 3$). **C**, Viability of Jeko-1 cells after 48 hours of treatment with 100 nmol/L ATRA ($n = 4$). **D**, Lysis of Jeko-1 cells after pretreatment with ATRA. Jeko-1 cells were treated with 100 nmol/L ATRA or medium control for 48 hours and cultured overnight with healthy donor-derived V γ 9V δ 2-T cells in a 1:2 E:T ratio in the presence of the indicated concentrations of the bispecific VHH ($n = 6$). **E**, CD1d expression on primary CLL cells after 48 hours of different concentrations of ATRA or medium control treatment ($n = 17$). **F**, Lysis of CLL cells after pretreatment with ATRA. Primary CLL cells were treated with 100 nmol/L ATRA or medium control for 48 hours and cultured overnight with healthy donor-derived V γ 9V δ 2-T cells in a 1:1 E:T ratio in the presence of the indicated concentrations of the bispecific VHH ($n = 6$). Specific lysis is calculated by correcting for background cell death in condition without V γ 9V δ 2-T cells. Data represent mean and SD. *, $P < 0.05$; **, $P < 0.001$; ****, $P < 0.0001$. (**C**: paired t test; **D**, **F**: two-way ANOVA followed by Šidák's *post hoc* test comparing control vs. ATRA-treated for each concentration; **E**: Friedman test followed by Dunnett's *post hoc* test compared with medium control).

As with Jeko-1 cells, ATRA pretreatment of primary CLL cells did not affect their sensitivity to V γ 9V δ 2-T cell-mediated lysis in the absence of the bispecific V γ 9V δ 2-T cell engager (Fig. 6F), but did sensitize primary CLL cells to bispecific V γ 9V δ 2-T cell engager-mediated cell death.

In summary, by upregulating CD1d expression ATRA increased the susceptibility of tumor cells toward V γ 9V δ 2-T cell-mediated lysis induced by the bispecific V γ 9V δ 2-T cell engager.

Discussion

V γ 9V δ 2-T cells hold strong immunotherapeutic potential, due to their potent intrinsic capacity to recognize and lyse malignant transformed cells (8–10). Although prior studies have demonstrated incidental clinical benefit and safety of V γ 9V δ 2-T cell-based therapy, the observed response rates and durations thereof have so far been unsatisfactory (18–23). The antitumor potential of V γ 9V δ 2-T cells can possibly be more efficiently employed by directing the V γ 9V δ 2-T cells toward the malignant cells.

We set out to create a novel bispecific antibody that would guide V γ 9V δ 2-T cells toward CLL cells, thereby enhancing their antileuke-

mic activity. For this purpose, we explored the possibility of targeting CD1d in CLL. In CLL, CD1d has primarily been studied in the context of its function as a glycolipid antigen-presenting molecule to type I NKT cells. Although all studies reported expression of CD1d on the surface of CLL cells, the expression levels are inconsistent between studies (35–40, 49). To confirm its suitability as target for bispecific V γ 9V δ 2-T cell engager therapy, we therefore analyzed CD1d expression in a large cohort of 78 untreated patients with CLL. Our analysis demonstrated homogenous surface CD1d expression within each sample, yet substantial interpatient variability with approximately two-thirds of patients expressing CD1d. Thus, CD1d appears to be a feasible target in the majority of patients with CLL, but selection based on CD1d expression prior to treatment may be necessary. In accordance with previous reports, we find that IGHV-status does not predict CD1d expression (49, 50) and CD1d expression increases according to disease stage (35), with the latter indicating that high CD1d levels may correspond with a higher treatment need.

We utilized the CD1d-specific VHH 1D7 (42) and coupled it to the V δ 2-specific VHH 5C8 (43) to create a bispecific V γ 9V δ 2-T cell engager. 1D7 has a high apparent affinity for CD1d, and 1D7 does not affect DC maturation or recognition of glycolipid-loaded CD1d by

type I NKT cells (42). A variable yet sometimes substantial part of $V\gamma 9^+$ cells use a non- $V\delta 2$ chain (51). Because >95% of $V\delta 2^+$ cells coexpress the $V\gamma 9$ chain (52), we used a $V\delta 2$ -specific VHH to specifically activate phosphoantigen-responsive $\gamma\delta$ -T cells. Prior characterization demonstrated that 5C8 can conditionally activate $V\gamma 9V\delta 2$ -T cells (43). VHHs offer advantages over conventional antibodies in terms of size (12–15 kDa), stability, and low immunogenicity (53, 54). The small size makes it possible to use VHHs in multivalent constructs while retaining tissue penetration capacity. In the presence of $CD1d^+$ tumor cells, this construct resulted in activation of $V\gamma 9V\delta 2$ -T cells that produced IFN γ and TNF. These proinflammatory cytokines are involved in the capacity of $V\gamma 9V\delta 2$ -T cells to induce maturation of DCs and other effector cells (55). Importantly, the bispecific $V\gamma 9V\delta 2$ -T cell engager was also capable of inducing activation and cytokine production by patient-derived $V\gamma 9V\delta 2$ -T cells in the presence of CLL cells, despite the fact that we and others have previously shown that $V\gamma 9V\delta 2$ -T cells from patients with CLL are functionally repressed (9, 45). Because *ex vivo* culture leads to alleviation of $V\gamma 9V\delta 2$ -T cell dysfunction the bispecific $V\gamma 9V\delta 2$ -T cell engager could be combined with such an approach (9, 56, 57).

The bispecific $V\gamma 9V\delta 2$ -T cell engager induced $V\gamma 9V\delta 2$ -T cell degranulation, allowing granzyme and perforin secretion (13). Correspondingly, the bispecific $V\gamma 9V\delta 2$ -T cell engager resulted in efficient and $CD1d$ -dependent tumor lysis by $V\gamma 9V\delta 2$ -T cells from healthy controls.

Notably, CLL patient-derived $V\gamma 9V\delta 2$ -T cells were also capable of lysing autologous leukemic cells at low effector to target ratios even in short-term assays. $V\gamma 9V\delta 2$ -T cells from a subset of patients with CLL were reported to inefficiently proliferate in response to phosphoantigens (45). Previously, we identified ratios of $V\delta 2$ -T cells to CLL cells in untreated patients with CLL of 1:50 in PB and 1:326 in lymph nodes (56). We report that the bispecific $V\gamma 9V\delta 2$ -T cell engager can induce enrichment of CLL patient-derived $V\gamma 9V\delta 2$ -T cells. The therapeutic efficacy of the bispecific $V\gamma 9V\delta 2$ -T cell engager approach could therefore potentially be further enhanced by increasing E:T ratios via adoptive transfer of $V\gamma 9V\delta 2$ -T cells, bispecific VHH-mediated expansion or prior debulking of CLL cells.

By using both a $BTN3A1$ -blocking antibody as well as pharmacologic agents known to modify intracellular phosphoantigen levels, we demonstrate that the level of activation of $V\gamma 9V\delta 2$ -T cells triggered by the bispecific $V\gamma 9V\delta 2$ -T cell engager is also affected by residual recognition of phosphoantigen- $BTN3A1$ molecules by the $V\gamma 9V\delta 2$ -TCR (11). Because the mevalonate pathway is critical for cholesterol synthesis, mevastatin could theoretically affect synapse formation through interfering with cholesterol synthesis. The fact that $CD1d$ blockade reduces target lysis more than $BTN3A1$ blockade indicates that target lysis predominantly depends on direct bispecific $V\gamma 9V\delta 2$ -T cell engager-induced triggering of the $V\gamma 9V\delta 2$ -TCR. In addition, we noted that combined blockade of $CD1d$ and $BTN3A1$ did not completely abrogate bispecific $V\gamma 9V\delta 2$ -T cell engager-induced lysis, which could result from incomplete blockade or indicate a role for additional receptors, such as $NKG2D$. Clinical trials have demonstrated that clinically relevant $V\gamma 9V\delta 2$ -T cell proliferation and objective antitumor responses can occur with systemic application of ABP in combination with IL2, both in solid (18, 58) and hematologic (10, 19) malignancies. The combination with ABP could therefore potentially improve the antitumor effects of the bispecific $V\gamma 9V\delta 2$ -T cell engager. Although it is known that $CD1d$ can also be expressed by several nonmalignant cell types, including B cells and monocytes (46, 47, 59), the risk of on-target off-tumor toxicity may be limited by the observed

preferential activity of the bispecific $V\gamma 9V\delta 2$ -T cell engager against malignant cells compared with, for example, healthy B cells and monocytes. This may be due to higher expression levels of $CD1d$ on CLL cells, but could also be related to a generally higher activity of the mevalonate pathway with higher phosphoantigen levels in malignant cells.

Our data indicate that the bispecific $V\gamma 9V\delta 2$ -T cell engager-induced lysis was dependent on the $CD1d$ expression levels, which triggered us to investigate methods to upregulate $CD1d$. As ATRA was reported to increase $CD1d$ levels on B cells, as well as CLL cells, through binding to an ATRA-responsive element in the $CD1d$ promoter (38, 48), we studied the effect of ATRA on $CD1d$ expression and susceptibility to bispecific $V\gamma 9V\delta 2$ -T cell engager-induced lysis on CLL cells. Our data confirm that ATRA increases $CD1d$ expression on CLL cells and in addition show that the higher $CD1d$ expression upon ATRA treatment translated into an enhancement of bispecific $V\gamma 9V\delta 2$ -T cell engager-induced lysis of leukemic cells. The numerous known biological effects of ATRA (60), make it conceivable that mechanisms other than $CD1d$ upregulation contribute to the increased lysis. For instance, ATRA increases the apoptotic sensitivity of CLL cells to chemotherapy (61) and can enhance the levels of $NKG2D$ ligands such as $MICA/B$ on malignant cells (62, 63), which could facilitate target recognition by $V\gamma 9V\delta 2$ -T cells. However, the fact that leukemic cell death did not increase substantially in the absence of $V\gamma 9V\delta 2$ -T cells or the bispecific $V\gamma 9V\delta 2$ -T cell engager strongly suggests that $CD1d$ upregulation is the dominant mechanism. Because ample experience in the context of promyelocytic leukemia has demonstrated that ATRA is tolerable in concentrations exceeding those used in this study (64), ATRA-induced enhancement of bispecific $V\gamma 9V\delta 2$ -T cell engager-induced killing could be clinically exploited. A recent report provided evidence that pharmacological manipulation of epigenetic regulation can further enhance ATRA-induced $CD1d$ upregulation in CLL (65), suggesting that an $EZH2$ inhibitor in combination with ATRA could perhaps be used to further enhance bispecific $V\gamma 9V\delta 2$ -T cell engager-induced cytotoxicity toward leukemic cells.

In conclusion, our study confirms that $CD1d$ is a feasible target for bispecific $V\gamma 9V\delta 2$ -T cell engager therapy in the majority of CLL patients, particularly in those with advanced disease. We have generated a bispecific $CD1d$ -directed $V\gamma 9V\delta 2$ -T cell engager that induces cytokine production and degranulation by $V\gamma 9V\delta 2$ -T cells from healthy controls and patients with CLL. In addition, this bispecific $V\gamma 9V\delta 2$ -T cell engager enables $V\gamma 9V\delta 2$ -T cell enrichment and potent $CD1d$ -dependent tumor lysis. ATRA provides a method to increase $CD1d$ expression on CLL cells, allowing enhanced bispecific $V\gamma 9V\delta 2$ -T cell engager-induced cytotoxicity of leukemic cells. Moreover, the antitumor effects and tumor specificity of the $V\gamma 9V\delta 2$ -T cell engager can be boosted by ABP. The $CD1d$ -specific $V\gamma 9V\delta 2$ -T cell engager is a promising candidate for CLL treatment and the expression of $CD1d$ by myeloid (31) and other lymphoid (32–34) malignancies indicates that the utility of this $CD1d$ -directed $V\gamma 9V\delta 2$ -T cell engaging strategy might extend beyond CLL.

Authors' Disclosures

All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. I. de Weerd reported research funding from LAVA Therapeutics during the conduct of the study; in addition, I. de Weerd had a patent for Novel $CD40$ -binding antibodies pending and a patent for Novel bispecific antibodies for use in the treatment of hematologic malignancies pending. R. Lameris reported grants from LAVA Therapeutics during the conduct of the study; in addition,

R. Lameris had a patent for Single domain antibodies targeting CD1d issued to Lava Therapeutics and a patent for Dual acting CD1d immunoglobulin pending. P.W.H.I. Parren reported other from Lava Therapeutics during the conduct of the study; in addition, P.W.H.I. Parren had a patent for CD1d gamma-delta T cell engagers pending to LAVA Therapeutics and a patent for gamma-delta T cell engagers pending to LAVA Therapeutics. T.D. de Gruijl reported other from LAVA Therapeutics during the conduct of the study and other from TILT Biotherapeutics, DCPrime, and Macrophage Pharma and grants from Idera Pharmaceuticals outside the submitted work; in addition, T.D. de Gruijl had a patent for Novel CD40-binding antibodies pending, a patent for Single domain antibodies targeting CD1d pending, a patent for Immunoglobulins binding human V γ 9V δ 2-TCRs pending, and a patent for Novel bispecific antibodies for use in the treatment of hematologic malignancies pending. A.P. Kater reported other from LAVA during the conduct of the study; grants, personal fees, and other from AbbVie; grants and other from Genentech/Roche, AstraZeneca, BMS; and grants from Celgene outside the submitted work; in addition, A.P. Kater had a patent for LAVA bispecific AB pending. H.J. van der Vliet reported grants and personal fees from Lava Therapeutics and grants from Worldwide Cancer Research and Cancer Center Amsterdam during the conduct of the study; in addition, H.J. van der Vliet had a patent for Single domain antibodies targeting CD1d issued and licensed to Lava Therapeutics, a patent for Immunoglobulins binding human V γ 9V δ 2-TCRs issued and licensed to Lava Therapeutics, a patent for Dual acting CD1d immunoglobulin pending, a patent for Novel CD40-binding antibodies pending, a patent for Novel bispecific antibodies for use in the treatment of hematological malignancies pending, and a patent for Treatment of cancer comprising administration of V γ 9V δ 2-TCR binding antibodies pending. No other disclosures were reported.

Authors' Contributions

I. de Weerd: Conceptualization, formal analysis, investigation, writing-original draft. **R. Lameris**: Formal analysis, investigation, writing-review and editing. **J.M. Ruben**: Formal analysis, investigation, writing-review and editing. **R. de Boer**: Formal analysis, investigation, writing-review and editing. **J. Kloosterman**: Formal analysis, investigation, writing-review and editing. **L.A. King**: Formal analysis, investigation, writing-review and editing. **M.-D. Levin**: Resources, writing-review and editing. **P.W.H.I. Parren**: Supervision, writing-review and editing. **T.D. de Gruijl**: Conceptualization, supervision, writing-review and editing. **A.P. Kater**: Conceptualization, supervision, writing-review and editing. **H.J. van der Vliet**: Conceptualization, supervision, writing-original draft.

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