# A bispecific single-domain antibody boosts autologous V gamma 9V delta 2-T cell responses toward CD1d in chronic lymphocytic leukemia

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# A Bispecific Single-Domain Antibody Boosts Autologous Vγ9Vδ2-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. Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cell engager induced robust activation and degranulation of Vγ9Vδ2-T cells, enabling Vγ9Vδ2-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 Vγ9Vδ2-T cell receptor retains responsiveness to phosphoantigens when the bispecific VHH is bound, and aminobisphosphonates can therefore enhance bispecific Vγ9Vδ2-T cell engager–mediated tumor-specific killing.

**Conclusions:** Collectively, our data demonstrate the immunotherapeutic potential of this novel CD1d-specific Vγ9Vδ2-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. Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cells to recognize malignant cells via stress ligands, such as the NKG2D ligands MICA/B and ULBP4 (13–15). Following activation, Vγ9Vδ2-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 Vγ9Vδ2-T cells, including ABP-based approaches to induce *in vivo* Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cells in CLL.

Vγ9Vδ2-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 Vγ9Vδ2-T cell therapy (19–22). However, these pilot studies demonstrated substantial variability in responses, indicating that novel strategies that more consistently trigger clinically relevant Vγ9Vδ2-T cell–mediated antitumor activity are needed.

We previously reported that Vγ9Vδ2-T cells are capable of recognizing and lysing patient CLL cells, although the function of Vγ9Vδ2-T cells from patients with CLL is suppressed, presumably by altered synapse formation between CLL cells and Vγ9Vδ2-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 Vγ9Vδ2-T cells and direct them toward CLL cells, we aimed to generate a Vγ9Vδ2-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 Vδ1-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 Vγ9Vδ2-T cell engager–based treatment in CLL, specifically in patients with advanced disease. We describe the generation and functional evaluation of a CD1d-specific Vγ9Vδ2-T cell engaging VHH. Our data demonstrate that the bispecific Vγ9Vδ2-T cell engager can activate Vγ9Vδ2-T cells from healthy controls and patients with CLL, leading to CD1d-specific lysis. We provide evidence that Vγ9Vδ2-T cells remain phosphoantigen responsive in the presence of the bispecific Vγ9Vδ2-T cell engager, and that ABP accordingly enhances the antileukemic activity mediated by the bispecific Vγ9Vδ2-T cell engager. Together, our data indicate the capacity of this CD1d-specific bispecific antibody to leverage the immunotherapeutic potential of Vγ9Vδ2-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.

Vγ9Vδ2-T cell lines were generated as described previously (28). In short, $Vδ2^+$-T cells were isolated from HC PBMCs using FITC-conjugated anti-Vδ2 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 Vγ9Vδ2-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). IFNγ (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 × $10^9$/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 Vγ9Vδ2-T cell engager

The CD1d-specific VHH 1D7 (42) and the Vγ9Vδ2-TCR–specific VHH 5C8 (43), binding to the Vδ2 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-Vδ2 bispecific VHH, VHH 5C8 (C-terminal) was joined to VHH 1D7 (N-terminal) with a $Gly_4Ser$ 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 (ÄKTAexplorer, GE Healthcare).

### Binding of the bispecific Vγ9Vδ2-T cell engager

To assess binding, CD1d-transduced MM.1s or Vγ9Vδ2-T cell lines were incubated with the bispecific Vγ9Vδ2-T cell engager for 30 minutes at 37°C. Bound bispecific Vγ9Vδ2-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

Vγ9Vδ2-T cell lines were incubated with the bispecific Vγ9Vδ2-T cell engager or medium control for 30 minutes at 37°C. Subsequently, Vγ9Vδ2-T cells were cocultured with Jeko-1 cells for 4 hours in a 1:1 ratio in the presence of Brefeldin A (10 μg/mL; B7651, Sigma-Aldrich), GolgiStop (554724, BD Biosciences) and anti-CD107a (Supplementary Table).

In the assays with autologous Vγ9Vδ2-T cells, CLL PBMCs were partially depleted of $CD19^+$ cells using magnetic beads (Miltenyi Biotec; after depletion ±50% of cells were $CD19^+$) and then cultured overnight with the bispecific Vγ9Vδ2-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 carboxyfluorescein succinimidyl ester (CFSE; C1157, Thermo Fisher Scientific) or Cell Trace Violet (CTV; C34557, Thermo Fisher Scientific) and incubated with the bispecific Vγ9Vδ2-T cell engager or medium control for 30 minutes at 37°C. Target cells were then cocultured overnight with Vγ9Vδ2-T cell lines in a 1:1 ratio unless otherwise indicated. For monocyte cytotoxicity assays only, γδ-T cells obtained by negative γδ-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 μmol/L mevastatin (M2537, Sigma-Aldrich), 50 μmol/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 5μg/mL anti-CD1d mAb (clone 51.1, 350304, BioLegend) and/or 10 μg/mL anti-BTN3A1 mAb (clone 103.2, Creative Biolabs) for 10 minutes.

For cytotoxicity assays performed with patient-derived Vγ9Vδ2-T cells, $CD3^+$ cells were enriched from patient PBMC by magnetic bead selection (130-050-101, Miltenyi Biotec; ≥90% purity). Total PBMCs were preincubated with the bispecific Vγ9Vδ2-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 (≤10% $CD19^+$) and purified $CD19^+$ (≥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 Vγ9Vδ2-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) × 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 Vγ9Vδ2-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 homogenously (**Fig. 1A**). However, the expression of CD1d was highly variable across the cohort (median relative MFI 131.2 ± 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 ± 21.7 (mean ± 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).

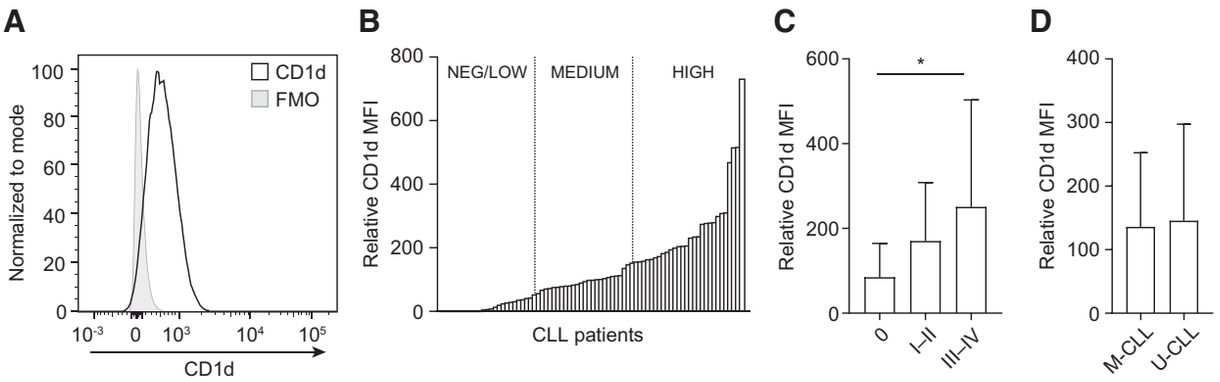


**Figure 1.**

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 γδ-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 Vγ9Vδ2-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-Vδ2 VHH induces effector responses of Vγ9Vδ2-T cells

Next, we constructed a bispecific anti-CD1d Vγ9Vδ2-T cell engager by joining the previously generated CD1d-specific VHH 1D7 and the Vδ2-specific VHH 5C8 with a $Gly_4Ser$ linker (43).

We first confirmed that binding to CD1d and Vγ9Vδ2-T cells is retained in the bispecific format. The bispecific Vγ9Vδ2-T cell engager bound with high apparent affinity to cultured Vγ9Vδ2-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 Vγ9Vδ2-T cell engager could induce Vγ9Vδ2-T cell activation. For this purpose, Vγ9Vδ2-T cells were cocultured with Jeko-1 cells, a malignant B-cell line that naturally expresses CD1d at moderate levels (Supplementary Fig. S2). Vγ9Vδ2-T cells did not appreciably produce IFNγ or degranulate in response to exposure to target cells or the bispecific Vγ9Vδ2-T cell engager alone, but this was dose dependently enhanced in cultures containing both Jeko-1 cells and the bispecific Vγ9Vδ2-T cell engager (**Fig. 2D**). The half-maximum activating effect on Vγ9Vδ2-T cells was reached around 3 pmol/L [$EC_{50}$ and 95% confidence interval IFNγ: 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-Vδ2 VHH that elicits target-dependent Vγ9Vδ2-T cell activation at low picomolar concentrations.

## The CD1d-specific Vγ9Vδ2-T cell engager promotes CD1d-dependent cytotoxicity

We then evaluated whether the activation of Vγ9Vδ2-T cells also led to lysis of $CD1d^+$ tumor cells. Less than 10% of Jeko-1 cells were lysed during overnight coculture with Vγ9Vδ2-T cells alone. The bispecific Vγ9Vδ2-T cell engager enhanced target lysis in a dose-dependent manner, lysing 75.2% ± 21.0 of the Jeko-1 cells in the presence of 10 nmol/L bispecific Vγ9Vδ2-T cell engager (**Fig. 3A**). The bispecific Vγ9Vδ2-T cell engager did not induce target cell lysis in the absence of Vγ9Vδ2-T cells.

Next, we analyzed the specificity of bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cells alone, less than 20% lysis of both the WT and $CD1d^+$ cells was observed during overnight coculture (**Fig. 3C**). The bispecific Vγ9Vδ2-T cell engager strongly increased lysis of $CD1d^+$ cells but not of $CD1d^-$ cells, demonstrating that the Vγ9Vδ2-T cell–mediated cytotoxicity triggered by the bispecific Vγ9Vδ2-T cell engager is CD1d specific.

We then tested the efficacy of the bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cells alone after overnight coculture (**Fig. 3D**). The bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cell engager–induced cell death than $CD1d^{MEDIUM}$ CLL cells, as 100 nmol/L bispecific Vγ9Vδ2-T cell engager–induced lysis

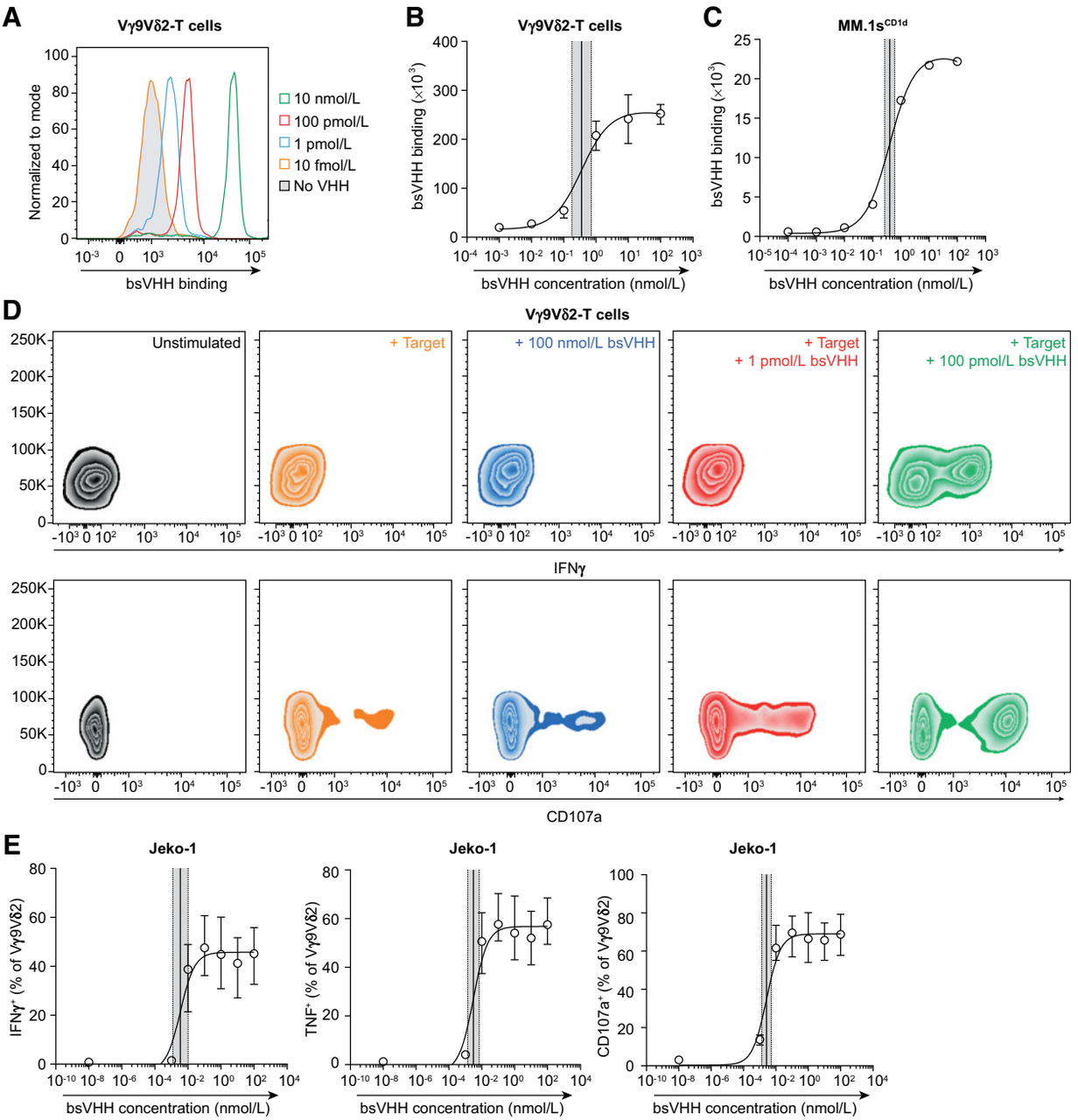


**Figure 2.**

The bispecific anti-CD1d-Vδ2 VHH activates Vγ9Vδ2-T cells. **A–C,** Binding of the bispecific VHH to healthy donor–derived Vγ9Vδ2-T cells and CD1d-transfected MM.1s cells by flow cytometric detection of anti-llama antibodies. **A,** Representative histograms of binding to Vγ9Vδ2-T cells. Summary of binding to Vγ9Vδ2-T cells (**B**) and CD1d-transfected MM.1s cells (**C**) for three experiments. **D** and **E,** Cytokine production and degranulation of Vγ9Vδ2-T cells in the presence of the bispecific VHH, brefeldin, monensin, and anti-CD107a as measured by flow cytometry. **D,** Healthy donor–derived Vγ9Vδ2-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 Vγ9Vδ2-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).

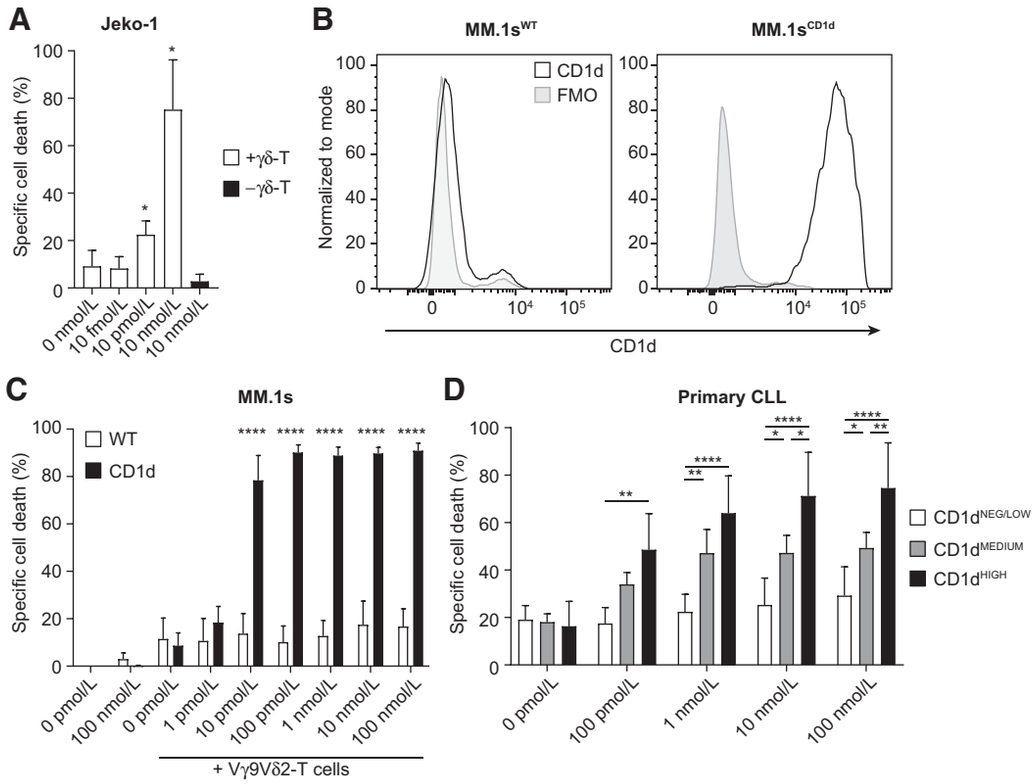


**Figure 3.**

The bispecific anti-CD1d-Vδ2 VHH induces lysis of malignant B cells. Lysis of target cells after overnight culture with healthy donor–derived Vγ9Vδ2-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 Vγ9Vδ2-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% ± 6.6 of $CD1d^{MEDIUM}$ cells versus 74.5% ± 19.1 of $CD1d^{HIGH}$ cells ($P = 0.0083$).

Taken together, the bispecific Vγ9Vδ2-T cell engager potently enhances Vγ9Vδ2-T cell–mediated cytotoxicity against tumor cells in a CD1d-dependent manner.

### The bispecific Vγ9Vδ2-T cell engager induces lysis of leukemic cells by autologous Vγ9Vδ2-T cells

Because Vγ9Vδ2-T cells from patients with CLL are functionally suppressed (9, 45), we assessed whether CLL patient-derived Vγ9Vδ2-T cells could be activated by the bispecific Vγ9Vδ2-T cell engager. Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cell engager also induced production of IFNγ and TNF by the patient-derived Vγ9Vδ2-T cells (**Fig. 4B**) and in addition triggered Vγ9Vδ2-T cell degranulation, comparable with ABP (**Fig. 4C**).

Next, we evaluated whether the induction of Vγ9Vδ2-T cell effector responses by the bispecific Vγ9Vδ2-T cell engager would also enable lysis of autologous leukemic cells. For this purpose, we enriched for Vγ9Vδ2-T cells by culturing magnetically isolated $CD3^+$ cells (≥90% purity) together with full PBMCs from the same donor in a CD3:PBMC ratio of 5:1 (±1:20 Vδ2:CLL) or 20:1 (±1:5 Vδ2:CLL) in the presence or absence of the bispecific Vγ9Vδ2-T cell engager. Patient-derived Vγ9Vδ2-T cells were capable of inducing cell death in autologous CLL cells, with a higher Vγ9Vδ2-T cell:tumor ratio leading to more leukemic cell death (**Fig. 4D**).

We then determined whether the bispecific Vγ9Vδ2-T cell engager could also promote enrichment of Vγ9Vδ2-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 Vγ9Vδ2-T cells within the T-cell fraction (baseline 1.2% ± 1.0) increased in all eight samples tested and was 3.1-fold higher with the bispecific VHH (5.6% ± 4.7) than without (1.8% ± 1.5, $P = 0.0353$; Supplementary Fig. S3).

Together, these data indicate that the bispecific Vγ9Vδ2-T cell engager is capable of activating CLL patient-derived Vγ9Vδ2-T cells and thereby enables autologous tumor lysis at relatively low effector to target (E:T) ratios.

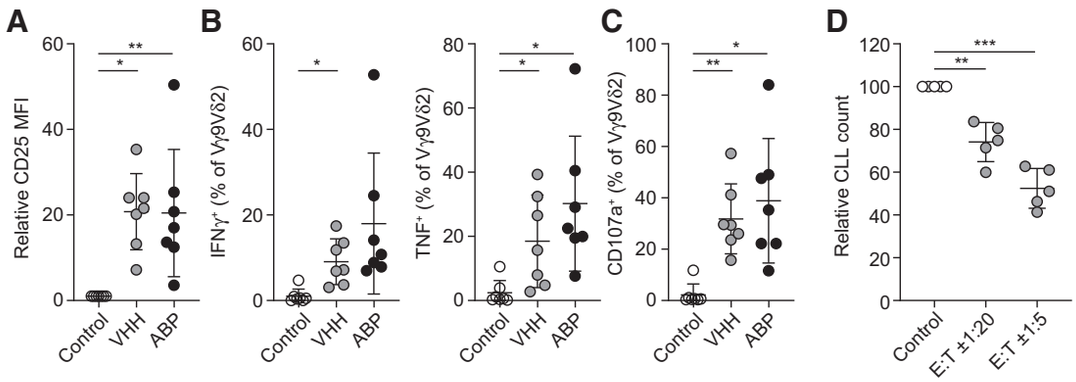


**Figure 4.**

The bispecific anti-CD1d-Vδ2 VHH activates Vγ9Vδ2-T cells from patients with CLL and induces autologous tumor lysis. **A–C,** Activation of and cytokine production and degranulation by patient-derived Vγ9Vδ2-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 Vγ9Vδ2-T cells. $CD3^+$ cells were isolated from CLL PBMCs and cultured with total CLL PBMCs in a low (5:1 CD3:PBMC, which equals ± 1:20 effector Vδ2:target CLL cells) or higher (20:1 CD3:PBMC, which equals ± 1:5 effector Vδ2: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 Vγ9Vδ2-T cells alters bispecific Vγ9Vδ2-T cell engager–induced target lysis

Next, we set out to assess whether residual recognition of the phosphoantigen-BTN3A1 complex by the Vγ9Vδ2-TCR contributes to the level of Vγ9Vδ2-T cell activation in the presence of the bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cells. We therefore tested whether ABP pretreatment of target cells could enhance bispecific Vγ9Vδ2-T cell engager–induced cytotoxicity against tumor cells.

Jeko-1 cells were more sensitive to Vγ9Vδ2-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 Vγ9Vδ2-T cell engager, where pamidronate pretreatment led to an increase in lysis of Jeko-1 cells, whilst mevastatin decreased bispecific Vγ9Vδ2-T cell engager–induced lysis. Together, this indicates that the Vγ9Vδ2-TCR can still detect phosphoantigens through sensing changes in the conformation of BTN3A1 when the bispecific Vγ9Vδ2-T cell engager is bound to the TCR.

To further confirm the ability to recognize BTN3A1 in the presence of the bispecific Vγ9Vδ2-T cell engager and to assess the relative contribution of bispecific Vγ9Vδ2-T cell engager–induced versus phosphoantigen/BTN3A1-Vγ9Vδ2 TCR-dependent target cell recognition, we evaluated the effect of blocking BTN3A1 and CD1d. The bispecific Vγ9Vδ2-T cell engager–induced cytotoxicity of Jeko-1 cells (29.6% ± 0.9, lysis without bispecific Vγ9Vδ2-T cell engager 3.7% ± 1.3) was reduced 11.4% on average by a BTN3A1-blocking antibody [clone 103.2 (11), 18.2% ± 2.0; 29.7% relative reduction in bispecific Vγ9Vδ2-T cell engager–induced cytotoxicity], confirming a remaining role for ligand recognition by the Vγ9Vδ2-TCR (**Fig. 5B**). Bispecific Vγ9Vδ2-T cell engager–induced lysis also decreased on average 15.9% with a CD1d-blocking antibody (to 13.7% ± 1.0; relative reduction 47.1%), and declined by 20.2% when BTN3A1 was simultaneously blocked (to 9.4% ± 0.8; relative reduction 63.7%).

Healthy B cells express low levels of CD1d (**Fig. 5C**). We therefore assessed the bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cells from a third donor in a 1:1:1 ratio in the presence of the bispecific Vγ9Vδ2-T cell engager. Lysis of healthy B cells by Vγ9Vδ2-T cells was minimal, irrespective of ABP pretreatment and the presence of the Vγ9Vδ2-T cell engager (10 pmol/L bispecific VHH; no ABP: 4.8% ± 0.6, with ABP: 5.4% ± 4.5; **Fig. 5D**). In contrast, while Vγ9Vδ2-T cell lysis of CLL cells was triggered by the bispecific Vγ9Vδ2-T cell engager, CLL cells were also more prone to Vγ9Vδ2-T cell lysis in the presence of the bispecific Vγ9Vδ2-T cell engager following pamidronate pretreatment (10 pmol/L bispecific Vγ9Vδ2-T cell engager; no ABP: 19.6% ± 4.8, with ABP: 25.1% ± 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 Vγ9Vδ2-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 Vγ9Vδ2-TCR of Vγ9Vδ2-T cells bound to bispecific Vγ9Vδ2-T cell engager can still recognize phosphoantigen-induced conformational changes in

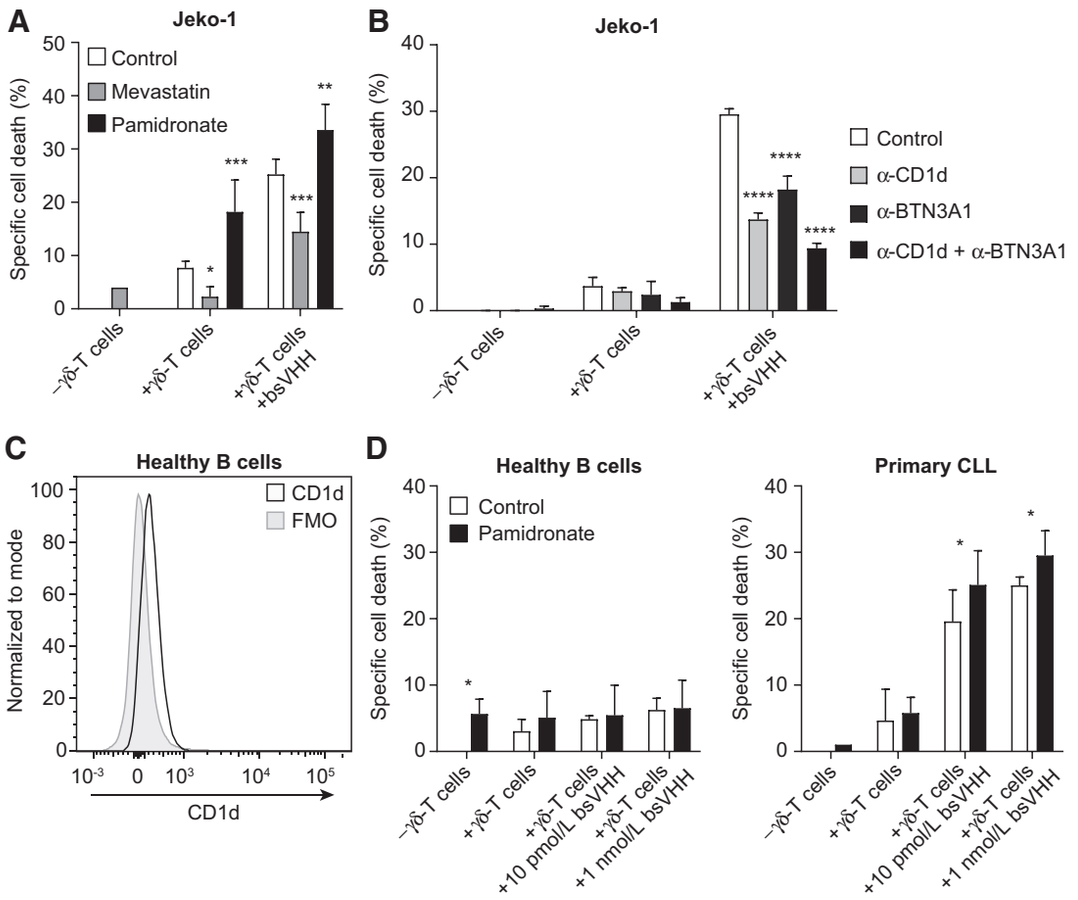


**Figure 5.**

Vγ9Vδ2-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 μmol/L mevastatin, 50 μmol/L pamidronate, or medium control for 2 hours and cultured for 6 hours with healthy donor-derived Vγ9Vδ2-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 μg/mL), anti-BTN3A1 antibody (10 μg/mL), anti-CD1d and anti-BTN3A1 antibodies, or medium control for 10 minutes. Target cells were then cultured overnight with healthy donor–derived Vγ9Vδ2-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 1:1:1 ratio and pretreated with 50 μmol/L pamidronate or medium control for 2 hours. Target cells were then cultured for 6 hours with healthy donor–derived Vγ9Vδ2-T cells in a 1:1:1 (Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cell engager–induced tumor cell lysis in the presence of ABP.

### ATRA upregulates CD1d expression and enhances bispecific Vγ9Vδ2-T cell engager–induced cytotoxicity

ATRA can increase the expression of CD1d on B cells (48). Since the capacity of the bispecific Vγ9Vδ2-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 Vγ9Vδ2-T cell–mediated cytotoxicity in the absence of the bispecific Vγ9Vδ2-T cell engager (**Fig. 6C** and **D**). However, Jeko-1 cells that were pretreated with ATRA were more sensitive to bispecific Vγ9Vδ2-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).

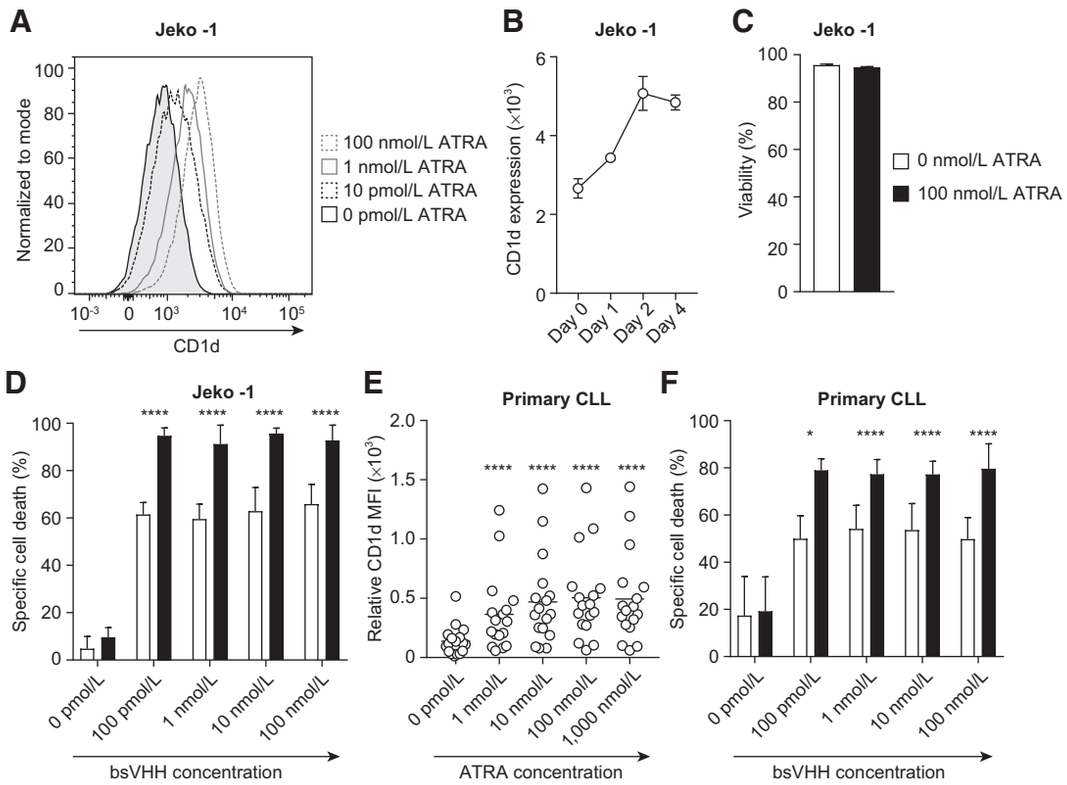


**Figure 6.**

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 curve 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 Vγ9Vδ2-T cells in a 1:2 E:T ratio in the presence of the indicated concentrations of the bispecific VHH ($n = 4$). **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 Vγ9Vδ2-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 Vγ9Vδ2-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 *post hoc* test compared with medium control).

As with Jeko-1 cells, ATRA pretreatment of primary CLL cells did not affect their sensitivity to Vγ9Vδ2-T cell–mediated lysis in the absence of the bispecific Vγ9Vδ2-T cell engager (**Fig. 6F**), but did sensitize primary CLL cells to bispecific Vγ9Vδ2-T cell engager–mediated cell death.

In summary, by upregulating CD1d expression ATRA increased the susceptibility of tumor cells toward Vγ9Vδ2-T cell–mediated lysis induced by the bispecific Vγ9Vδ2-T cell engager.

## Discussion

Vγ9Vδ2-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 Vγ9Vδ2-T cell–based therapy, the observed response rates and durations thereof have so far been unsatisfactory (18–23). The antitumor potential of Vγ9Vδ2-T cells can possibly be more efficiently employed by directing the Vγ9Vδ2-T cells toward the malignant cells.

We set out to create a novel bispecific antibody that would guide Vγ9Vδ2-T cells toward CLL cells, thereby enhancing their antileukemic 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 Vγ9Vδ2-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 Vδ2-specific VHH 5C8 (43) to create a bispecific Vγ9Vδ2-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 $V\gamma9^+$ cells use a non-Vδ2 chain (51). Because >95% of $V\delta2^+$ cells coexpress the Vγ9 chain (52), we used a Vδ2-specific VHH to specifically activate phosphoantigen-responsive γδ-T cells. Prior characterization demonstrated that 5C8 can conditionally activate Vγ9Vδ2-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 Vγ9Vδ2-T cells that produced IFNγ and TNF. These proinflammatory cytokines are involved in the capacity of Vγ9Vδ2-T cells to induce maturation of DCs and other effector cells (55). Importantly, the bispecific Vγ9Vδ2-T cell engager was also capable of inducing activation and cytokine production by patient-derived Vγ9Vδ2-T cells in the presence of CLL cells, despite the fact that we and others have previously shown that Vγ9Vδ2-T cells from patients with CLL are functionally repressed (9, 45). Because *ex vivo* culture leads to alleviation of Vγ9Vδ2-T cell dysfunction the bispecific Vγ9Vδ2-T cell engager could be combined with such an approach (9, 56, 57).

The bispecific Vγ9Vδ2-T cell engager induced Vγ9Vδ2-T cell degranulation, allowing granzyme and perforin secretion (13). Correspondingly, the bispecific Vγ9Vδ2-T cell engager resulted in efficient and CD1d-dependent tumor lysis by Vγ9Vδ2-T cells from healthy controls.

Notably, CLL patient-derived Vγ9Vδ2-T cells were also capable of lysing autologous leukemic cells at low effector to target ratios even in short-term assays. Vγ9Vδ2-T cells from a subset of patients with CLL were reported to inefficiently proliferate in response to phosphoantigens (45). Previously, we identified ratios of Vδ2-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 Vγ9Vδ2-T cell engager can induce enrichment of CLL patient-derived Vγ9Vδ2-T cells. The therapeutic efficacy of the bispecific Vγ9Vδ2-T cell engager approach could therefore potentially be further enhanced by increasing E:T ratios via adoptive transfer of Vγ9Vδ2-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 Vγ9Vδ2-T cells triggered by the bispecific Vγ9Vδ2-T cell engager is also affected by residual recognition of phosphoantigen-BTN3A1 molecules by the Vγ9Vδ2-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 Vγ9Vδ2-T cell engager–induced triggering of the Vγ9Vδ2-TCR. In addition, we noted that combined blockade of CD1d and BTN3A1 did not completely abrogate bispecific Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cells. However, the fact that leukemic cell death did not increase substantially in the absence of Vγ9Vδ2-T cells or the bispecific Vγ9Vδ2-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 Vγ9Vδ2-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 Vγ9Vδ2-T cell engager–induced cytotoxicity toward leukemic cells.

In conclusion, our study confirms that CD1d is a feasible target for bispecific Vγ9Vδ2-T cell engager therapy in the majority of CLL patients, particularly in those with advanced disease. We have generated a bispecific CD1d-directed Vγ9Vδ2-T cell engager that induces cytokine production and degranulation by Vγ9Vδ2-T cells from healthy controls and patients with CLL. In addition, this bispecific Vγ9Vδ2-T cell engager enables Vγ9Vδ2-T cell enrichment and potent CD1d-dependent tumor lysis. ATRA provides a method to increase CD1d expression on CLL cells, allowing enhanced bispecific Vγ9Vδ2-T cell engager–induced cytotoxicity of leukemic cells. Moreover, the antitumor effects and tumor specificity of the Vγ9Vδ2-T cell engager can be boosted by ABP. The CD1d-specific Vγ9Vδ2-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 Vγ9Vδ2-T cell engaging strategy might extend beyond CLL.

## Authors' Disclosures

I. de Weerdt reported research funding from LAVA Therapeutics during the conduct of the study; in addition, I. de Weerdt 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 Vγ9Vδ2-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, and 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 Vy9Vd2-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 Vγ9Vδ2-TCR binding antibodies pending. No disclosures were reported by the other authors.

## Authors' Contributions

**I. de Weerdt:** 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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