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The CD226-ERK1/2-LAMP1 pathway is an important mechanism for Vγ9Vδ2 T cell cytotoxicity against chemotherapy-resistant acute myeloid leukemia blasts and leukemia stem cells

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Abstract

Vγ9Vδ2 T cells are attractive effector cells for immunotherapy with potent cytotoxic activity against a variety of malignant cells. However, the effect of Vγ9Vδ2 T cells on chemotherapy-resistant acute myeloid leukemia (AML) blasts, especially highly refractory leukemia stem cells (LSCs) is still unknown. In this study, we investigated the effect of cytotoxicity of allogeneic Vγ9Vδ2 T cells on chemotherapy-resistant AML cell lines, as well as on primary AML blasts and LSCs obtained from refractory AML patients. The results indicated that Vγ9Vδ2 T cells can efficiently kill drug-resistant AML cell lines in vitro and in vivo, and the sensitivity of AML cells to Vγ9Vδ2 T cell–mediated cytotoxicity is not influenced by the sensitivity of AML cells to chemotherapy. We further found that Vγ9Vδ2 T cells exhibited a comparable effect of cytotoxicity against LSCs to primary AML blasts. More importantly, we revealed that the CD226–extracellular signal–regulatory kinase1/2 (ERK1/2)–lysosome-associated membrane protein 1 (LAMP1) pathway is an important mechanism for Vγ9Vδ2 T cell–induced cytotoxicity against AML cells. First, Vγ9Vδ2 T cells recognized AML cells by receptor-ligand interaction of CD226–Nectin-2, which then induced ERK1/2 phosphorylation in Vγ9Vδ2 T cells. Finally, triggering the movement of lytic granules toward AML cells induced cytolysis of AML cells. The expression level of Nectin-2 may be used as a novel marker to predict the susceptibility/resistance of AML cells to Vγ9Vδ2 T cell treatment.

Wu and Wang contributed equally to this work.

KEYWORDS
acute myeloid leukemia, CD226, extracellular signal–regulatory kinase1/2, lysosome-associated membrane protein 1, Vγ9Vδ2 T cell

1 | INTRODUCTION

Acute myeloid leukemia (AML), the most common form of acute leukemia in adults, remains a life-threatening disease and a challenge for clinical doctors. Despite significant advances in the treatment of AML, most patients with AML ultimately have a relapse and die from progressive disease, regardless of initial sensitivity to chemotherapy. Furthermore, prognosis in the elderly AML patients who account for the majority of newly diagnosed cases remains poor. Even with current treatments, as much as 70% of patients 65 years or older will die of their disease within 1 year of diagnosis.[1] Patients who are in complete remission generally receive consolidation treatment with high-dose cytarabine–based consolidation chemotherapy or allogeneic hematopoietic stem cell transplantation (allo-HSCT).[2-5] However, limited by comorbidities, advanced age or lack of suitable human leukocyte antigen (HLA)-matched donors, a large number of AML patients are not eligible for intensive consolidation chemotherapy or allo-HSCT.[6] Besides, by chemotherapy alone, it is extremely difficult to cure AML due to the presence of drug-resistant and highly refractory leukemia stem cells (LSCs). Although allo-HSCT offers the most effective antileukemic therapy and has curative potential in AML, enhanced nonrelapse mortality (NRM) may compromise that favorable effect. At the same time, relapse after transplantation is still a major clinical problem and is associated with particularly poor outcomes. Therefore, new AML therapeutic approaches are still highly desired to offer the promise of effective antileukemic activity with reduced toxicity from off-target effects, particularly for patients with relapsed or refractory diseases.[7]

Without many commonly seen adverse effects for strategies based on total $CD3^+$ T cell activation, such as graft-versus-host disease (GVHD), cellular immunotherapy using allogeneic innate lymphocytes, such as Vγ9Vδ2 T cells, has become the most attractive strategy to prevent relapse for solid tumors and hematologic malignancies.[8-10] Vγ9Vδ2 T cells are one of the most widely studied immune-cell populations, as they are readily accessible in human blood, easy to be cultured and expanded in vitro, and have potent cytotoxic activity against a wide variety of malignant cells in a T cell receptor (TCR)-dependent yet major histocompatibility complex (MHC)-independent process.[11,12] Julie Gertner-Dardenne et al have reported that Vγ9Vδ2 T cells efficiently killed autologous AML blasts.[13] We have established induction of allogeneic Vγ9Vδ2 T cells in a long-term induction ex vivo environment, which also can efficiently kill AML cells.[14] Those suggested that Vγ9Vδ2 T cell–based cellular immunotherapy is a promising treatment option for AML patients. However, the effect of Vγ9Vδ2 T cells on chemotherapy-resistant AML blasts, especially the highly refractory LSCs are still unknown. In this study, we investigated the therapeutic efficiency of allogeneic Vγ9Vδ2 T cells on chemotherapy-resistant AML blasts and LSCs in vitro and in vivo and further revealed its underlying molecular mechanisms.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

The following FITC-, PE-, PE-Cy5–, or APC-conjugated mAbs were obtained from BD Bioscience, eBioscience, or Biolegend: anti-NKG2D, anti-LFA-1 (CD11a), anti–DNAM-1 (CD226), anti-MICA/B, anti-ULBP1, anti-ULBP2, anti-ULBP4, anti-ICAM-1 (CD54), anti-ICAM-2 (CD102), anti-PVR (CD155), and anti-Nectin-2 (CD112). Additionally, the following blocking mAbs were also used: anti-NKG2D (1D11 from eBioscience), anti-CD11a (HI111 from Biolegend), anti-CD226 (DX11 from Abcam), and mouseIgG1κ isotype control (P3.6.2.8.1 from eBioscienc). Specific extracellular signal–regulatory kinase1/2 (ERK1/2) inhibitor (PD098059) was purchased from Sigma-Aldrich.

2.2 | In vitro culture of AML cell lines and primary AML samples

HL-60, NB4, and K562 cells were routinely cultured in our lab. Doxorubicin-resistant AML cells (HL-60/ADR and K562/AO2) and retinoic acid-resistant AML cells (NB4/R1) were obtained from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. All AML cells were grown in RPMI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin.

Primary AML cells were obtained from 11 refractory AML patients followed at the Bone Marrow Transplantation Center of The First Affiliated Hospital, Zhejiang University School of Medicine in China. Refractory AML was defined as fulfilling at least one of the following criteria: (1) primary induction failure (PIF) defined as[15,16] (i) bone marrow blast percentage above 25% or in the case of initial blast percentage below 50%, less than a 50% reduction in the blast percentage after the first cycle of induction therapy and in the case of achieving partial response after the first cycle of induction therapy, persistence of >5% blasts in the bone marrow at blood count recovery after the second cycle of induction therapy; (2) first early relapse after a remission duration of fewer than 6 months; (3) relapse refractory to at least one course of salvage combination chemotherapy containing high-dose AraC; and (4) second or subsequent relapse. The mononuclear cells from AML patients were isolated by density gradient centrifugation. We further obtained $CD34^+$ cells with purity

>95% from specimens from AML patients by positive-selection magnetic bead sorting using a VarioMACS device (Miltenyi Biotec). Cells were cultured in IMDM medium (Gibco) supplemented with 5% FBS, 1 mM L-glutamine, and 50 mg/mL penicillin/streptomycin.

The study was approved by the ethical committee of The First Affiliated Hospital of Zhejiang University School of Medicine. Informed consents were obtained from all patients according to the Declaration of Helsinki.

2.3 | In vitro culture of human Vγ9Vδ2 T cells from healthy volunteers

The details of the protocol for expansion of allogeneic Vγ9Vδ2 T cells have been described previously.[14] In brief, peripheral blood mononuclear cells (PBMCs) isolated from healthy adult volunteers by density gradient centrifugation were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics in the presence of 2 μM zoledronate (Novartis Pharma) and recombinant human IL-2 (Peprotech; 250 IU/mL final concentration), added at day 0.[17] The cells were supplemented with IL-2 every 3 days. Following 10-14-day culture, about 90% of the cells expressed the Vγ9Vδ2 TCR, as determined by fluorescence-activated cell sorting (FACS) analysis. The cell viability exceeded 95% as determined by trypan blue exclusion.

2.4 | Cytotoxicity assays and blocking experiments

Cytotoxicity analysis was measured by FACS analysis using the carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) and propidium iodide (PI, from BD-Pharmingin). AML cell lines or primary AML cells as target cells were labeled with 2 μM CFSE for 10 minutes at 37°C and gently mixed every 5 minutes. The reaction was stopped by adding ice-cold FBS to the cells for 5 minutes on ice and washed twice with fresh culture media. Then, the concentration of target cells was adjusted to 5×10^5 cells/mL, and 100 μL cell suspension was added into each 5-mL sterile polystyrene tube. Purified Vγ9Vδ2 T cells used as effector cells were added to AML cells at different E:T ratios of 5:1, 10:1, or 20:1 for coculture for 4 hours in a humidified atmosphere of 5% CO2 and 37°C. Control tubes containing only labeled target cells without Vγ9Vδ2 T cells were set as self-control for exclusion of dead cells. After 4 hours coculture, both of the two cells were washed with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA). Then, 1 mg/mL PI (Sigma-Aldrich) was added to stain dead cells and analyzed by FACS. Target cells killed by Vγ9Vδ2 T cells were measured as follows: percentage of Vγ9Vδ2 T cells cytotoxicity = (specific lysis − spontaneous lysis) × 100, in which specific lysis was defined as the CFSE and PI double-positive cells ($CFSE^+PI^+$)/total target cells ($CFSE^+$) cocultured with effector cells, and spontaneous lysis was that ratio from negative control.[18]

In blocking experiments, blocking mAbs (at the final concentration of 10 μg/mL) were used to evaluate the mechanisms of Vγ9Vδ2 T cell–mediated recognition and cytotoxicity of AML cell lines. Isotype-matched antibodies were used to determine nonspecific blocking effect. All blocking experiments were performed at the ratio of effector cells/target cells of 10:1.

2.5 | Adoptive immunotherapy in an animal model

All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experiments. Healthy 4- to 5-week-old female NOD-SCID mice received a single dose of 2×10^6 K562/AO2 cells (iv on day 0); then mice were randomly assigned to the treatment group or control group. The mice in the treatment group received a single dose of 2×10^7 purified Vγ9Vδ2 T cells (iv on day +3). On day +7, they received intraperitoneal injections of 30000 IU IL-2 and 2 μg zoledronate, and thereafter, they received intraperitoneal injections of 30 000 IU IL-2 per week. The mice in the control group were injected with PBS with or without 2 μg zoledronate on day +7 and 30 000 IU IL-2 per week. Peripheral blood samples with 10-20 μl were collected from the tail vein of the mice every week, and the number of human $CD45^+$ cells was detected by flow cytometry. We set the gate according to CD45/SSC to discriminate leukemia cells and Vγ9Vδ2 T cells. The weight, activity status, and survival status were also compared.

2.6 | CD107 mobilization assays

Vγ9Vδ2 T cells (5×10^6/mL) were activated by coculture with AML cells at an effector to target (E/T) ratio of 1:1 at 37°C in complete RPMI 1640 medium with or without FITC-conjugated anti-CD107a/b mAbs. After 4 hours, cells were harvested, stained with a TCRγδ-specific mAb, fixed, and then analyzed by flow cytometry.

2.7 | FACS analysis

Expression of surface markers on Vγ9Vδ2 T cells or on AML cells was determined by FACS analysis. Cells were collected, washed twice with PBS containing 2% FBS, stained for 30 minutes at 4°C with labeled Abs according to manufacturers' recommendations, washed, and analyzed by FACS. Viable cells were gated by forward- and side-scatter, and the analysis was performed on 100 000 acquired events for each sample. The results were presented as rate of positive cells or median fluorescence intensity (MFI). Quantification of the expression level of surface molecules on AML cells was evaluated using the marker MFI/isotype control MFI.

2.8 | Fixation of target cells and Western blotting

K562, K562/AO2, or HL60 cells were washed with PBS once and incubated with 1% paraformaldehyde (methanol-free) in PBS (pH

7.4) on ice for 30 min. The cells were then washed four times with PBS to remove all paraformaldehyde and resuspended at a final concentration of 2×10^7/mL in serum-free medium. Vγ9Vδ2 T cells were cultured in serum-free medium for 4 hours at 37°C before use in order to reduce the background phosphorylation. The rested Vγ9Vδ2 T cells (2×10^7/mL) were then mixed with an equal number of paraformaldehyde-fixed target AML cells. Then the mixture cells were washed and centrifuged at 1000 g at 4°C, followed by incubation for 0-15 minutes at 37°C. The cells were lysed in ice-cold lysis buffer containing 1% NP-40, 10 mmol/L Tris, 140 mmol/L NaCl, 0.1 mmol/L PMSF, 10 mmol/L iodoacetamide, 50 mmol/L Na fluoride, 1 mmol/L EDTA, 0.4 mmol/L Na orthovanadate, 10 μg/mL leupeptin, 10 μg/pepstatin, and 10 μg/mL aprotinin. Cell lysates were centrifuged at 12 000 g for 15 minutes to remove nuclei and cell debris. Samples were then boiled for 5 minutes in loading buffer and separated by 10% SDS-PAGE, followed by Western blot analysis with the desired antibody. The proteins were detected by Odyssey infrared laser two-color image analysis system. In blocking experiments, Vγ9Vδ2 T cells were pretreated with either blocking antibody or isotype for 1 hour at 37°C.

2.9 | Data and statistical analysis

Statistical analyses were performed by SPSS 17.0. Data were presented as mean ± standard error of the mean (SEM). Two groups were compared by Student's *t*-test. Three or more different groups were analyzed by one-way ANOVA with Dunnett's post hoc multiple comparison tests or two-way ANOVA with Bonferroni post-tests. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Vγ9Vδ2 T cells efficiently kill drug-resistant AML cell lines in vitro and in vivo

We compared the effect of cytotoxicity of Vγ9Vδ2 T cells against three chemotherapy-resistant AML cell lines, including anthracycline-resistant HL-60/ADR and K562/AO2 cells, and retinoic acid–resistant NB4/R1 cells, as well as their parental cell lines, HL-60, K562, and NB4, by FACS analysis. The results indicated that Vγ9Vδ2 T cells efficiently killed drug-resistant AML cell lines in a dose-dependent manner. In comparison with control groups, at the E/T ratio of 20:1, the cytotoxic efficiency of Vγ9Vδ2 T cells against K562/AO2 cells, NB4/R1 cells, and HL-60/ADR cells were 32.4% ± 1.5%, 30.8% ± 11.1%, and 30.4% ± 5.6%, respectively. Furthermore, the sensitivity of AML cells to Vγ9Vδ2 T cell–mediated cytotoxicity was not influenced by the sensitivity of AML cells to chemotherapy. The killing effect of Vγ9Vδ2 T cells on K562/AO2 was comparable with that on K562 (at the E/T ratio of 5:1, 10:1, 20:1: 21.9% ± 2.9% vs. 25.2% ± 5.5%; 27% ± 0.7% vs. 25.2% ± 4.6%; 32.4% ± 0.9% vs. 29% ± 3.1%; $P > .05$) and the killing effect on NB4/R1 was inferior to that on NB4 (at the E/T ratio of 5:1, 10:1, 20:1: 20.1% ± 2.8% vs. 25.9% ± 3.8%; 21.5 ± 4.2% vs. 35.3% ± 1.7%; 30.8% ± 6.4% vs. 46.5% ± 2.7%; $P < .05$), while the killing effect on HL-60/ADR was superior to that on HL-60 (at the E/T ratio of 5:1, 10:1, 20:1: 13.4% ± 0.6% vs. 7.6% ± 3.2%; 26.5% ± 3.4% vs. 11.6% ± 5%; 30.2% ± 3.2% vs.14.5% ± 6.5%; $P < .05$) (Figure 1A).

To confirm the therapeutic effects of Vγ9Vδ2 T cells in vivo, we established a humanized chemotherapy-resistant AML model in NOD/SCID mice by injection of K562/AO2 cell lines into the tail vein. We found the AML mice treated with Vγ9Vδ2 T cells showed unchanged body weight (Figure 1B), lower engraftment rate of leukemia cells at 8 weeks post injection of K562/AO2 cells (Figure 1C), and longer survival (Figure 1D) compared with untreated control or those treated with zoledronic acid and IL-2 alone.

3.2 | Vγ9Vδ2 T cells exhibit a comparable cytotoxic effect on LSCs to primary AML blasts

We further tested the cytotoxicity of Vγ9Vδ2 T cells on primary AML blasts obtained from AML patients. Bone marrow samples were collected from 11 refractory AML patients, among whom three patients had primary refractory AML and eight had relapsed AML. We found that Vγ9Vδ2 T cells exerted potent ex vivo cytotoxic activity against these refractory blasts, although with obvious individual differences. The cytotoxicity was enhanced as the E/T increased. In comparison with the control group (8.7% ± 8.5%), the cytotoxic efficiency of Vγ9Vδ2 T cells against primary AML blasts was 25.9% ± 12.5% ($P < .05$), 25.9% ± 14.3% ($P < .05$), 30.6% ± 17.7% ($P < .05$), and 39.5% ± 24% ($P < .05$) at the E/T ratio of 25:1, 50:1, 100:1, and 200:1, respectively (Figure 2A).

Taking into consideration that LSCs are usually considered to be the root cause of resistance and relapse of AML,[19] we further analyzed the anti-LSC effect of Vγ9Vδ2 T cells. $CD34^+$ AML cells were enriched by positive selection using magnetic bead sorting (MACS). The results showed that Vγ9Vδ2 T cells exhibited a comparable effect of cytotoxicity against LSCs to common AML blasts. In comparison with the control group (25.9% ± 14.3%), the cytotoxic efficiency of Vγ9Vδ2 T cells against LSCs was 42.1% ± 17.1% ($P < .05$), 48.3% ± 14% ($P < .05$), and 56.8% ± 19.3% ($P < .05$) at the E/T ratio of 50:1, 100:1, and 200:1, respectively (Figure 2B). More importantly, Vγ9Vδ2 T cells showed no cytotoxicity effect against normal bone marrow primary monocytes or fibroblasts (Figure 2C).

3.3 | Receptor-ligand interaction of CD226-Nectin-2 is a key pathway for Vγ9Vδ2 T cells to recognize AML cells

The potent cytotoxic activity of Vγ9Vδ2 T cells toward chemotherapy-resistant AML blasts encouraged us to further explore how Vγ9Vδ2 T cells can recognize and kill the drug-resistant cells. Flow cytometric analysis showed high expressions of three

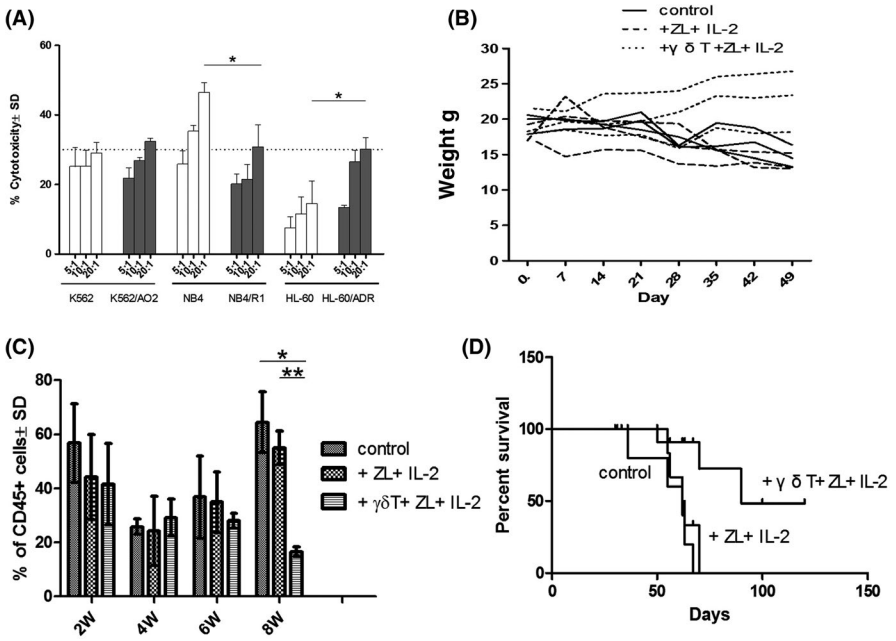
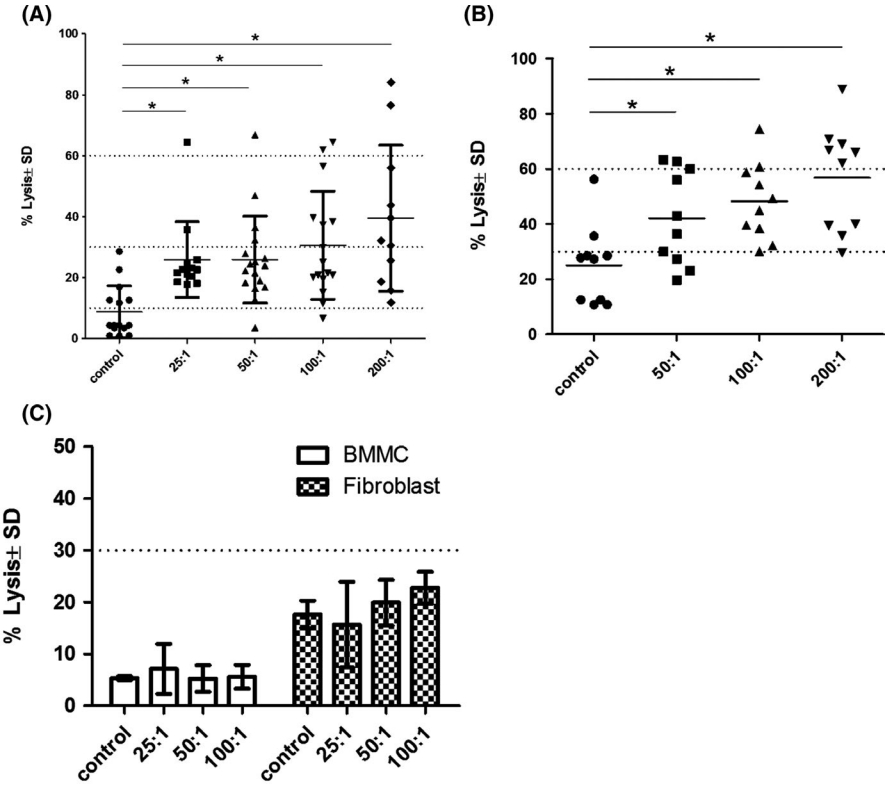


FIGURE 1 The cytotoxicity effect of Vγ9Vδ2 T cells against acute myeloid leukemia (AML) cell lines in vitro and in vivo. A, The cytotoxicity effect of Vγ9Vδ2 T cells with different E/T ratios against three chemotherapy-resistant AML cell lines and their parental cell lines after being cocultured for 4 h. B-D, In humanized chemotherapy-resistant AML model in NOD/SCID mice, compared with untreated control or those treated with zoledronic acid and IL-2 alone, AML mice that received treatment with Vγ9Vδ2 T cells combined with zoledronic acid and IL-2 showed unchanged body weight (B), lower engraftment rate of leukemia cells (C), and longer survival (D). All data were expressed as mean ± SD, n = 3. $^{*}P < .05$, $^{**}P < .01$. ZL, zoledronic acid

FIGURE 2 The cytotoxicity effect of Vγ9Vδ2 T cells against primary acute myeloid leukemia (AML) blasts and AML leukemia stem cells (LSCs). The cytotoxicity effect of Vγ9Vδ2 T cells with different E/T ratios against primary AML blasts (A) and $CD34^{+}$ AML LSCs (B) after being cocultured for 8 h. C, Vγ9Vδ2 T cells showed no cytotoxicity effect against normal bone marrow primary monocytes or fibroblasts. $^{*}P < .05$, $^{**}P < .01$



cytotoxicity-associated receptors on the surface of Vγ9Vδ2 T cells, including NKG2D, CD11a, and CD226 (Figure S2A). The levels of the expression of the respective ligands for NKG2D (ie, MICA/B, ULBP-1, ULBP-2, ULBP-4), ligands for CD11a (ie, ICAM-1, ICAM-2), and ligands for CD226 (ie, Nectin-2, PVR) on series of AML cell lines including drug-resistant cell lines were calculated by the method of MFI/isotype (Figure S1A). We found that those ligands were expressed at different levels on different subsets of AML cell lines, and

the levels of expression also varied on pairs of drug-resistant cell lines and their parental cell lines (Figure 3A). Correlation analysis using linear regression model revealed that the expression level of Nectin-2 on AML cell lines was highly relevant to the sensitivity of AML cells to Vγ9Vδ2 T cell cytotoxicity, regardless of whether the AML cells are chemotherapy-sensitive or chemotherapy-resistant (Figure 3B).

We further blocked the CD226–Nectin-2 pathway by anti-CD226 antibody, which significantly reduced the cytotoxicity of Vγ9Vδ2 T cells against Nectin-2–expressing AML cell lines including Kasumi-1 (41.5% ± 13.5% vs. 30.8% ± 8.2%, $P < .05$), K562 (34% ± 4.6% vs. 25.4% ± 5.3%, $P < .05$), NB4 (26.8% ± 12.6% vs. 18.5% ± 11.9%, $P < .05$), and K562/AO2 (28.5% ± 14.2% vs. 22.4% ± 10.8%, $P < .05$) (Figure 3C). Although we found that the expression level of ULBP-4 on AML cells was related to the sensitivity of AML cells to Vγ9Vδ2 T cell cytotoxicity ($r = 0.88$) by linear regression model analysis (Figure S1B), the correlation was not confirmed after blocking NKG2D of Vγ9Vδ2 T cells by anti-NKG2D antibody (Figure S2C). Meanwhile, blocking the CD11a-ICAM1/2 pathway by anti-CD11a antibody also did not significantly attenuate the cytotoxicity of Vγ9Vδ2 T cells (Figure S2B). These results demonstrated that CD226-Nectin-2 is one of the pivotal molecules determining the killing efficiency of Vγ9Vδ2 T cells toward AML cells.

Taking into account of the importance of TCR-dependent recognition in T cells, we asked the role of TCR-dependent recognition in Vγ9Vδ2 T cells against AML cells. The published studies have identified that atorvastatin has immunosuppressive effect by abolishing isopentenyl diphosphate (IPP) accumulation.[20] To answer this question, first, we blocked the composition of IPP, the ligand of TCRγδ, by atorvastatin in AML cell lines Kasumi-1, K562, NB4, and K562/AO2. We found that blockage of the TCR by 5 μmol/L atorvastatin significantly impaired the cytotoxicity effects of Vγ9Vδ2 T cells. The cytotoxic efficiency of Vγ9Vδ2 T cells against Kasumi-1 reduced from 30.1 ± 17.6% to 22.2 ± 17.2% ($P < .01$), against NB4 from 21.3 ± 9.1% to 14.8 ± 9.8% ($P < .05$), and against K562 and K562/AO2 it also showed decreasing trend (19.2 ± 8.5% vs 14.1 ± 2% and 20.5 ± 21.2 vs 12.3 ± 8.9%, respectively) (Figure 3D). TCR-mediated activation results in elevated intracellular $Ca2^+$ and activation of the $Ca2^+$–calmodulin-dependent serine–threonine phosphatase calcineurin. We further blocked the TCR-mediated activation pathway by calcineurin inhibitor cyclosporin A (CSA) and then detected the impact on degranulation of Vγ9Vδ2 T cells. $CD107a^+$ Vγ9Vδ2 T cells cocultured with NB4 reduced from 28.9 ± 5.7% to 13.8 ± 6.0% when pretreated with 10ug/ml CSA, and $CD107a^+$ Vγ9Vδ2 T cells cocultured with NB4/R1 cells reduced from 36.4 ± 10.1% to 14.6 ± 5.5% ($P < .01$) (Figure 3E). These results indicated that the TCR recognition

(A)

	Kasumi-1	K562	NB4	HL-60	K562/AO2	NB4/R1	HL-60/ADR
MICA/B	2.641873	2.455621	1.710843	2.466125	1.9076923	2.816867	1.45205479
ULBP-1	2.989362	2.96114	3.294355	7.914286	7.4373089	2.50996	3.24657534
ULBP-2	9.153203	6.542781	2.02356	5.089888	7.4484848	23.01205	1.16173913
ULBP-4	4.678947	1.053533	0.892473	1.080605	1.2708861	1.103226	0.86885246
ICAM-1	9.232446	20.51282	6.446281	0.838275	23.449735	4.233546	2.88985149
ICAM-2	2.765079	3.133787	1.135266	1.172702	7.2929936	1.049133	2.08879023
Nectin-2	20.1107	16.1435	8.800866	1.002283	5.2900901	1.223529	8.61757426
PVR	5.448649	5.384501	3.980938	1.029478	4.3172542	4.683544	5.16259542

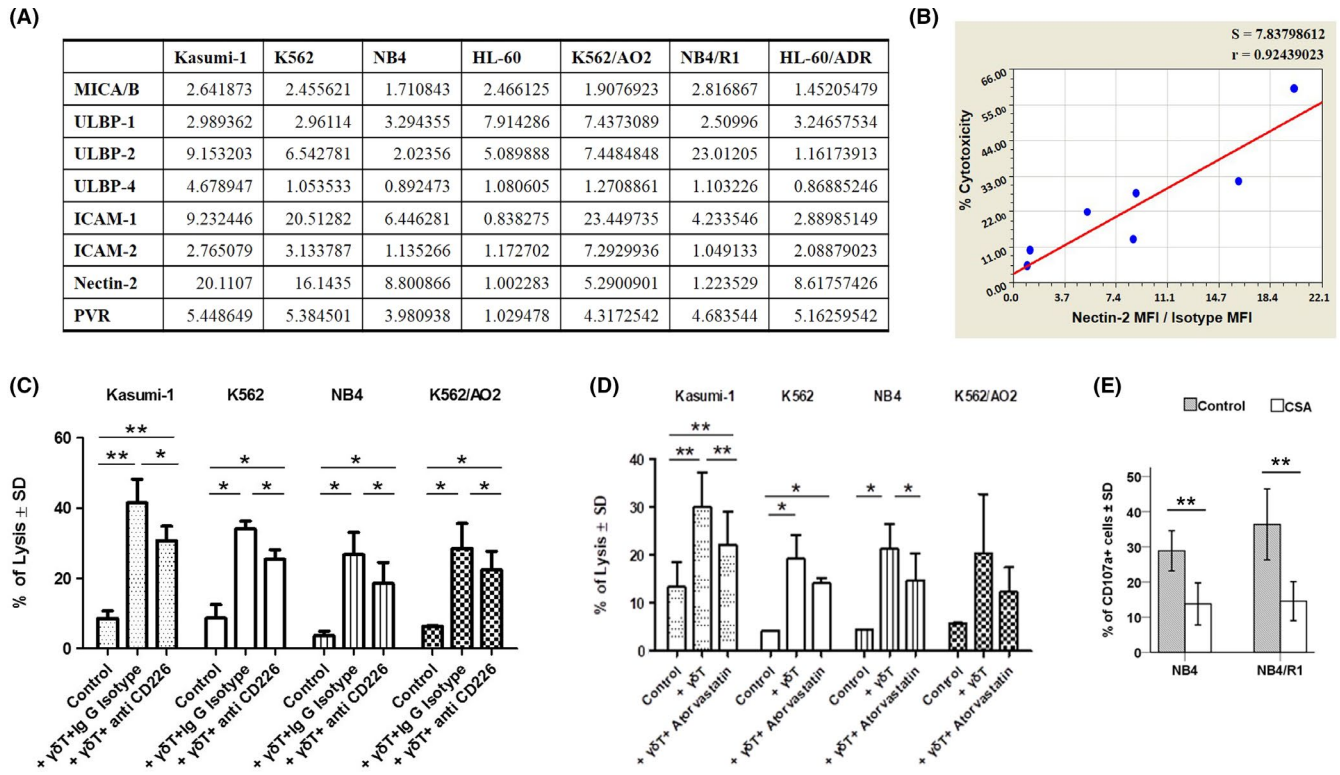


FIGURE 3 Vγ9Vδ2 T cells recognize acute myeloid leukemia (AML) cells by receptor-ligand interaction of CD226–Nectin-2. A, The expression levels of ligands for NKG2D (ie, MICA/B, ULBP-1, ULBP-2, ULBP-4), CD11a (ie, ICAM-1, ICAM-2), and CD226 (ie, Nectin-2, PVR) on series of AML cell lines determined by flow cytometric analysis. B, Correlation analysis of the expression level of Nectin-2 on AML cells and the sensitivity of AML cells to the cytotoxic effect of Vγ9Vδ2 T cells using linear regression model. C, Blocking the CD226–Nectin-2 pathway by anti-CD226 antibody significantly reduced the cytotoxicity of Vγ9Vδ2 T cells against K562/AO2 cells, Kasumi-1, K562, and NB4 cells. D, Blockage of the TCRγδ pathway by atorvastatin impaired the cytotoxicity effects of Vγ9Vδ2 T cells. E, Blockage of the TCR-mediated activation pathway by cyclosporin A impaired the degranulation effect of Vγ9Vδ2 T cells. *$P < .05$, **$P < .01$

pathways also may take part in the cytotoxicity of Vγ9Vδ2 T cells against AML cells, especially.

3.4 | Vγ9Vδ2 T cells induce cytotoxicity against AML cells via the CD226-ERK1/2-LAMP1 pathway

Finally, we explored how the CD226–Nectin-2 interaction triggered the cytolysis of AML cells. We hypothesized that the mechanism of cytotoxicity of Vγ9Vδ2 T cells is similar to that of NK- and $CD8^+$ T cell–mediated cytotoxicity, where TCR, NKG2D, or FcR directly bound to Syk/Zap70, rapidly activated the phosphoinositide 3-kinase (PI3K)-Rac-PAK-MEK-ERK pathway, and finally triggered the release of cytotoxic granules.[21,22] Vγ9Vδ2 T cells were mixed with an equal number of paraformaldehyde-fixed targeted cells, and the level of ERK1/2 activation was analyzed by Western blotting analysis as previously reported.[23] The results revealed that the level of ERK phosphorylation in Vγ9Vδ2 T cells was quickly induced after being cocultured for 5 to 20 minutes with K562 cells or with drug-resistant K562/AO2 cells, which have both high Nectin-2 expression. However, the induction of ERK phosphorylation was not observed in Vγ9Vδ2 T cells when being cocultured with HL-60 cells, which have low Nectin-2 expression (Figure 4A and Figure S3A). Furthermore, blocking the CD226–Nectin-2 pathway by anti-CD226 antibody inhibited ERK1/2 phosphorylation in Vγ9Vδ2 T cells after being cocultured with K562 or K562/AO2 cells (Figure 4B and Figure S3B).

FACS analysis showed that compared with the control group, the expression of lysosome-associated membrane protein 1 (LAMP1)/CD107a, a protein reflecting exocytosis of lytic granules, on Vγ9Vδ2 T cells was significantly increased after Vγ9Vδ2 T cells had been cocultured with Kasumi-1 (0.58% ± 0.6% vs. 22.9% ± 6.2%, $P < .01$), K562 (0.58% ± 0.6% vs. 17% ± 10.2%, $P < .05$), or even drug-resistant K562/AO2 cells (0.58% ± 0.6% vs. 11.8% ± 3.3%, $P < .01$) for 4 hours (Figure 4C). Importantly, the level of expression of LAMP1/CD107a on Vγ9Vδ2 T cells was closely related to the level of expression of Nectin-2 on targeted AML cells, regardless of whether AML cells are drug-resistant or not (Figure 4D). Moreover, blocking the ERK pathway by pretreating Vγ9Vδ2 T cells with specific ERK1/2 inhibitor PD098059 1 hour before Vγ9Vδ2 T cells were cocultured with K562, K562/AO2, NB4 or NB4-R1 cells attenuated the expression of LAMP1/CD107a on Vγ9Vδ2 T cells, which was not found in non–Nectin-2–expressing HL-60 cells (Figure 4E).

4 | DISCUSSION

Our results first revealed that allogeneic Vγ9Vδ2 T cells can efficiently kill chemotherapy-resistant AML cell lines as well as AML

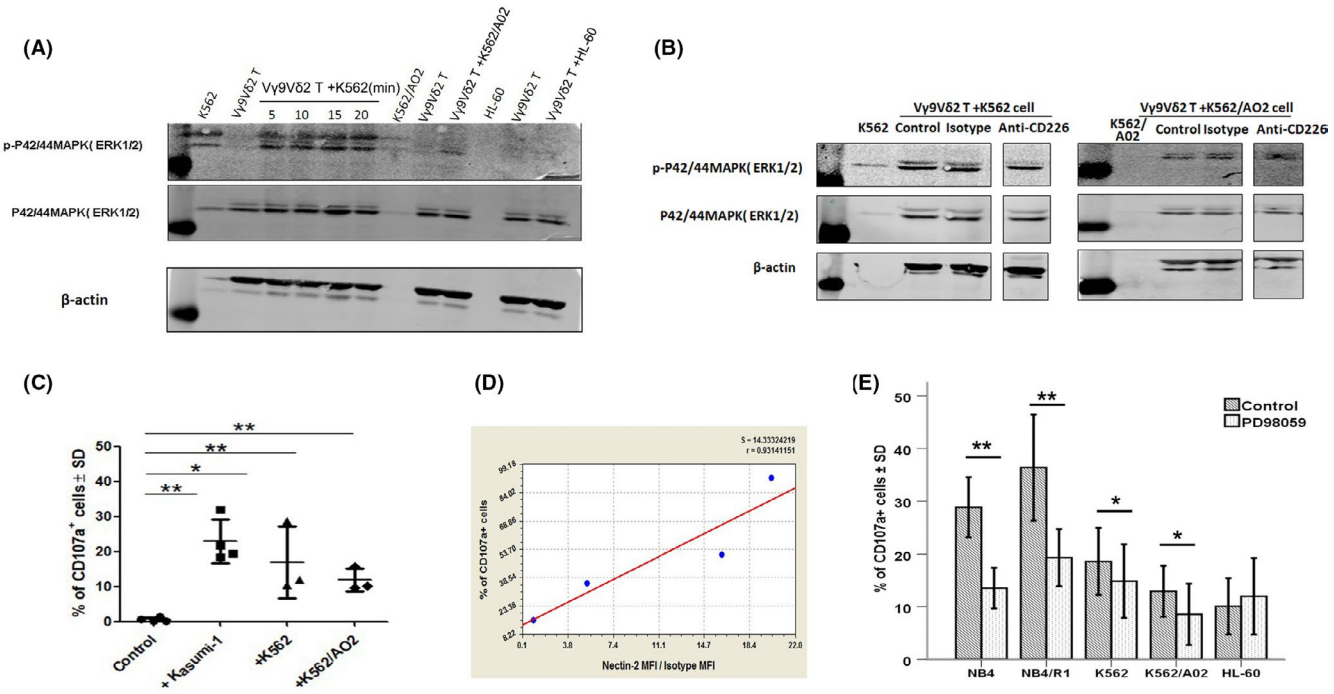


FIGURE 4 A, The levels of ERK phosphorylation inVγ9Vδ2 T cells after being cocultured with acute myeloid leukemia (AML) cell lines, which have high Nectin-2 expression, including K562 cells and drug-resistant K562/AO2, and with HL-60 cells, which have low Nectin-2 expression. B, Effect of blocking the CD226–Nectin-2 pathway on the levels of ERK1/2 phosphorylation in Vγ9Vδ2 T cells after being cocultured with K562 and K562/AO2 cells. C, The expression levels of lysosome-associated membrane protein 1 (LAMP1)/CD107a on Vγ9Vδ2 T cells after being cocultured with Kasumi-1, K562, and drug-resistant K562/AO2 for 4 h by FACS analysis. D, Correlation analysis of the expression level of LAMP1/CD107a on Vγ9Vδ2 T cells and the expression levels of Nectin-2 on drug-resistant and nonresistant AML cells. E, The effect of blocking the ERK pathway on the expression levels of CD107a on Vγ9Vδ2 T cells after being cocultured with NB4, NB4/R1, K562, K562/AO2 cells, and HL-60 cells. *$P < .05$, **$P < .01$

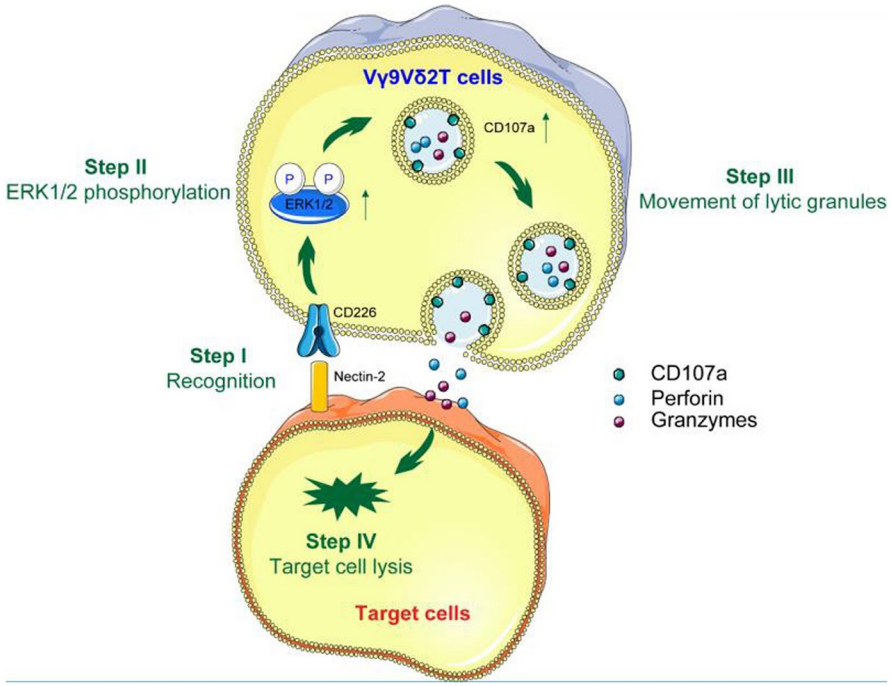


FIGURE 5 Schematics of Vγ9Vδ2 T cell–induced cell cytolysis of chemotherapy-resistant acute myeloid leukemia (AML) cells

blasts and even LSCs in vitro and in vivo, which may be determined by the expression levels of CD226–Nectin-2. We also demonstrated that activation of CD226-ERK1/2-LAMP1 signaling in Vγ9Vδ2 T cells to induce the movement of lytic granules may play a pivotal role in the cytotoxic activity of Vγ9Vδ2 T cells against AML cells.

Vγ9Vδ2 T cells are attractive effector cells for immunotherapy with potent cytotoxic activity against a variety of malignant cells. It has been reported that infusion of Vγ9Vδ2 T cells along with or immediately after the chemotherapeutic agents, substantially enhanced antitumor effect against clones of cancer-initiating cells and hence provided reliable clinical benefits to cancer patients.[24] However, clinical application of autologous Vγ9Vδ2 T cells seems to be hampered by the observed dysfunction of Vγ9Vδ2 T cells.[25] In this study, we demonstrated that allogeneic Vγ9Vδ2 T cells can efficiently kill drug-resistant AML blasts and even LSCs, which were highly resistant to the conventional chemotherapeutic drugs and some other innovative therapies and are the root cause of AML relapse. Recently, the fact that γδ T cells do not cause GVHD but can exert strong graft-versus-leukemia (GVL) effects and can mediate strong antiviral and antibacterial activity predisposes γδ T cells to achieve increasing interest in the context of allo-HSCT.[26] In vivo activation of γδ T cells post transplant by the repetitive infusion of zoledronate has shown promising results in pediatric leukemia.[27] Our results and previous reports suggested that allogeneic Vγ9Vδ2 T cell–based cellular immunotherapy could be an effective therapeutic approach for refractory AML and a beneficial clinical strategy to prevent AML relapse post HSCT when this cell population was combined with chemotherapy or allo-HSCT. Yet, we found that when cocultured with primary AML cells, Vγ9Vδ2 T cells needed a high E:T to achieve a remarkable cytotoxic effect. It reminded us that Vγ9Vδ2 T cells and other treatments such as chemotherapy should be applied sequentially and in combination in clinical treatment. First, the tumor load should be reduced by chemotherapy, and then Vγ9Vδ2 T cells should be used to eliminate drug-resistant residual tumor cells. Although further research involving patient-derived xenograft (PDX) models and serial transplantation experiments are necessary to identify the efficacy, optimal dose, and treatment schedule of Vγ9Vδ2 T cells killing AML blasts and even LSCs, it is reasonable to surmise that the future of immunotherapy in AML lies in the rational combination of complementary immunotherapeutic strategies with chemotherapeutics or other oncogenic pathway inhibitors. More promising, the introduction of chimeric antigen receptor (CAR)-modified T cells has revolutionized immunotherapy and cancer treatment as a whole. The principal advantage of γδ T cells is that they possess cytotoxic capacity, but none express an endogenous TCR; therefore, they do not mediate GVHD when administered to MHC-mismatched hosts.[28] Investigators have successfully transduced γδ T cells with CARs, which have demonstrated activity in preclinical models.[29,30]

Although Vγ9Vδ2 T cells have been attracting increasing attention in the field of cellular immunotherapy, its killing mechanisms remains poorly understood. In this study, we revealed that the CD226-ERK1/2-LAMP1 pathway is an important mechanism for Vγ9Vδ2 T cell–induced cytotoxicity against AML cells. First, Vγ9Vδ2 T cells recognized AML cells by receptor-ligand interaction of CD226–Nectin-2, which then induced ERK1/2 phosphorylation in Vγ9Vδ2 T cells, finally triggering the movement of lytic granules toward AML cells to induce cytolysis of AML cells (Figure 5). Anguille et al[31] reported that a panel of 10 genes encoding cell surface proteins that can segregate "Vγ9Vδ2 T cell–susceptible" from "Vγ9Vδ2 T cell–resistant" lymphoblastic leukemia or lymphoma cells. Among MICA/B, ULBP-1, ULBP-2, ULBP-4, ICAM-1, ICAM-2, PVR, and Nectin-2 on target cells, only Nectin-2 was closely related to Vγ9Vδ2 T cell cytotoxicity against AML cells, which is consistent with our results and suggested that the expression level of Nectin-2 may be used as a novel marker to predict the

susceptibility/resistance of AML cells to Vγ9Vδ2 T cell treatment and select optimal patients to receive Vγ9Vδ2 T cell–based immunotherapy. However, the expression level of Nectin-2 varied among several pairs of drug-resistant AML cell lines and parental cell lines (Figure 3A and Figure S1), which suggested that mechanisms for chemotherapy-resistance and immune escape are independent. The predictive value of the level of the expression of Nectin-2 should then be evaluated in further preclinical and clinical studies.

In addition, our results indicated that the TCR recognition pathways also may take part in the cytotoxicity of Vγ9Vδ2 T cells against AML cells. There are indeed some deficiencies in this study. The mechanism of Vγ9Vδ2 T cells recognizing AML cells has not been fully elucidated. Whether the recognition pathways of TCRγδ-IPP and CD226–Nectin-2 are independent or synergistic is not yet proved in this study. Further studies should be performed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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