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ORIGINAL ARTICLE

Overcoming antigen loss in CAR T therapy with Vγ9Vδ2 CAR T-cells

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Background: Vγ9Vδ2 T-cells demonstrate potent antitumor activity *in vitro* but, despite successful safety studies, the clinical benefit of Vγ9Vδ2 in adoptive cell therapy has been limited. One approach to enhance the therapeutic potential of Vγ9Vδ2 T-cells while maintaining their safety profile is genetic engineering to express a chimeric antigen receptor (CAR). Vγ9Vδ2 CAR T-cells retain the ability to target tumor cells even after target antigen loss, a major cause of CAR treatment relapse.

Methods: Vγ9Vδ2 T-cells were expanded from peripheral blood mononuclear cells in the presence of high levels of interleukin 2 (IL-2) or IL-2 in combination with IL-15. Cells were then virally transduced with a CD19-directed CAR and underwent antigen-specific stimulation to enrich CAR-expressing cells.

Results: Vγ9Vδ2 CAR T-cells showed similar cytotoxic activity to conventional αβ-CAR T-cells against CD19-positive tumor cells. They demonstrated superior responses against CD19-negative tumor cells, however, particularly when IL-15 was included during expansion. This enhanced function was further confirmed in co-culture assays with mixed CD19-positive and CD19-negative tumor populations, simulating antigen loss.

Conclusions: Vγ9Vδ2 CAR T-cell therapy presents a promising strategy for B-cell malignancies, offering sustained antitumor activity even after antigen loss. This approach may help overcome a major limitation of conventional CAR T-cell therapy, potentially improving clinical outcomes.

Key words: Vγ9Vδ2 T-cells, CAR T-cells, antigen loss, B-cell malignancies

INTRODUCTION

Chimeric antigen receptor (CAR) T-cell therapy is an effective, cell-based cancer treatment consisting of the infusion of autologous, conventional alpha-beta (αβ) T-cells genetically modified *ex vivo* to target tumor cells.[1] Its success against selected hematologic malignancies has led to the approval of anti-CD19 CAR T-cell therapy by the United States Food and Drug Administration (FDA) in 2017 as the first ever T-cell therapy.[2] Currently, six different CAR T-cell products have been FDA approved, expanding this therapeutic opportunity to include multiple B-cell malignancies[3] (Supplementary Table S1, available at https://doi.org/10.1016/j.iotech.2025.101053). CAR T-cell therapy, however, faces limitations that include severe life-threatening toxicities, limited antitumor activity, low persistence, and relapse.[4,5] In the case of acute lymphoblastic leukemia, although most patients experience a clinical response,[6] relapse rates within the first year range from 40% to 60%,[7] with antigen loss being the cause in 20% of treated patients.[4,7,8] The use of gamma-delta (γδ) T-cells as carriers of CARs, instead of conventional αβ T-cells, could help in overcoming these obstacles.[9]

γδ T-cells are a group of unconventional lymphocytes that constitute 1%-5% of all CD3-positive T-cells.[10] In human peripheral blood, the most abundant subset is known as Vγ9Vδ2 T-cells,[11] which can recognize a broad spectrum of tumor antigens. Vγ9Vδ2 T-cells can directly interact with tumor cells through their γδ T-cell receptors (TCR) by recognizing phosphoantigens (pAgs) bound to a butyrophilin complex.[12] These pAgs are produced by the mevalonate pathway, which is up-regulated in many cancer types.[13] *In vitro*, administration of zoledronic acid (ZOL) can mimic this process, allowing the specific expansion of Vγ9Vδ2 T-cells from peripheral blood mononuclear cells (PBMCs) from healthy donors.[14,15] Beyond Vγ9Vδ2 TCR signaling, γδ T-cells are also activated through natural killer (NK)-cell receptors including NK group 2 member D (NKG2D)[16] and DNAX accessory molecule-1 (DNAM-1).[17] Vγ9Vδ2 T-cells elicit cytotoxicity by release of granzyme B and perforin, as well as through Fas/FasL and TRAIL

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interactions.[18] This unique MHC independence supports not only endogenous responses against HLA class I negative tumors,[19] but also supports potential therapeutic transfer of allogeneic Vγ9Vδ2 T-cells.

Multiple clinical trials have evaluated the therapeutic potential of γδ T-cells,[20] either through *in vivo* stimulation of autologous γδ T-cells,[21,22] or adoptive cell therapy (ACT) of *ex vivo* expanded autologous[23,24] or allogeneic[25,26] γδ T-cells. Despite the limited therapeutic effect, these trials demonstrated the safety and feasibility of Vγ9Vδ2-based ACT. To increase the response rates of this approach, several studies since 2004 have engineered Vγ9Vδ2 T-cells to express CARs[27] (Supplementary Table S2, available at https://doi.org/10.1016/j.iotech.2025.101053). These efforts have been primarily focused on CD19-[28] and GD2-directed[29] CARs to address B-cell malignancies and neuroblastoma, respectively. In these studies, Vγ9Vδ2 CAR T-cells exhibited a strong antitumor effect that was comparable to αβ CAR T-cells *in vitro* and were able to kill tumor cells through CAR-mediated mechanisms as well as through their innate antitumor mechanisms.[28] Notably, cross-presentation abilities of tumor antigens to αβ T-cells were retained by Vγ9Vδ2 T-cells after CAR endowment.[29,30]

Although CAR specificity can direct antitumor immunity, γδ T-cells are equipped with a diverse library of endogenous tumor recognition receptors, giving them a broad scope of CAR-independent antitumor ability. Here, we demonstrate that Vγ9Vδ2 T-cells can be used to address antigen loss in CD19-focused CAR T therapies. These T-cells can be expanded, armed with anti-CD19 CAR, and kill tumor cells both through CAR-dependent and CAR-independent mechanisms, an ability lacking in directly compared αβ CAR T-cells. Further, the functionality of γδ CAR T-cells is ameliorated when produced with interleukin 2 (IL-2) and IL-15 dual-cytokine expansion, consistent with our previous work.[31] These findings demonstrate the feasibility of γδ T-cells to combat antigen loss, which can lead to relapse in patients receiving CAR T-cell therapy.[32]

MATERIALS AND METHODS

PBMC isolation

PBMCs were isolated using Lymphoprep™ density gradient media (Alere Technologies, Waltham, MA) from buffy coats from healthy donors collected at the blood bank at Rigshospitalet, Copenhagen University Hospital, Denmark. Once isolated, PBMCs were cryopreserved in fetal bovine serum (FBS, Gibco, Grand Island, NY) with 10% dimethyl sulfoxide (DMSO, Honeywell, Charlotte, NC).

Expansion of Vγ9Vδ2 T-cells

For the expansion of Vγ9Vδ2 T-cells cryopreserved PBMCs were thawed, and 1×10^6 cells were seeded per well in 2 ml of X-VIVO 15 medium (Lonza, Basel, Switzerland) with 5% human serum (Sigma Aldrich, Burlington, MA) and 10 μM ZOL. After 2 days, cytokines were added to the cultures: 1000 U/ml IL-2 (Peprotech, Cranbury, NJ) or 100 U/ml IL-2 and 100 U/ml IL-15 (Peprotech). From day 4, fresh media and cytokines were provided every 2-3 days.

Cancer cell cultures

The CD19-negative leukemia cell lines Molm-14 (acute monocytic leukemia) and K562 (chronic myelogenous leukemia), and the CD19-positive leukemia cell lines Daudi (Burkitt's lymphoma), Nalm-6 (acute lymphoblastic leukemia) and K562-CD19 (K562 previously transduced to express CD19 on the surface) used in this study were cultured in RPMI 1640 GlutaMAX-ITM medium (RPMI, Gibco) supplied with 10% FBS (R10 media).

Production of lentiviral particles

Human embryonic kidney (HEK) 293T cells were used to produce lentiviral particles. HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Some 50% confluent HEK 293T cells were transfected using a traditional $CaCl_2$ method (Promega, Madison, WI) following manufacturer instructions with a third-generation lentiviral system using packaging and envelope plasmids pCMV-VSV-G, pMDLg-pRRE and pRSV-Rev (Addgene, Watertown, MA), and a transfer plasmid encoding for an anti-CD19-41BB-CD3z CAR.[33] Some 48 h after transfection, the lentiviral-containing supernatant (LV-SN) was collected, filtered using a 0.44 μm filter and used immediately for transduction of T-cells. Fresh media was added to transfected HEK 293T cells and LV-SN was harvested again after 24 h for a second round of T-cell transduction.

T-cell transduction

Fresh LV-SN was used for transduction of both γδ and αβ T-cells. Vγ9Vδ2 T-cells were expanded from frozen PBMCs and cultured for 5 days before transduction, while PBMCs from the same donor were activated with CD3/CD28 Dynabeads (Gibco) for 2 days to assure proliferation of αβ T-cells. For transduction, 2 ml of fresh LV-SN was added to a coated Retronectin (Takara, Shiga, Japan) non-tissue culture (NTC) treated 24-well plate. The NTC treated plate was centrifuged for 1 h (1800*g*, 32°C), the LV-SN was removed and 0.5×10^6 cells were added to each well. The plate was again centrifuged for 1 h (900*g*, 32°C) and incubated overnight at 37°C and 5% CO_2. The same process was repeated the next day for a second transduction. Cells were incubated for 24 h before being transferred to a tissue culture 24-well plate. Flow cytometry analysis to assess the GFP/CAR expression was carried out on day 3 after transduction, and every week after that. After 1 week, the cells were transferred to a plate coated with a recombinant human CD19 protein (1 μg/ml, R&D Systems, Minneapolis, MN) for 2 weeks to specifically expand the engineered CAR T-cells. Fresh media and cytokines were provided every 2-3 days.

Extracellular staining of Vγ9Vδ2 T-cells

All used antibodies are listed in Supplementary Table S3, available at https://doi.org/10.1016/j.iotech.2025.101053. Flow cytometry was used for the phenotyping of Vγ9Vδ2 T-cell cultures, and the detection of the genetically modified cells. For phenotyping, 0.5×10^6 cells were washed twice with PBS + 2% FBS (FACS buffer) and incubated with 50 µl of antibody mix for 20-30 min at 4°C. For the detection of the anti-CD19 CAR, cells were first stained with an anti-mouse F(ab)'2 specific antibody for 20-30 min at 4°C in the dark. To avoid unspecific binding, cells were washed four times with FACS buffer before adding the rest of the surface markers. Finally, cells were washed twice with FACS buffer before acquisition on the NovoCyte Quanteon (Agilent, Santa Clara, CA). LIVE/DEAD Near IR Fixable Stain was used for dead cell exclusion. Flow cytometry data were analyzed using NovoExpress software (version 1.5.0).

Intracellular staining

To study the production of cytokines and degranulation markers upon stimulation, co-cultures with 0.45×10^6 CAR T-cells and 0.15×10^6 tumor cells were set up (E : T ratio of 3 : 1). Vγ9Vδ2 and αβ CAR T-cells without stimuli were included as a negative control. For data analysis, the baseline levels (negative control condition) were subtracted from the values of the different experimental conditions. Brefeldin A (BioLegend, San Diego, CA) and anti-CD107a antibody were added to the co-cultures and incubated for 5 h at 37°C. Then, the cells were stained with the extracellular antibodies for 20-30 min at 4°C, washed and incubated overnight with 200 µl/well of Fixation/Permeabilization buffer (Invitrogen, Waltham, MA) at 4°C. The next day, cells were washed with 150 µl of Permeabilization buffer (Invitrogen) and stained with the intracellular antibodies for 20-30 min at 4°C. After two final washes with the Permeabilization buffer, cells were resuspended in FACS buffer before acquisition on the Novocyte Quanteon.

Cytotoxicity assays

The ability of Vγ9Vδ2 or αβ CAR T-cells to kill CD19-positive or CD19-negative tumor cell lines was assessed by the xCELLigence real-time cell analysis (RTCA) assay. First, the E96 plate (Agilent) was coated with 4 µg/ml of anti-CD29 or anti-CD19 tethering reagent (Agilent) for 3 h. Then, the tumor cells were added at a density of 0.05×10^6 cells/well for the K562 and K562-CD19 tumor cell lines, or 0.1×10^6 cells/well for the Daudi, Nalm-6 and Molm-14. The plate was inserted into the xCELLigence SP system (Agilent) for the cells to attach and initiate proliferation. After 2-3 h, Vγ9Vδ2 and αβ CAR T-cells were added to the plate at an E : T ratio of 2 : 1, and the readout resumed for 72 h. A condition with tumor cells alone was included for each tumor cell line. The cell index of tumor cells plus CAR T-cells was normalized by the values of the tumor alone for each condition.

Proliferation assay

To determine differences in the proliferation of CAR T-cell cultures when co-cultured with CD19-positive or CD19-negative tumor cells, CAR T-cells were dyed with CellTraceViolet (CTV, Thermo Fisher Scientific, Waltham, MA) for 20 min at 37°C. A positive control consisting of cytokine stimulation (1000 U/ml IL-2 or 100 U/ml IL-2 + 100 U/ml IL-15 for the Vγ9Vδ2 CAR T-cell cultures and 500 U/ml IL-2 for the αβ CAR T-cell cultures), and a negative control of CAR T-cells alone, were included in this assay. Cells were seeded in a 24-well plate at a concentration of 0.5×10^6 CAR T-cells/well together with 0.5×10^6 tumor cells (E : T ratio of 1 : 1). The plate was incubated at 37°C and 5% CO_2 for 5 days, when flow cytometry analysis was carried out as described above. Flow cytometry data were analyzed using the proliferation tool of FlowJo software (version 10.6.1).

RESULTS

Zoledronic acid treatment expands Vγ9Vδ2 T-cells from PBMCs

Six different Vγ9Vδ2 T-cell cultures were expanded from healthy donor PBMCs by adding 10 µM of ZOL in combination with either high-level IL-2 (IL-2-Vγ9Vδ2 T-cells) or low-level IL-2 plus IL-15 (IL-2/IL-15-Vγ9Vδ2 T-cells). Flow cytometry analysis was carried out daily from day 0 to day 9 and on day 14 to investigate the expression of CD3, Vγ9, CD19 and CD56 (gating strategy shown in Supplementary Figure S1, available at https://doi.org/10.1016/j.iotech.2025.101053). No differences in proliferation or purity were observed between the two different cytokine conditions. Both cultures were started with $0.13\text{-}0.31 \times 10^6$ Vγ9-positive cells on day 0, corresponding to an average of 2.6% [standard deviation (SD) ± 1.8%] Vγ9Vδ2 T-cells, and reached an average of 73×10^6 (SD ± 40×10^6) cells by day 14, which comprised 93.5%-98.6% of the cultures, corresponding to a 738 (SD ± 447) fold expansion (Figure 1A-C). Vγ9Vδ2 T-cell expansion pattern was sigmoidal, with the linear phase starting 5 days after ZOL administration. Figure 1D showcases how αβ T-cells, B-cells and NK-cells, present at the beginning of the expansion decreased over time, while the composition of Vγ9Vδ2 T-cells increased to as much as 90% of all living cells.

Further phenotype analysis showed that the majority of Vγ9-positive cells were double-negative for CD4 and CD8 markers throughout the whole expansion (Supplementary Figure S2A, available at https://doi.org/10.1016/j.iotech.2025.101053). From day 7 of expansion, however, the Vγ9-positive CD8-positive population started increasing up 10.3% (SD ± 6.7%) of the cultures by day 14. A small fraction of Vγ9-positive CD4-positive cells (3%-7%) could be detected at the beginning but slightly decreased over the 14-day expansion down to 1.5% (SD ± 0.4%). Moreover, building on the interesting findings reported in our previous study,[31] we investigated the expression of various surface molecules of interest, including NKG2D, programmed cell death protein 1 (PD-1) and CD56, which are known to play a

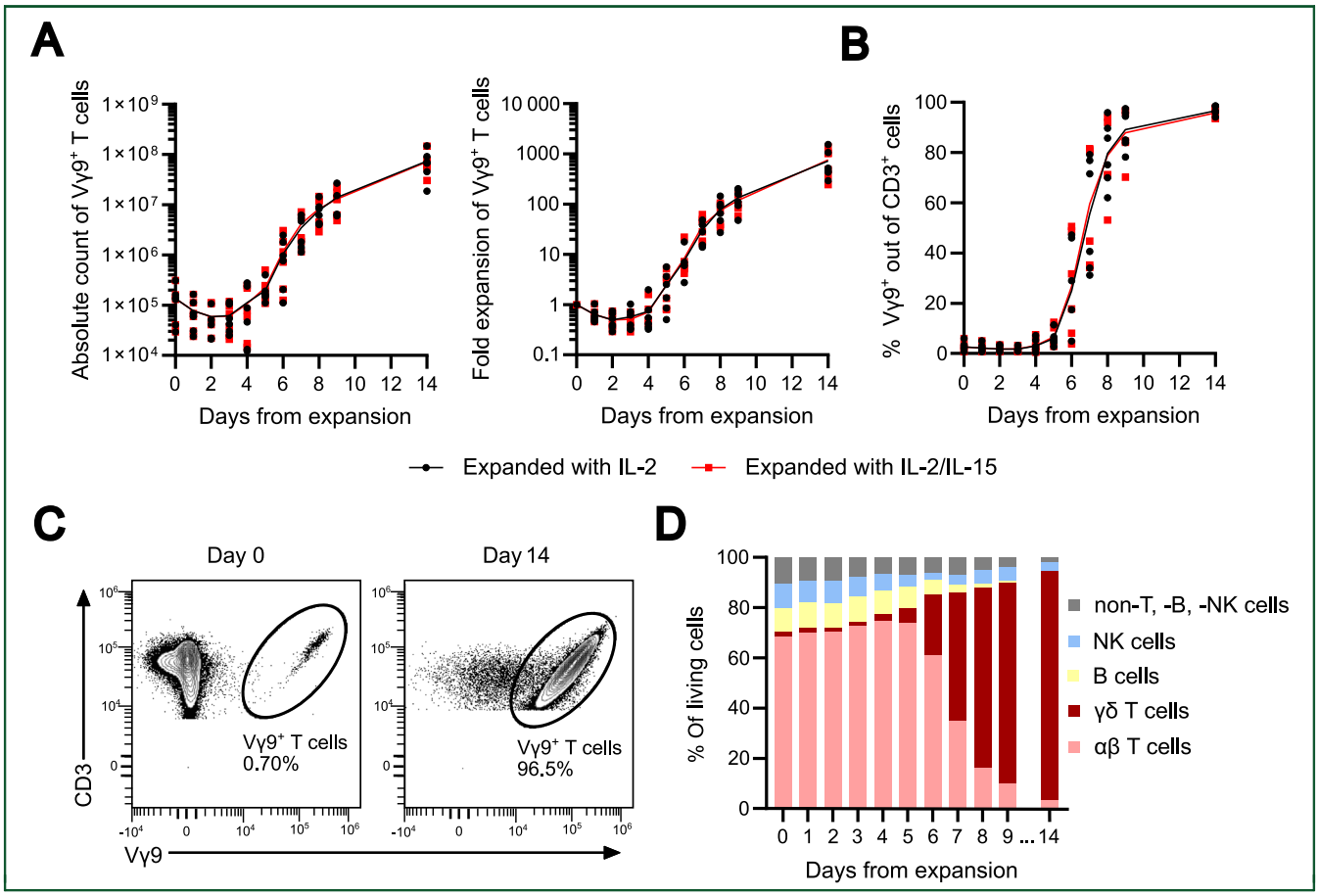

Figure 1. Proliferation and purity analysis of Vγ9Vδ2 T-cell cultures. Vγ9Vδ2 T-cells were expanded with 10 μM zoledronic acid and either 1000 U/ml IL-2 (black) or 100 U/ml IL-2 + 100 U/ml IL-15 (red) from peripheral blood mononuclear cells. (A) Absolute cell count and fold expansion of Vγ9Vδ2 T-cell cultures. Fold expansion was calculated based on the number of Vγ9-positive cells present in the culture at day 0. (B) Purity analysis of Vγ9Vδ2 T-cell cultures with flow cytometry every day from day 0 to 9 and on day 14. (C) Representative contour plots of the percentage of CD3-positive Vγ9-positive cells on day 0 and day 14 of expansion. (D) Mean percentages of the different cell subsets in the cultures expanded with either IL-2 or IL-2/IL-15 throughout the 14-days expansion. Lymphocytes were gated first then doublets were excluded before gating on living cells, and then on CD3-positive cells. Lines represents means of all cultures ($n = 6$, two independent experiments).
IL, interleukin; NK, natural killer.

role in the functionality of Vγ9Vδ2 T-cells. NKG2D expression was high and constant for all six Vγ9Vδ2 T-cell cultures with an average of 93.3% (SD ± 7.5%) of positive cells (Supplementary Figure S2B, available at https://doi.org/10.1016/j.iotech.2025.101053), and the CD56-positive cells increased from 19.5% (SD ± 5.8%) on day 0 to 53.2% (SD ± 7.3%) by day 14 (Supplementary Figure S2C, available at https://doi.org/10.1016/j.iotech.2025.101053). An opposite trend was observed for PD-1 expression, which decreased from 58.6% (SD ± 14.6%) at day 5 to 7.6% (SD ± 3.4%) at the end of the 14-day *in vitro* expansion (Supplementary Figure S2D, available at https://doi.org/10.1016/j.iotech.2025.101053).

Production of anti-CD19 Vγ9Vδ2 T-cells by lentiviral transduction

IL-2-Vγ9Vδ2, IL-2/IL-15-Vγ9Vδ2 and αβ T-cells from the same donor were included in all experiments to ensure comparability of the antitumor effect between the different engineered CAR T-cells. Given the Vγ9Vδ2 T-cell proliferation starts on day 5 of expansion, this timepoint was chosen for lentiviral transduction. Days 7 and 10 of Vγ9Vδ2 T-cell expansion were also evaluated as time points for lentiviral transduction. Transduction at these later stages, however, did not enhance efficiency and instead hindered the proliferation and expansion of the Vγ9Vδ2 cultures (data not shown). Three days after transduction, Vγ9Vδ2 T-cell cultures exhibited 3.7% (SD ± 1.3%) CAR-positive cells, whereas the αβ T-cell cultures showed 30.8% (SD ± 3.2%) CAR-positive cells (Figure 2A, top row).

To increase the percentages of CAR-positive cells in the cultures, a 2-week antigen-specific stimulation in a CD19-coated plate was included after lentiviral transduction. Successful expansion was achieved in six transduced cultures, while four cultures failed due to low CAR expression or outgrowth of αβ T-cells in Vγ9Vδ2 T-cell cultures. After 7 days in the CD19-coated plate, Vγ9Vδ2 T-cell cultures were comprised of 18.7% (SD ± 17.1%) CAR-positive cells, while αβ T-cells displayed 54.3% (SD ± 20.8%) CAR-positive cells (fold increase of 5.1 and 1.8, respectively). By day 14, all cultures exhibited between 72% and 97% CAR-positive cells, corresponding to an average increase of 24.8-fold for Vγ9Vδ2, and 2.7-fold for αβ CAR T-cells (Figure 2A and B), ending with three final CAR T culture productions: IL-2-Vγ9Vδ2 CAR T-cells, IL-2/IL-15-Vγ9Vδ2 CAR T-cells and αβ CAR T-cells.

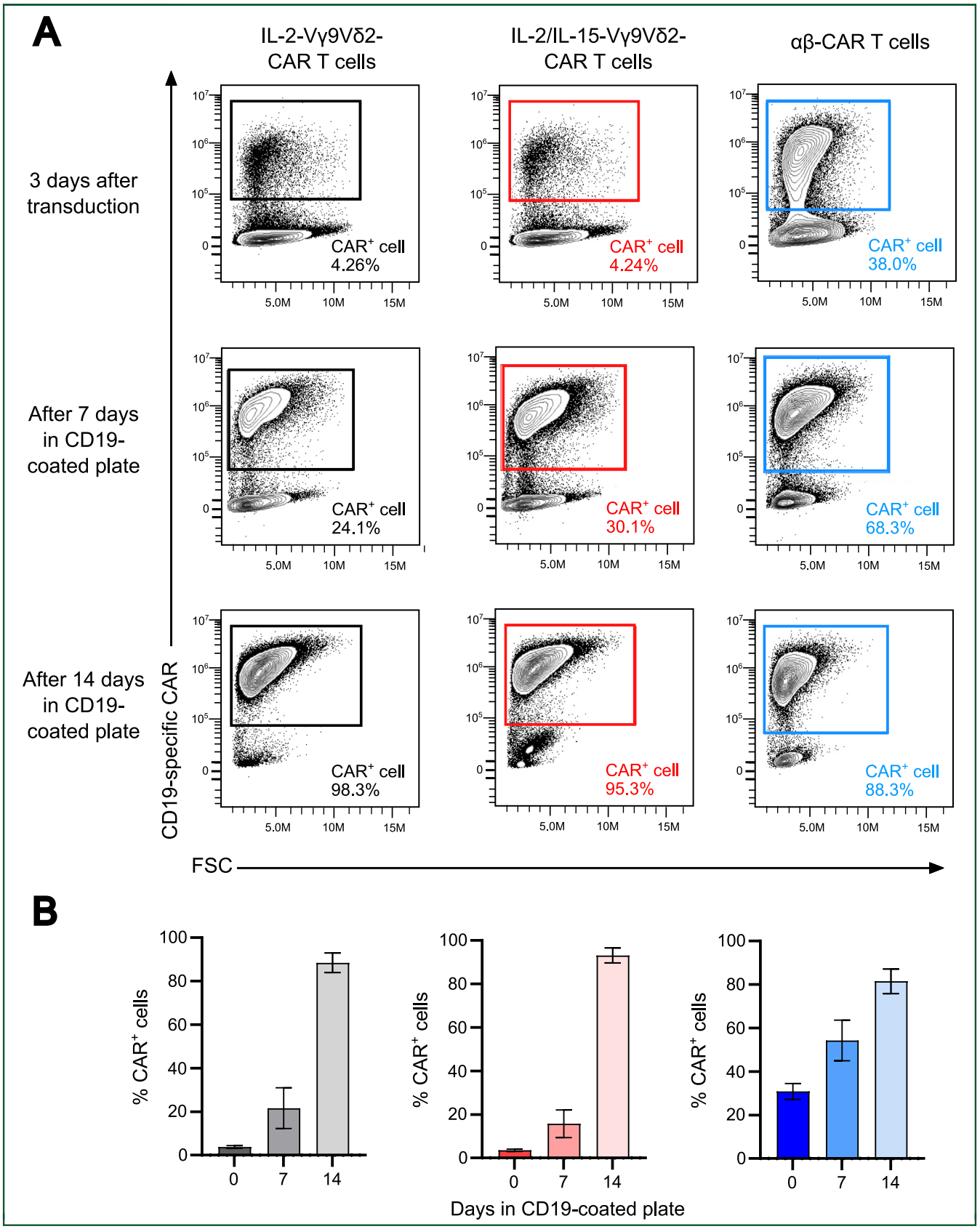

Figure 2. Antigen-specific expansion of Vγ9Vδ2 and αβ T-cells transduced with a CD19-specific CAR. (A) Contour plots of a representative donor showing the percentages of CAR-positive cells in Vγ9Vδ2 T-cells expanded with either IL-2 (black) or IL-2/IL-15 (red) and αβ T-cell (blue) cultures before the Ag-specific expansion started, and after 7 and 14 days in a CD19-coated plate. (B) Percentages of CAR-positive T-cells in all cultures through the 14-day Ag-specific expansion. Bars represent the mean value, and error bars show the standard deviation ($n = 6$, three independent experiments).
Ag, antigen; CAR, chimeric antigen receptor; IL, interleukin.

Vγ9Vδ2 CAR T-cells exhibit potent cytotoxicity against CD19-positive tumor cell lines

The target-specific efficacy of Vγ9Vδ2 CAR T-cells was compared with αβ-CAR T-cells by co-culture with CD19-positive tumor cell lines Daudi, Nalm-6 and CD19-transduced K562 (K562-CD19). Cytokine production and degranulation were assessed by intracellular staining and flow cytometry. All three CAR T-cell productions expressed high levels of the degranulation marker CD107a when tested against the different CD19-positive cell lines (average of 29.6%, 36.6% and 37.2%, respectively), as well as a high tumor necrosis factor-α (TNFα) production (23.9%, 26.3% and 23.9%, respectively). Interferon-γ (IFN-γ) levels were lower across all CAR T-cell cultures, with the highest levels observed by IL-2/IL-15-Vγ9Vδ2 CAR T-cells (8.5% of reactive cells), followed by IL-2-Vγ9Vδ2 CAR T-cells (6.3%), and αβ CAR T-cells (3.8%, Figure 3A).

An xCELLigence real-time cell analysis assay was carried out to study long-term tumor cell killing. All three CAR T-cell productions demonstrated efficient killing of the CD19-positive tumor cells, reaching between 80% and 95% of tumor cell killing after 50 h. Cytotoxicity against K562-CD19 and Daudi target cells was even between the three cultures, and more rapid than the anti-Nalm-6 activity, against which IL-2/IL-15-Vγ9Vδ2 CAR T-cells were moderately most efficient (Figure 3B). This is further demonstrated by the IL-2/IL-15-Vγ9Vδ2 condition being the fastest to reach 50% of tumor cell killing (KT50, Figure 3C).

Considering the clinical importance of post-administration expansion of CAR T-cells,[34] the proliferative capacity upon encountering CD19-positive tumor cells was assessed. IL-2-Vγ9Vδ2, IL-2/IL-15-Vγ9Vδ2 and αβ CAR T-cells all presented strong proliferation upon tumor recognition, with ∼80% of proliferating cells (Figure 3D).

Vγ9Vδ2 CAR T-cells harbor antitumor reactivity with CD19 independence

To explore the potential of Vγ9Vδ2 CAR T-cells to address antigen loss, their interactions with CD19-negative tumors, K562 and Molm-14, were compared with that of αβ-CAR T-cells. IL-2/IL-15-Vγ9Vδ2 CAR T-cells demonstrated the highest reactivity, with an average of 7.6% CD107a-positive, 8.2% TNFα-positive and 6.4% IFN-γ-positive cells. IL-2-Vγ9Vδ2 CAR T-cells presented similar levels of CD107a and TNFα, but only 3.7% IFN-γ-producing cells, while only 1%-3% αβ CAR T-cells were reactive (Figure 4A). The cytotoxicity assays supported the superiority of IL-2/IL-15-Vγ9Vδ2 CAR T-cells yielding 60%-80% tumor cell lysis, followed by IL-2-Vγ9Vδ2 CAR T-cells with 40%-60% of tumor cell killing after 50 h. αβ CAR T-cells exhibited a substantially lower cytotoxicity with only 10%-30% cytolysis after 50 h (Figure 4B). KT20 analysis also illustrates the significance between Vγ9Vδ2 and αβ CAR T-cells against CD19-negative tumors (Figure 4C). The proliferation of all three CAR T-cell cultures upon encountering CD19-negative tumor was decreased, compared with when CD19-positive tumor cell lines were used. A higher percentage of IL-2/IL-15-Vγ9Vδ2 CAR T-cells, however, was found in the second and third generations, compared with the other conditions (Figure 4D).

Vγ9Vδ2 CAR T-cells retain their ability to kill tumor cells during antigen loss

To simulate the progressive phenomenon of antigen loss, mixed-target cell killing assays were conducted using CD19-positive and CD19-negative target cells, K562 and K562-CD19. The composition of antigen-bearing target cells was decreased by 25% intervals, and the CAR T-cell reactivity was assessed at each interval. The consistency and comparability of CD19-positive and CD19-negative ratios throughout the experiments were ensured by flow cytometry analysis and comparison of cell indexes (Supplementary Figure S3A and S3B, respectively, available at https://doi.org/10.1016/j.iotech.2025.101053). All three CAR T-cell cultures achieved 81%-92% cytolysis after 50 h at 100% CD19-positive target cell composition (Figure 5A). As the CD19 composition in the target cell population decreased, both Vγ9Vδ2 CAR T cultures retained partial cytotoxic control even at 0% CD19-positive. The antitumor effect of αβ CAR T-cells, however, was substantially decreased when the target cell population was <50% CD19-positive (Figure 5A). KT20 analysis of the different cultures confirmed this pattern (Figure 5B). Moreover, although the differences were not significant, a trend indicates that the cytotoxic capability of IL-2/IL-15-Vγ9Vδ2 CAR T-cells consistently surpassed that of IL-2-Vγ9Vδ2 CAR T-cells. Collectively, these experiments demonstrate that IL-2/IL-15-Vγ9Vδ2 CAR T-cells can kill selected tumor cells that have lost CD19 expression.

DISCUSSION

Vγ9Vδ2 T-cells are potent anticancer immune cells that exhibit strong *in vitro* cytotoxicity against tumor cells,[35,36] but their clinical efficacy has been limited, despite the proven safety and feasibility of Vγ9Vδ2-ACT.[25,26,37] The potential for CAR-based γδ therapies has been proven as their functionality was comparable to conventional αβ CAR T-cells, consistent with previous studies.[27-29] Presented here are insights into the expansion and production of CD19-directed γδ-CAR T-cells that could contribute valuable benefits for successful clinical translation. Additionally, the advantage of CAR-independent antitumor abilities of γδ CAR T-cells are made evident to address antigen loss, frequently experienced during conventional αβ CAR T therapy.[32]

The expansion of Vγ9Vδ2 T-cells from healthy donor PBMCs by ZOL treatment is a well-established method,[15] however, there is limited knowledge regarding the kinetics of the expansion. Considering the sensitive correlation between cell proliferation and transduction efficiency,[38,39] identification of log-phase expansion of Vγ9Vδ2 T-cells was crucial for CAR T production. Through daily flow

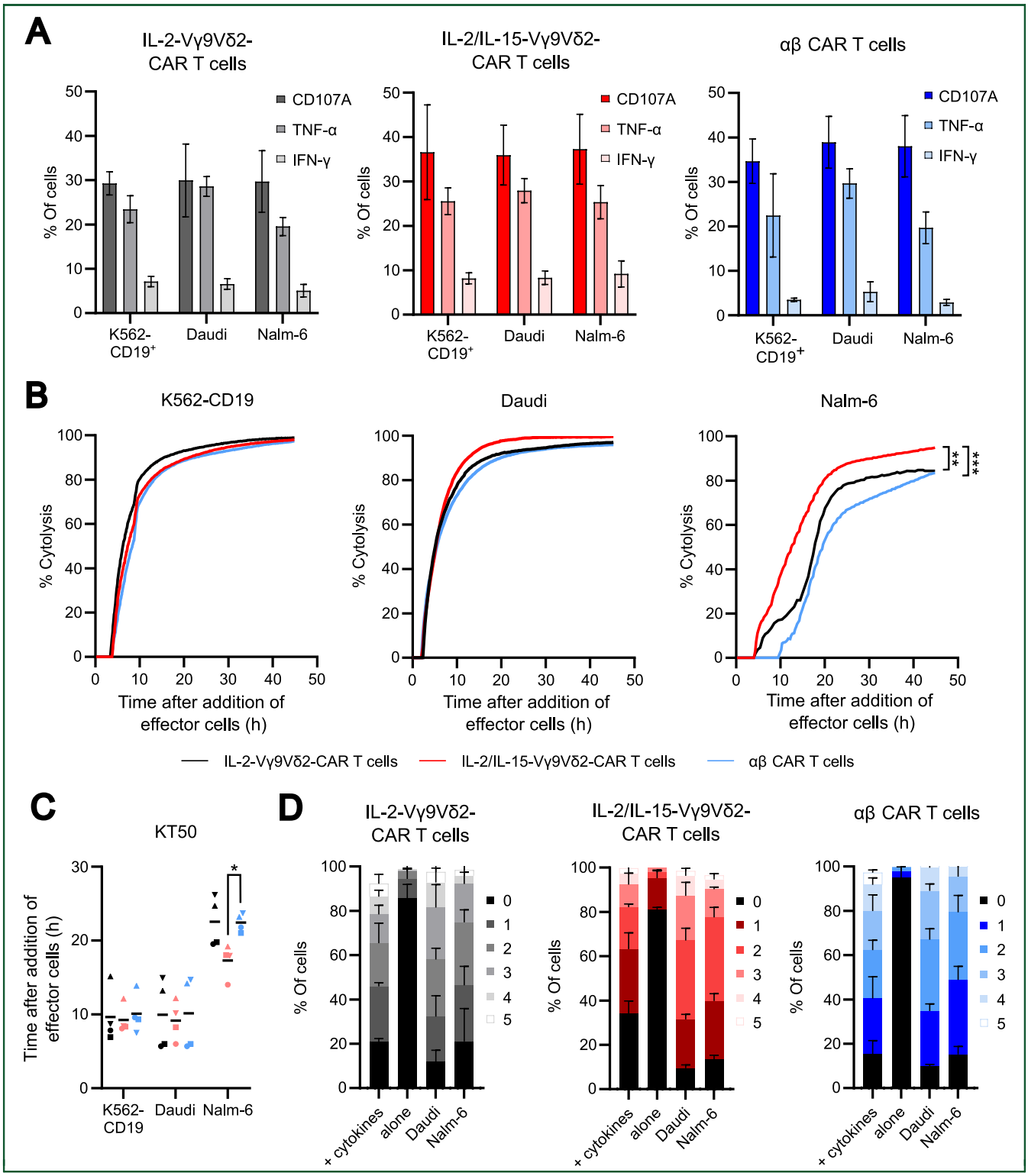

Figure 3. Antitumor effect of Vγ9Vδ2 and αβ CAR T-cells against CD19-positive tumor cell lines. (A) Intracellular staining analysis was used to determine the levels of the degranulation marker CD107a and the cytokines TNF-α and IFN-γ in Vγ9Vδ2 T-cells expanded with either IL-2 (black) or IL-2/IL-15 (red) and αβ T-cell (blue) cultures after a 5-h co-culture with the CD19-positive tumor cell lines K562-CD19, Daudi and Nalm-6 ($n = 4$, three independent experiments). (B) Representative plot for long-term cytotoxicity of Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures against the CD19-positive cell lines K562-CD16 (left plot), Daudi (middle plot) and Nalm-6 (right plot). Data are presented as the average of triplicates. One-way analysis of variance (ANOVA) with Tukey multiple comparisons test was used to compare the different curves. (C) Time to kill 50% of the tumor cells (KT50) was compared between Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures. Each donor is represented with a different symbol, and lines represent the average of all ($n = 4$, three independent experiments). Two-way ANOVA with Tukey multiple comparisons test was used to compare the different cultures. (D) Proliferation of Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T cell upon encountering the cell lines Daudi or Nalm-6. Stacked bar graphs represent the percentage of cells in each generation after a 5-day co-culture with the tumor cells. Bars represent the mean value, and error bars show the standard deviation ($n = 4$, three independent experiments).
CAR, chimeric antigen receptor; IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor-α.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

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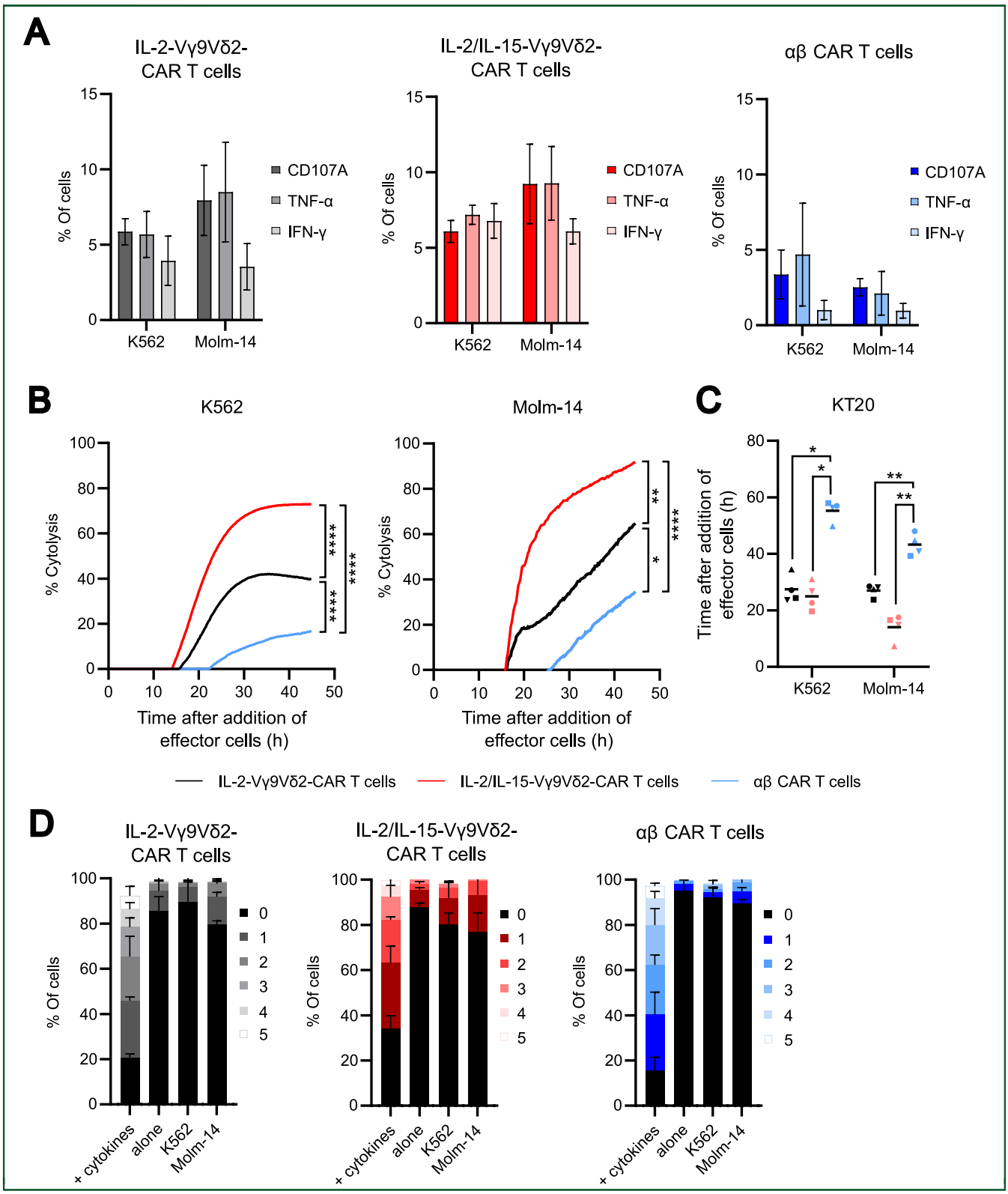

Figure 4. Antitumor effect of Vγ9Vδ2 and αβ CAR T-cells against CD19-negative tumor cell lines. (A) Intracellular staining analysis was used to determine the levels of the degranulation marker CD107a and the cytokines TNF-α and IFN-γ in Vγ9Vδ2 T-cells expanded with either IL-2 (black) or IL-2/IL-15 (red) and αβ T-cell (blue) cultures after a 5-h co-culture with the CD19-negative tumor cell lines K562 and Molm-14 ($n = 4$, three independent experiments). (B) Representative plot for long-term cytotoxicity of Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures against the CD19-negative cell lines K562 (left plot) and Molm-14 (right plot). Data are presented as the average of triplicates. One-way analysis of variance (ANOVA) with Tukey multiple comparisons test was used to compare the different curves. (C) Time to kill 20% of the tumor cells (KT20) was compared between Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures. Each donor is represented with a different symbol, and lines represent the average of all ($n = 4$, three independent experiments). One-way ANOVA with Tukey multiple comparisons test was used to compare the different curves. (D) Proliferation of Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell upon encountering the cell lines K562 and Molm-14. Stacked bar graphs represent the percentage of cells in each generation after a 5-day co-culture with the tumor cells. Bars represent the mean value, and error bars show the standard deviation ($n = 4$, three independent experiments).
CAR, chimeric antigen receptor; IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor-α.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

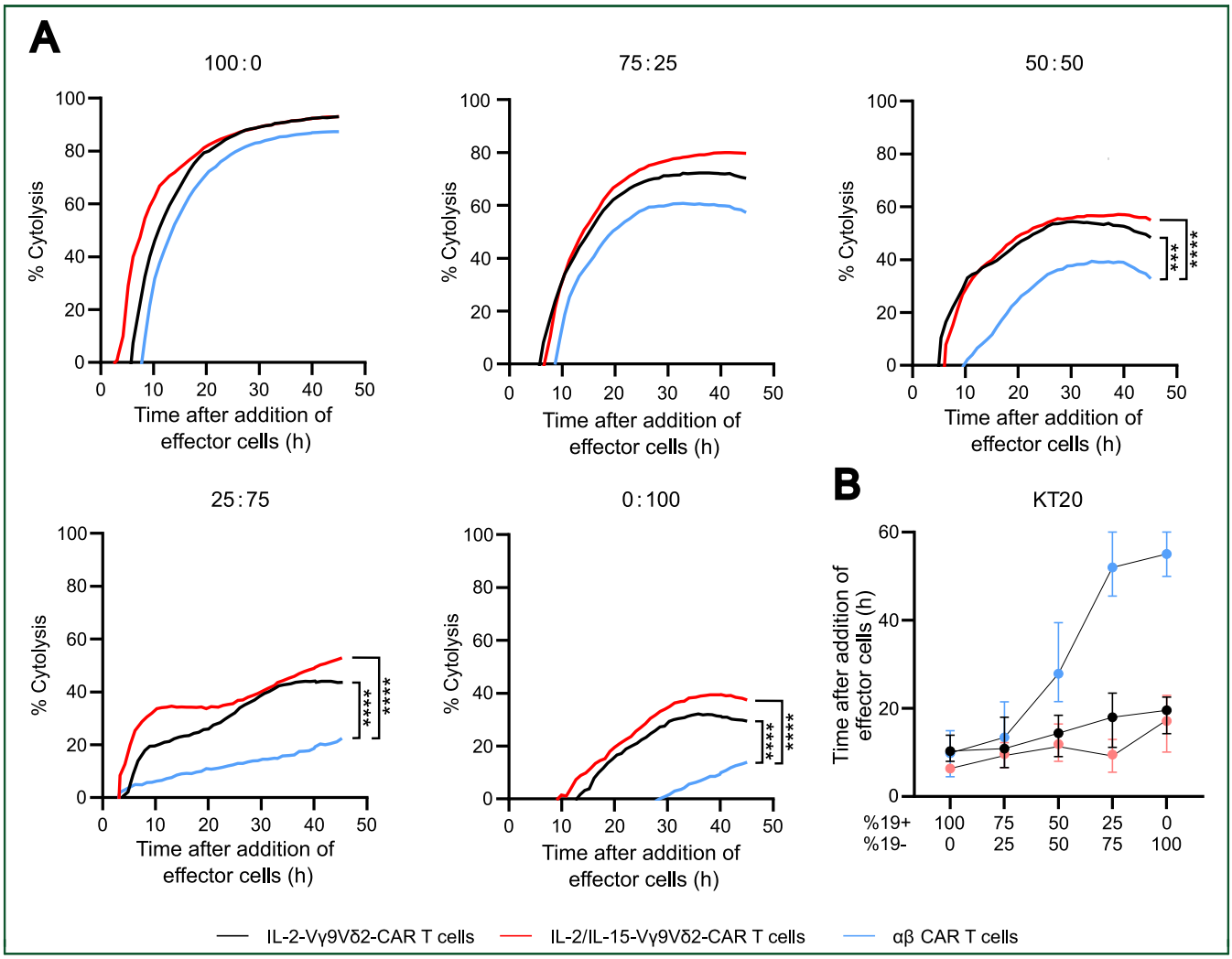

Figure 5. Antitumor effect of Vγ9Vδ2 and αβ CAR T-cells during antigen loss. (A) Long-term cytotoxicity of Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures against mixed tumor population of the CD19-negative cell line K562 and the CD19-positive cell line K562-CD19. Data are presented as the average of duplicates. One-way analysis of variance with Tukey multiple comparisons test was used to compare the different curves. (B) Time to kill 20% of the tumor cells (KT20) was compared between Vγ9Vδ2 T-cells expanded with either IL-2 or IL-2/IL-15 and αβ T-cell cultures. Dots represent average, and error bars represent the range ($n = 3$, two independent experiments).
CAR, chimeric antigen receptor; IL, interleukin.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

cytometry proliferation experiments, the optimal time point for lentiviral transduction was identified as 5 days after ZOL treatment. Still, CAR expression by γδ T-cells ranged between only 3% and 5%. To increase this fraction, an antigen-specific expansion phase was included after transduction, resulting in >70% CAR-positive cells in both αβ and γδ T-cell cultures. Notably, CAR-specific expansion in Vγ9Vδ2 T-cell cultures was observed in only 6 out of 10 donors. In two cases, αβ T-cells outcompeted the γδ population, while in two other donors, the CAR-positive population failed to expand. This limitation was mitigated by implementing a 1-week resting period between transduction and antigen-specific stimulation, as both processes impose significant stress on the cells. The dependency on antigen stimulation for successful γδ-CAR T production, however, foreshadows a limitation for future therapeutic application as an ACT. Therefore, further production optimization is necessary for clinical translation. A potential strategy to enhance transduction efficiency involves characterizing the expression of the low-density lipoprotein receptor on Vγ9Vδ2 T-cells, as its up-regulation has been associated with increased transduction efficiency in various cell types.[40] Additionally, further optimization is required to determine the optimal protocol for antigen-specific expansion on the CD19-coated plate. It is essential to consider that membrane-bound (or plate-bound) antigen is necessary for activation through the CAR, as receptor aggregation is required to induce signaling.[41,42]

γδ CAR T-cells bear robust antitumor abilities, which may help mitigate antigen loss-driven relapse. Upon encounter with CD19-positive tumor cell lines, Vγ9Vδ2 CAR T-cells were as capable of activation and cytotoxicity as conventional αβ CAR T-cells. The most notable advantage of Vγ9Vδ2 CAR T-cells was seen against tumor cells that lack the CAR-specific antigen. While αβ CAR T-cells were minimally reactive and elicited negligible cytotoxic effect against CD19-negative tumor cells, Vγ9Vδ2 CAR T-cells proved to be effective in these parameters, although diminished

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compared with CD19-positive reactivity. To mimic antigen loss, a spectrum of CD19-positive and CD19-negative mixed-target cell co-culture was established ranging from 100% to 0% CD19-positive composition. Cytotoxicity of αβ CAR T-cells was directly correlated with CD19 composition, showing almost no cytotoxicity at the lowest levels. Conversely, Vγ9Vδ2 CAR T-cells did exhibit cytotoxic effects against mixed CD19-positive and CD19-negative tumor populations, throughout the gradient. Further, while differences between IL-2- and IL-2/IL-15-Vγ9Vδ2 CAR T-cells were negligible against tumor cells bearing CAR-targeted antigen, the functional benefit of IL-2/IL-15-expanded cells was clear when tested against CD19-negative cell lines. Not only did they have higher IFN-γ secretive potential, but they also demonstrated markedly higher cytotoxic activity against K562 and Molm-14 cell lines, as well as maintain moderate cytotoxic superiority in the mixed-target experiments. This supports our previous findings that the functional qualities of γδ T-cells can be greatly impacted by methods of expansion[31] which are retained after CAR endowment.

The superiority of γδ CAR T-cells during antigen loss can be partly explained by their multi-faceted ability to recognize cancer cells. While interactions between the γδ TCR and tumor-derived pAgs are likely a driving mechanism behind CAR-independent cytotoxicity, Vγ9Vδ2 T-cells also present an extensive library of NK-cell activation receptors, including NKG2D,[16] NKG2C[43] or DNAM-1,[17] which recognize the stress ligands MICA/B and ULBP1-6,[44,45] HLA-E and Nectin-like-5. The expression of natural cytotoxicity receptors like NKp44 has also been shown on Vγ9Vδ2.[31,46] This collection of receptors illustrates the broad range of tumor cell ligands that Vγ9Vδ2 T-cells can recognize, providing an advantage over conventional αβ CAR T-cells to clear tumor cells after antigen loss. Besides activating receptors, Vγ9Vδ2 T-cells also express inhibitory counterparts such as NKG2A,[43] which competes with NKG2C for binding to HLA-E, or TIGIT[47], which help to dampen Vγ9Vδ2 T-cell activation. Additionally, these cells express varying levels of inhibitory KIRs,[48,49] CD161,[50] 2B4 and ILT2.[51] Their presence could improve the safety of CAR treatment by preventing excessive immune responses.

In conclusion, this study demonstrates the feasibility of generating CD19-specific IL-2/IL-15-Vγ9Vδ2 CAR T-cells from healthy PBMCs through lentiviral transduction. Vγ9Vδ2 CAR T-cells exhibited strong antitumor effect against CD19-positive tumor cell lines, with comparable antitumor effects to conventional αβ CAR T-cells. Moreover, Vγ9Vδ2 CAR T-cells displayed superior efficacy against CD19-negative tumor cell lines, which was augmented by IL-15 inclusion during production, indicating their potential to target tumor cells even after loss of CAR-specific antigen. Therefore, this study lays the groundwork for IL-2/IL-15-Vγ9Vδ2 CAR T therapy as an off-the-shelf alternative treatment of patients with B-cell malignancies, that remains effective after the loss of CD19 expression.

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DISCLOSURE

The authors have declared no conflicts of interest.

DATA SHARING

Data supporting the results and conclusions presented are available upon reasonable request. Requests should be directed to the corresponding authors, and access will be provided according to the institutions applicable policies and laws.

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