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CD19 antigen: from tumor target to safety switch in CAR-T cell therapy

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Authors' Disclosures

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Author Contributions

SM, BDA, CQ and FL designed the experimental studies and supervised the project conduction.
SM, BDA, CQ and FL analyzed the data and wrote the manuscript.
SM, LI, MCQ, MA, MG, PDF and AS developed the *in vitro* models and performed the *in vitro* experiments.
SM, BDA, CQ, LI, MCQ, MA, MG, PDF and AS performed the *in vivo* experiments.
MADI, FQ, CR, PM, MB, FDB and FL provided their expertise to define the clinical translation perspective.
SM, BDA, CQ and FL revised the final version of the manuscript.

All authors read and approved the final manuscript.
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be perceived as a potential conflict of interest.

Data sharing statement

The data analyzed during the current study are available from the corresponding author upon request.

To the Editor,

Chimeric antigen receptor (CAR) T-Cell therapy has transformed the therapeutic landscape of selected hematologic malignancies, demonstrating potent and durable anti-tumor activity in patients with relapsed/refractory B-cell precursor acute lymphoblastic leukemia (BCP-ALL), B-cell Non-Hodgkin Lymphoma (B-NHL), and Multiple Myeloma (MM),(1) and its therapeutic role is increasingly being explored in solid neoplasia. However, inflammatory toxicity remains a possible, undesired consequence of CAR T-Cell activation, as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS)(2-4) are not sporadic complications, but rather frequently diagnosed events following CAR T-Cell expansion. In addition, efforts to translate CAR T-Cell therapy to solid tumors are constrained by on-target, off-tumor toxicity (OTOT).(5, 6) Because many candidate tumor antigens are also expressed in normal tissues, CAR T-Cells may target non-malignant cells expressing the same antigen, thereby increasing the risk of clinically significant adverse effects. Corticosteroids and antibodies-neutralizing specific cytokines such as interleukin-6 (IL-6), IL-1 or γ-interferon (IFNγ)(7) remain the only effective options for severe cases, but they are not always capable to fully control these toxicities and their prolonged use can increase the risk of clinically significant infections in immunocompromised patients.(8)

These limitations underscore an unmet critical need: the identification of reliable inducible safety switches able to inhibit CAR T-Cell activity when toxicity becomes life-threatening.

The most extensively validated safety switch systems in the hematopoietic stem cell transplantation (HSCT) setting are the herpes-simplex thymidine kinase (HSV-TK) and inducible caspase 9 (iCasp9). While HSV-TK has demonstrated clinical efficacy, its viral origin may elicit immune responses in patients treated with HSV-TK transduced T-Cells, (9) and its reliance on ganciclovir, a drug frequently used for cytomegalovirus/HHV-6 treatment in immunocompromised patients, may result in unintended activation of the suicide system, thereby limiting its broader applicability. In addition, the time needed to trigger apoptosis of the target cells may be incompatible with the urgent need of obtaining their elimination. By contrast, iCasp9 provides rapid and efficient apoptosis induction through administration of a synthetic dimerizing agent (rimiducid), with reduced immunogenicity due to its human protein backbone.(10)

We conducted an academic phase I-II clinical trial on the use of GD2-targeting CAR T-Cells in children with relapsed or refractory high-risk neuroblastoma (NB),(11, 12) demonstrating the significant therapeutic potential of this therapy. Nevertheless, CRS occurred in 79.6% of treated patients (43 out of 54),(12) underscoring the high incidence of systemic inflammatory toxicity associated with CAR T-Cell activation also in the setting of solid tumors. Although the iCasp9

system was successfully implemented in this context, the dimerizing agent rimiducid is currently not commercially available, this potentially limiting its broad clinical accessibility.

The lack of an accessible pharmacologic trigger for an otherwise effective suicide platform represents a relevant translational bottleneck. Therefore, alternative, clinically feasible strategies to ensure prompt CAR T-Cell elimination are needed.

In a previously published preclinical study,(13) we demonstrated that the expression of the human truncated CD19 (hΔCD19, extracellular and transmembrane regions of hCD19) on T-Cells may be used as a suicide gene in donor lymphocyte infusion (DLI) approaches, enabling the selective depletion of CD19+/CD3+ T-Cells through exposure to a bispecific T-Cell engager (TCE) targeting hCD19 and CD3 in the event of graft-versus-host disease (GvHD). Here, we extend this approach to CAR T-Cells with the goal of providing a rapid and pharmacologically controllable mechanism for their elimination in the event of undue severe systemic toxicity, with potential applicability also to ICANS, where inflammation-driven blood brain barrier (BBB) dysfunction and altered immune cell trafficking may enable the anti-hCD19-hCD3 TCE to cross BBB despite its limited penetrance under physiological conditions. Indeed, bispecific molecules such as blinatumomab (~54 kDa) have limited ability to cross an intact BBB. However, ICANS is characterized by central nervous system (CNS) inflammation associated with endothelial activation and functional disruption, resulting in increased BBB permeability and altered immune cell trafficking. In this context, inflammatory cytokines such as IL-1β (~17 kDa), IL-6 (~24 kDa), IL-15 (~13 kDa), and GM-CSF (~15 kDa), have been detected in the cerebrospinal fluid of patients with ICANS, consistent with BBB dysfunction compared to physiological conditions. Importantly, a recent clinical report describing the efficacy of blinatumomab in treating CNS leukemia support the possibility of therapeutic effects in the CNS in the setting of inflammatory disease and altered barrier integrity.(14) Specifically, we generated a novel retroviral bicistronic ΔCD19.GD2 CAR construct (Figure 1a, upper panel) by cloning the extracellular region of hΔCD19 antigen in frame with the third-generation CAR.GD2 construct, already in late clinical development.(11, 12, 15)

This design enables the co-expression of the ΔCD19 and CAR construct on T-Cells and, thus, the selective depletion of ΔCD19.GD2 CAR T-Cells upon exposure to the anti-hCD19-hCD3 TCE as shown in Figure 1a, bottom panel. The single-chain variable fragments (scFvs) of this TCE are structurally identical to those of Blinatumomab, the first-in-class TCE molecule approved for relapsed/refractory B-ALL. While Blinatumomab normally binds CD3 on T-Cells and CD19 on B-Cells, in our system, the forced expression of hCD19 on CAR T-Cells allows the TCE to mediate CAR T-Cell elimination through fratricide among T-Cells.

To test this strategy, T-Cells obtained from healthy donors (HDs) were activated, transduced with the ΔCD19.GD2 CAR construct and expanded in the presence of CD3/CD28 antibodies and pleiotropic cytokines (i.e., IL7/IL15). The handling of HDs was conducted in accordance with the Institutional Review Board (IRB) of Bambino Gesù Children's Hospital, IRCCS, Rome, Italy (OPBG; Ethics Committee Approval N°969/2015 prot. N°669LB, and N°1422/2017 prot.N°810).

To exclude the possibility that insertion of the ΔCD19 cassette might impair CAR surface expression, the ΔCD19.GD2 CAR T-Cells were evaluated for CAR expression. By flow-cytometry, using the specific idiotype of the anti-ganglioside GD2 antibody 14G2a (1A7) and the anti-hCD19 antibody, no significant difference in the detection of either of the 2 antigens was observed on Day +5 post-transduction (Figure 1b). Furthermore, the ΔCD19.GD2 CAR expression did not induce any change, in terms of fold expansion (Supplementary Figure 1a) and in CD4+/CD8+ ratio (Supplementary Figure 1b) as compared to control un-transduced (NT) T-Cells. Moreover, ΔCD19.GD2 CAR T-Cells were evaluated for their anti-tumor activity in the context of NB model by long-term (5 days) co-culture cytotoxic assay with SHSY5Y GD2+ NB cell lines. As shown in Figure 1c, ΔCD19.GD2 CAR T-Cells were able to *in vitro* eliminate GD2+ tumor cells at 1:1 Effector:Target (E:T) ratio. Lastly, in order to obtain preliminary *in vitro* evidence of the functionality of the ΔCD19 suicide gene in the context of CAR T-Cell therapy, we exploited the ability of anti-hCD19-hCD3 to recognize and eradicate ΔCD19.GD2 CAR T-Cells in culture assays at pre-defined timepoints. For this purpose, ΔCD19.GD2 CAR T-Cells were exposed either to the bispecific anti-hCD19-hCD3 (an antibody characterized by the scFv of blinatumomab joined by a glycine-serine linker and a hexahistidine-tag (His6)), or to the control bispecific anti-βGal-hCD19 antibody binding hCD19 and E. coli β-galactosidase (an antibody characterized by a scFv specific for human CD19 joined by a glycine-serine linker and a His6 to the scFv of an antibody binding E. coli β-galactosidase). The concentration of anti-hCD19-hCD3 or anti-βGal-hCD19 was 10 ng/mL, as already established in our previous DLI/GvHD model.(13) As shown in Figure 1d, anti-hCD19-hCD3 significantly increased the percentage of dead and apoptotic ΔCD19.GD2 CAR T-Cell levels as compared to anti-βGal-hCD19 treatment or untreated T-Cell condition after both 24 (left panel) and 72 (right panel) hours of exposure, highlighting a direct correlation between the time of anti-hCD19-hCD3 exposure and the ability to eliminate ΔCD19.GD2 CAR T-Cells from the culture. In contrast, anti-hCD19-hCD3 did not affect the corresponding control NT T-Cells (Supplementary Figure 1c). Moreover, the analysis of apoptotic cells at 24 hours demonstrated that the suicide-switch strategy based on ΔCD19 targeting through anti-hCD19-hCD3 TCE induces a rapid elimination of CAR T-Cells, displaying a kinetics and efficacy comparable to those achieved with the more established iCasp9/AP1903 system (Supplementary Figure 1d).

To investigate whether CD19 expression could be an effective safety switch also in a more complex system, we tested this approach in an *in vivo* animal model. The experimental design, described in Supplementary Figure 2, consists of multiple steps to address key safety and efficacy evaluation.

First, we tested the anti-tumor capacity of the novel ΔCD19.GD2 CAR T-Cells against SHSY5Y GD2+ NB xenograft mouse model. As shown in Supplementary Figure 2, mice were intraperitoneally (*i.p.*) engrafted with $0.5x10^6$ SHSY5Y FF-LUC cells on Day -3 and tumor burden was monitored by *in vivo* imaging system (IVIS). On Day 0, after tumor engraftment, mice were randomized in two cohorts, receiving intravenously (*i.v.*) $10x10^6$ NT (n=3) or $10x10^6$ ΔCD19.GD2 CAR (n=8) T-Cells/mouse. As shown in Figure 2a-b, ΔCD19.GD2 CAR T-Cells exerted a significant tumor control compared to NT T-Cells by Day +17 (p-value=0.04). At this point, to evaluate whether the anti-hCD19-hCD3 exposure could be effective in reducing the level of ΔCD19.GD2 CAR T-Cells, half of ΔCD19.GD2 CAR T-Cell treated mice (n=4) were infused with the anti-hCD19-hCD3 TCE (1ug/ day) for a 5-day cycle.(13) A short-term anti-hCD19-hCD3 treatment schedule was adopted to model a potential clinical application, in which transient targeting of CD19+ CAR T-Cells is envisioned to minimize prolonged B-Cell depletion in already immunocompromised patients. Mice were also monitored by IVIS for three weeks until Day +45 to detect possible tumor reemergence. As shown in Figure 2c, flow-cytometry analysis of peripheral blood showed that treatment with the anti-hCD19-CD3 TCE resulted in a significant reduction of circulating ΔCD19.GD2 CAR T-Cells three days after the last TCE dose (Day+24 post CAR T-Cell infusion; $p=2.39\times10^{-6}$) as compared to mice that did not receive the treatment (Figure 2c). Importantly, the anti-hCD19-hCD3 treatment was not associated to the re-emergence of the tumor and we observed a significant re-expansion of the hΔCD19.CAR.GD2 T-Cells in 2 of the mice (#35 and #37; Figure 2c), without a concurrent elevation of the IFNγ level (Figure 2d). Indeed, overall, IFNγ levels measured in peripheral blood of all mice over the course of the experiment, showed that mice infused with of hΔCD19.GD2 CAR T-Cells and treated with anti-hCD19-hCD3 TCE had significantly lower IFNγ level compared to mice that did not receive the TCE (Figure 2d).

At this stage, we evaluated the capacity of residual hΔCD19.GD2 CAR T-Cells to re-expand and control tumor re-challenge after anti-hCD19-hCD3 TCE exposure. For this purpose, $0.5x10^6$ SHSY5Y FF-LUC cells were inoculated again into all surviving mice (all hΔCD19.CAR.GD2 T-Cell-treated mice) and three other untreated mice used as tumor re-challenge control cohort (i.e. mice never inoculated with tumor, effector T-Cells or the drug; Supplementary Figure 2 and Figure 2a, Day+48 post CAR T-Cell infusion). Interestingly, bioluminescence values at day +89 post CAR T-Cell infusion (Figure 2b) showed that a significant anti-tumor activity persists in the cohort of mice receiving anti-hCD19-hCD3 TCE as compared to the control cohort of mice. Moreover,

tumor re-challenge triggered robust activation of hΔCD19.CAR.GD2 T-Cells in the cohort of mice that had not received the anti-hCD19-hCD3 TCE, leading to fatal toxicity in 4/4 mice. Conversely, mice treated with anti-hCD19-hCD3 TCE and re-challenged with the tumor administration did not develop fatal acute toxicity. Indeed, three out of four mice survived until the end of the study, while one mouse succumbed to tumor progression at day 82 (Figure 2e).

Overall, our results suggest that the hΔCD19 suicide gene approach may represent a valid and robust strategy to increase the safety profile of CAR T-Cell therapy, allowing successful control of toxicities.

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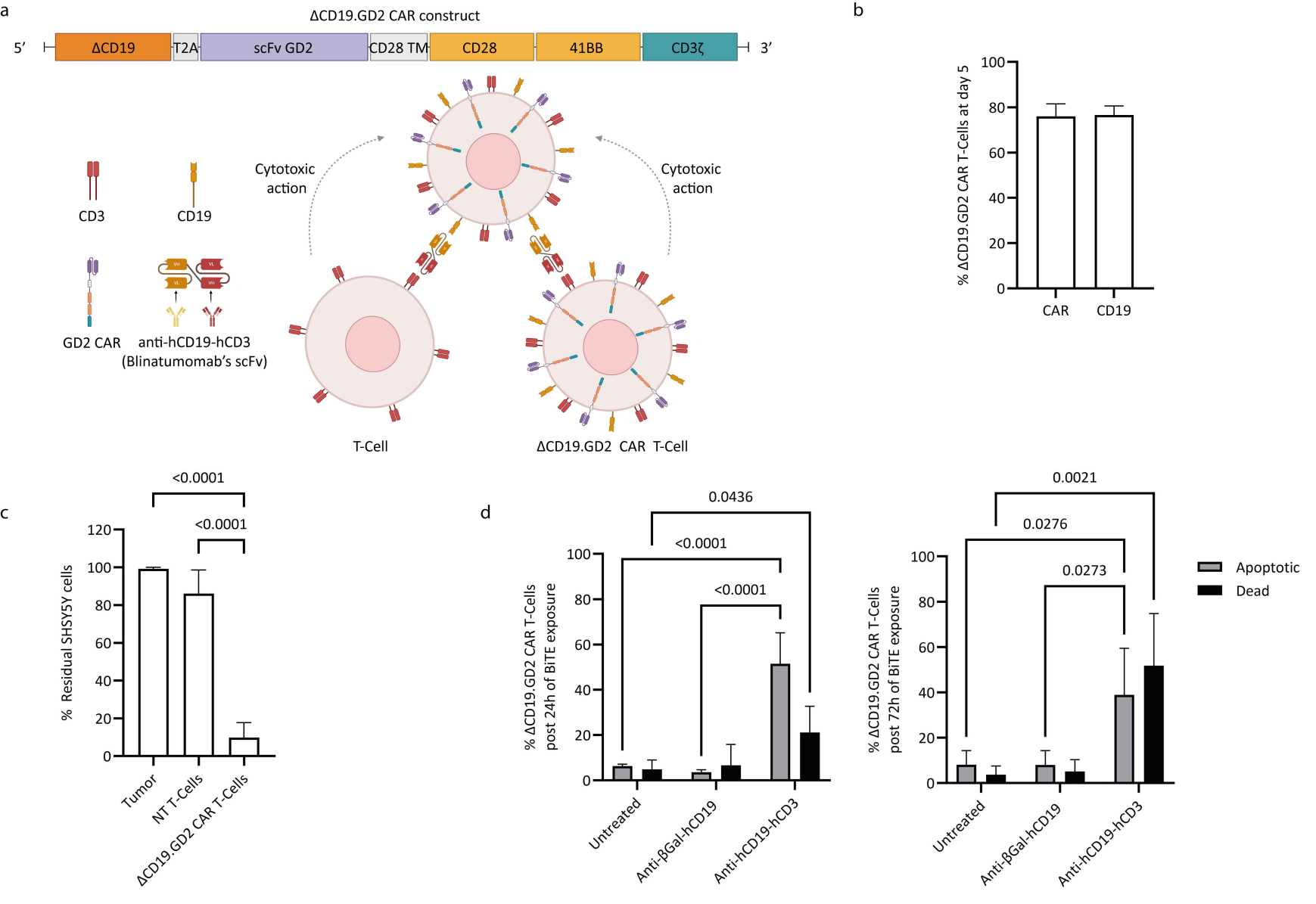
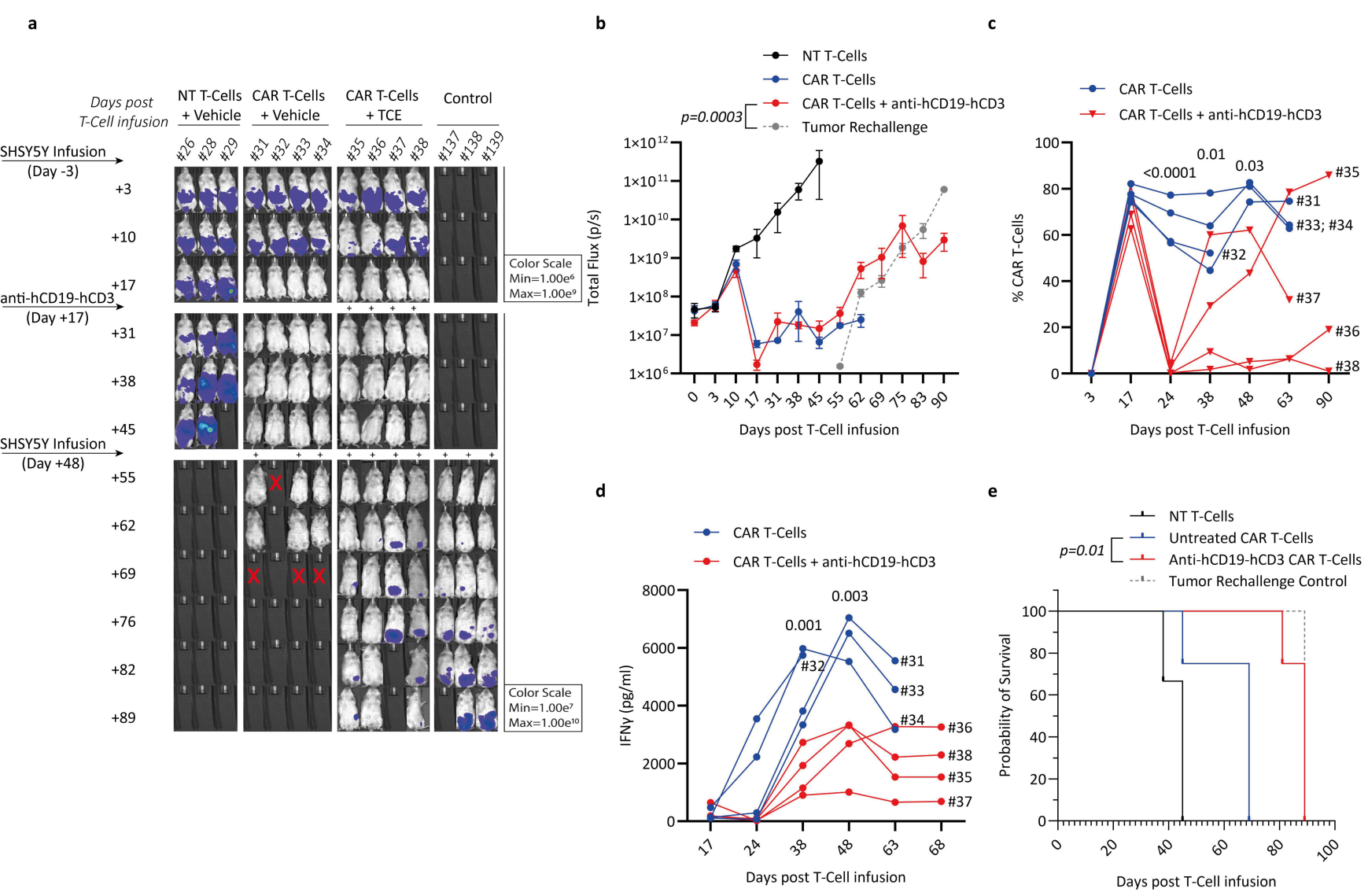
Figure Legends

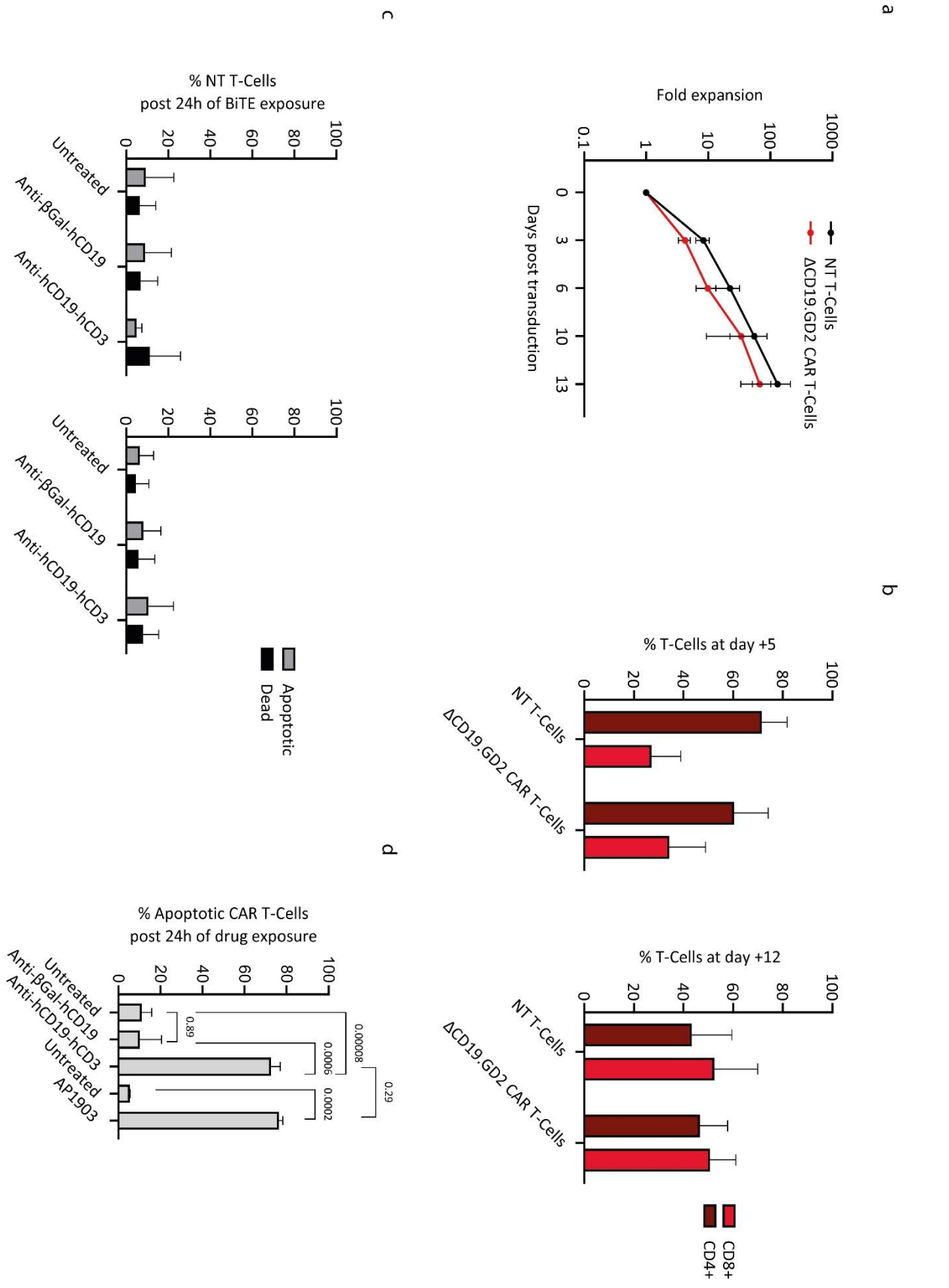
Figure 1. Anti-hCD19-hCD3 significantly recognize and eradicate ΔCD19.GD2 CAR T-cells. (a) Schematic representation of the ΔCD19.GD2 CAR construct (top) and the mechanism of action of the hΔCD19 suicide gene (bottom). Created in https://BioRender.com. **(b)** CAR expression assessed by flow-cytometry using the anti-idiotype antibody 1A7, specific for GD2 CAR scFv, or an anti-CD19 antibody recognizing ΔCD19. Paired t-Test was used as comparison test. **(c)** Cytotoxic activity of NT or ΔCD19.GD2 CAR T cells against GFP^+ SH-SY5Y $GD2^+$ NB cells following 5-day co-culture at a 1:1 E:T ratio. Residual GFP^+ tumor cells were quantified by flow-cytometry. One-way Anova was used as multi comparison test. **(d)** Induction of apoptosis in ΔCD19.GD2 CAR T cells following *in vitro* exposure to anti-hCD19-hCD3 TCE, anti-βGal-hCD19 (negative control recognizing hCD3 and *E. coli* β-galactosidase), or untreated conditions. Early (24 hours, left panel) and late (72 hours, right panel) apoptotic and dead cells were quantified by flow-cytometry. Two-way Anova was used as multi comparison test. All data are expressed in percentage, as mean ± standard deviation (SD).

Figure 2. hΔCD19.GD2 CAR T cell toxicity is controlled by anti-hCD19-hCD3 treatment without impairing anti-tumor activity. (a) Representative bioluminescence imaging of mice in each cohort. **(b)** Quantification of bioluminescence signal for individual mice. Paired t-Test was used as comparison test. **(c)** Flow-cytometry analysis of mice's peripheral blood showing circulating hΔCD19.GD2 CAR T cell levels over the course of the experiment. Paired t-Test was used as comparison test. **(d)** IFNγ levels measured in mice's peripheral blood over the course of the experiment. Paired t-Test was used as comparison test. **(e)** Kaplan-Meier survival analysis showing a statistically significant survival advantage in mice treated with hΔCD19.GD2 CAR T cells and anti-hCD19-hCD3 TCE compared with CAR T cells alone. The log-rank test was used to measure differences between groups.


a
ΔCD19.GD2 CAR construct
5'
ΔCD19
T2A
scFv GD2
CD28 TM
CD28
41BB
CD3ζ
3'
CD3
CD19
GD2 CAR
anti-hCD19-hCD3
(Blinatumomab's scFv)
Cytotoxic action
Cytotoxic action
T-Cell
ΔCD19.GD2 CAR T-Cell
b
% ΔCD19.GD2 CAR T-Cells at day 5
CAR
CD19
c
% Residual SHSY5Y cells
<0.0001
<0.0001
Tumor
NT T-Cells
ΔCD19.GD2 CAR T-Cells
d
% ΔCD19.GD2 CAR T-Cells post 24h of BiTE exposure
0.0436
<0.0001
<0.0001
Untreated
Anti-βGal-hCD19
Anti-hCD19-hCD3
% ΔCD19.GD2 CAR T-Cells post 72h of BiTE exposure
0.0021
0.0276
0.0273
Apoptotic
Dead

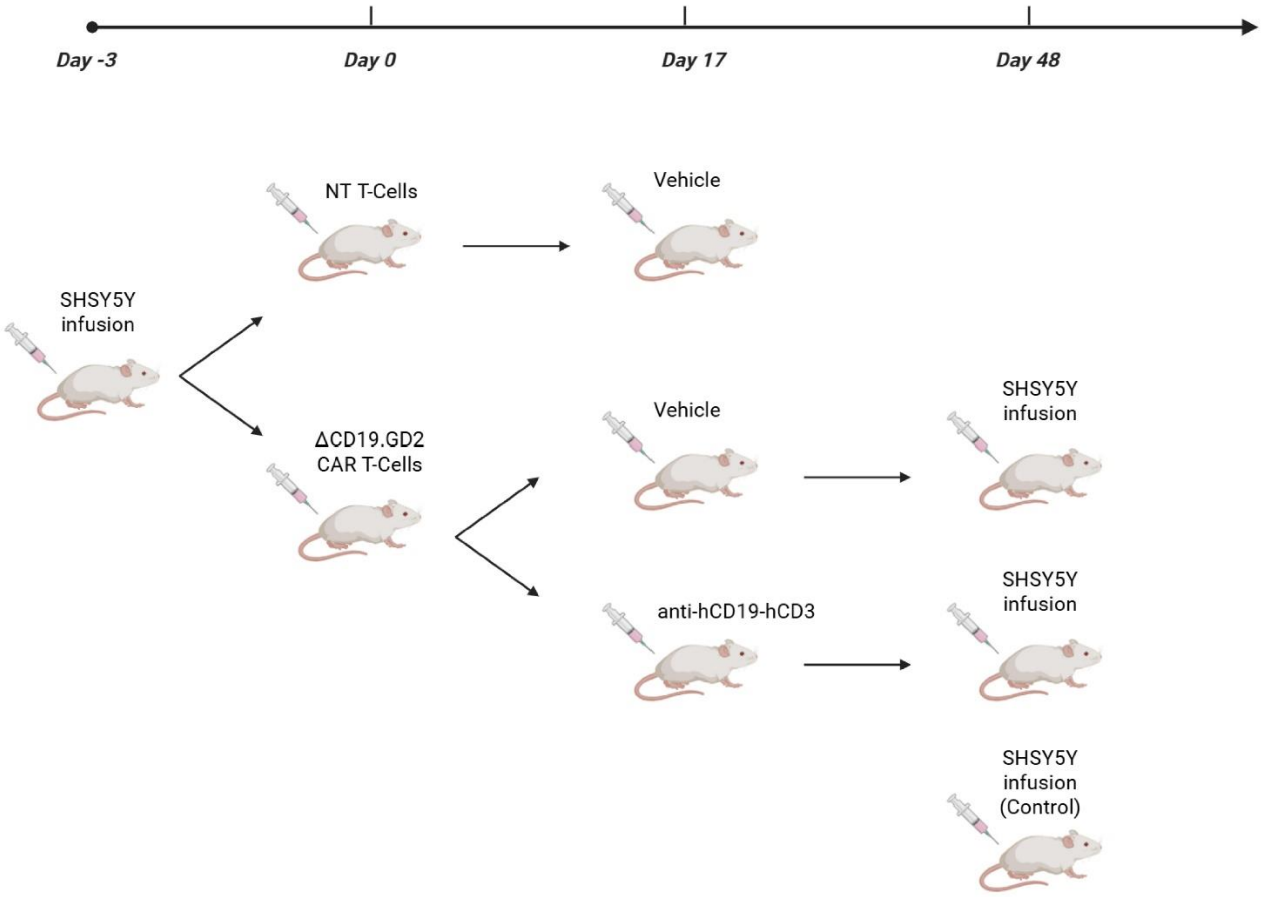

a
Days post T-Cell infusion
NT T-Cells + Vehicle
CAR T-Cells + Vehicle
CAR T-Cells + TCE
Control
#26 #28 #29
#31 #32 #33 #34
#35 #36 #37 #38
#137 #138 #139
SHSY5Y Infusion (Day -3)
anti-hCD19-hCD3 (Day +17)
SHSY5Y Infusion (Day +48)
+3
+10
+17
+31
+38
+45
+55
+62
+69
+76
+82
+89
Color Scale Min=1.00e6 Max=1.00e9
Color Scale Min=1.00e7 Max=1.00e10
b
NT T-Cells
CAR T-Cells
CAR T-Cells + anti-hCD19-hCD3
Tumor Rechallenge
p=0.0003
Total Flux (p/s)
Days post T-Cell infusion
c
CAR T-Cells
CAR T-Cells + anti-hCD19-hCD3
% CAR T-Cells
<0.0001
0.01
0.03
#35
#31
#33; #34
#32
#37
#36
#38
Days post T-Cell infusion
d
CAR T-Cells
CAR T-Cells + anti-hCD19-hCD3
IFNγ (pg/ml)
0.003
0.001
#31
#32
#33
#34
#36
#38
#35
#37
Days post T-Cell infusion
e
NT T-Cells
Untreated CAR T-Cells
Anti-hCD19-hCD3 CAR T-Cells
Tumor Rechallenge Control
p=0.01
Probability of Survival
Days post T-Cell infusion

Supplementary Figure 1



Supplementary Figure 1. Anti-hCD19-hCD3 TCE spares un-transduced T cells and mirrors iCasp9/AP1903 kinetics and efficacy. (a) Expansion kinetics of un-transduced T cells and ΔCD19.GD2 CAR T cells cultured in the presence of IL-7 and IL-15. Paired t-Test was used as comparison test. **(b)** Flow-cytometry analysis of $CD4^+$ and $CD8^+$ T cell subsets in un-transduced T cells (NT) and ΔCD19.GD2 CAR T cells at day 5 (left panel) and day 12 (right panel) of culture. Two-way Anova was used as multi comparison test. **c)** Induction of apoptosis in un-transduced (NT) T-cells following *in vitro* exposure to anti-hCD19-hCD3, anti-βGal-hCD19 (negative control recognizing hCD3 and *E. coli* β-galactosidase). Early (24 hours, left panel) and late (72 hours, right panel) apoptotic and dead cells were quantified by flow-cytometry. Two-way Anova was used as multi comparison test. **d**) Kinetic comparison between the ΔCD19/anti-hCD19-hCD3 TCE system and the iCasp9/AP1903 platform, in which AP1903 acts as the pharmacologic dimerizing agent activating the iCasp9 suicide switch. Apoptosis was evaluated 24h after treatment of hΔCD19.GD2 CAR T cells with 10 ng/mL anti-hCD19-hCD3 TCE or iC9.GD2 CAR T cells with 10 nM AP1903. Data are shown as mean ± SD (n=3). Statistical analysis was performed using a paired t-test.

Supplementary Figure 2



Supplementary Figure 2. Experimental design. Experimental design. Mice were *i.p.* engrafted with SH-SY5Y NB cells on day -3 and tumor burden was monitored by bioluminescence imaging (IVIS). After tumor establishment, mice received *i.v.* infusion of un-transduced (NT) T cells or hΔCD19.GD2 CAR T cells. At day +17, when tumor control was achieved, half of the CAR-treated mice received anti-hCD19–hCD3 TCE (1 µg/day) for 5 days to eliminate circulating hΔCD19.GD2 CAR T cells. Mice were then re-challenged with SH-SY5Y cells to evaluate the ability of residual hΔCD19.GD2 CAR T cells to re-expand and control tumor growth after TCE exposure. Created in https://BioRender.com.