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FULL-LENGTH ARTICLE

Targeting of low ALK antigen density neuroblastoma using AND logic-gate engineered CAR-T cells

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ABSTRACT

Background aims: The targeting of solid cancers with chimeric antigen receptor (CAR) T cells faces many technological hurdles, including selection of optimal target antigens. Promising pre-clinical and clinical data of CAR T-cell activity have emerged from targeting surface antigens such as GD2 and B7H3 in childhood cancer neuroblastoma. Anaplastic lymphoma kinase (ALK) is expressed in a majority of neuroblastomas at low antigen density but is largely absent from healthy tissues.
Methods: To explore an alternate target antigen for neuroblastoma CAR T-cell therapy, the authors generated and screened a single-chain variable fragment library targeting ALK extracellular domain to make a panel of new anti-ALK CAR T-cell constructs.
Results: A lead novel CAR T-cell construct was capable of specific cytotoxicity against neuroblastoma cells expressing low levels of ALK, but with only weak cytokine and proliferative T-cell responses. To explore strategies for amplifying ALK CAR T cells, the authors generated a co-CAR approach in which T cells received signal 1 from a first-generation ALK construct and signal 2 from anti-B7H3 or GD2 chimeric co-stimulatory receptors. The co-CAR approach successfully demonstrated the ability to avoid targeting single-antigen-positive targets as a strategy for mitigating on-target off-tumor toxicity.
Conclusions: These data provide further proof of concept for ALK as a neuroblastoma CAR T-cell target.
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Introduction

Chimeric antigen receptor (CAR) T cells represent a major advance in cancer immunotherapy for hematological malignancies. Targeting solid tumors has proven a significant challenge, especially with regard to identifying target antigens with strong differential expression between cancer and healthy tissues. Moreover, the failure of CAR T cells to persist and proliferate within the suppressive solid tumor environment has limited success in many disease types. Neuroblastoma is the most common extracranial childhood solid tumor and, similar to many childhood cancers, is immunologically cold with limited evidence of adaptive immune response as well as a well-described suppressive tumor microenvironment [1–3].

The targeting of neuroblastoma with CAR T cells is a promising solid tumor application for CAR T-cell technology. Recent clinical trials using anti-GD2 CAR T-cell platforms have demonstrated proof of concept that CAR T cells have the ability to infiltrate solid masses of neuroblastoma to effect at least partial tumor shrinkage [4–7]. Thus far, there have been no reports of significant neurotoxicity in patients targeted with anti-GD2 CAR T cells. However, some pre-clinical studies have suggested that high-affinity anti-GD2 antibodies in CAR T-cell format have the capacity for neurotoxicity [8], a conclusion that has been refuted by other studies [9]. Nevertheless, the theoretical risk of the emergence of toxicity if GD2 CAR T-cell function is boosted by modifications to increase persistence or reduce antigen threshold for activation is strong motivation for seeking alternate and

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complementary neuroblastoma surface antigens. For example, the emergence of GD2-low antigen loss variants, as previously described [10–12], could be countered by a dual targeting approach capable of recognizing antigen-low variants. The toxicity profile of B7H3 CAR T cells following systemic administration is not yet reported.

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase with physiological expression in mice and *Xenopus* that is limited to early development with resultant assumed low levels of expression in post-natal tissues [13,14], although detailed immunohistochemistry studies of cell surface expression comparing healthy tissues with neuroblastoma are lacking. ALK is a recognized driver oncogene of neuroblastoma, as evidenced by genetic studies [15] and by the presence of both inherited and sporadic kinase mutations in neuroblastoma tumors [16–18]. The strong differential expression of ALK between normal and malignant tissues has identified it as a putative tumor antigen and a target for CAR T cells, although its absolute expression in neuroblastoma cells is low [14]. Previous studies for the generation of anti-ALK CAR T cells confirmed proof of concept that anti-ALK CAR T cells can distinguish neuroblastoma from healthy tissues but also established the paradigm that antigen-low tumor targets below a critical activation threshold suboptimally activate CAR T cells and hence fail to elicit durable immune responses [19].

Herein we describe an effort to identify alternate binders for ALK that, in CAR T-cell format, might overcome the limitations of low antigen expression. Although some enhancement in CAR T-cell reactivity was observed compared with a previously established model, it was insufficient for a meaningful response to antigen-low neuroblastoma. However, when ALK CAR was combined with anti-B7H3 or anti-GD2 chimeric co-stimulatory receptors (CCRs) in an AND gate configuration, effector function against ALK-dim neuroblastoma target cells was enhanced, whereas reactivity against B7H3 or GD2 single-positive target cells was avoided.

Methods

Production of anti-ALK single-chain variable fragment phage display library

K562 cells were stably transduced using retroviral transduction to produce recombinant protein comprising the entire ALK ectodomain fused to mouse Fc. Protein was produced in a bioreactor and purified on protein A columns. RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) from splenocytes from SJL/J mice that had seroconverted to specific staining of SupT1-ALK cells following prime and boost vaccinations. Messenger RNA was reverse transcribed (SuperScript III Reverse Transcriptase; Thermo Fisher Scientific, Waltham, MA, USA) and then amplified (AmpliTaq DNA Polymerase; Thermo Fisher Scientific) via polymerase chain reaction (PCR). Fusion of heavy and light chain DNA with a serine–glycine linker was achieved through fusion PCR. Amplified single-chain variable fragment (scFv) library DNA was first cloned into an intermediary pSP73 vector before cloning into a pHEN phagemid vector. *Escherichia coli* TG1 was transduced using electroporation.

Panning of scFv phage display library

Panning of the scFv display library was carried out after helper phage infection using recombinant ALK ectodomain protein generated by OriGene Technologies (Rockville, MD, USA) and coated on selection immunotubes. After two rounds of panning, eluted phages were used to infect fresh bacteria. Soluble scFvs were induced from individual colonies and used to screen for ALK binding specificity. A total of 21 clones with specificity for SupT1-ALK cell staining were identified, and sequencing analysis identified six different binders

Cell lines and cell culture

The cancer cell lines used in the study as well as growth conditions and source are shown in supplementary Table 1. Healthy human peripheral blood mononuclear cells (PBMCs) were isolated from purchased whole blood leukocyte cones via density gradient centrifugation using Lymphoprep (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. Red blood cells were lysed by resuspending in 5 mL of ammonium–chloride–potassium lysis buffer (Lonza, Basel, Switzerland) for 10 min at room temperature. PBMCs were resuspended in 50 mL of Roswell Park Memorial Institute (RPMI) complete medium. The neuroblastoma cell lines used harbored the ALK mutations LAN1 and Kelly (have F1174L) and LAN-5 (has R1275Q) and were obtained from American Type Culture Collection (Manassas, VA, USA).

Molecular cloning

New CARs were assembled from scFv fragments cloned into CAR backbones. Phusion High-Fidelity PCR (New England Biolabs, Ipswich, MA, USA) was used for amplification of products for ligation by restriction digestion and blunt end ligation using standard protocols. New CARs were cloned into an SFG gammaretroviral vector with EF1α promoter, which has been previously described [20].

Retroviral production and T-cell transduction

For ALK and GD2 constructs, 293T cells were plated at 1.5×10^6 in 10-cm plates (Corning, Corning, NY, USA) in 10 mL of Iscove's Modified Dulbecco's Medium prior to transient transfection at 70% confluence using GeneJuice (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Triple plasmid transient transfection incorporated the SFG gammaretroviral vactor, gag-pol and RD114 envelope at an equimolar ratio. Retroviral supernatant was harvested at 48 h and 72 h following transfection and used immediately for T-cell transduction. Briefly, untreated 24-well Costar plates (Corning) were coated with RetroNectin reagent (Takara Bio, Kusatsu, Japan) in phosphate-buffered saline (final concentration of 1 mg/mL) and incubated at 4°C for 24 h. RetroNectin was removed and 1.5 mL of retroviral supernatant was added to each RetroNectin-coated well. Next, the authors added 3×10^5 expanded T cells in 500 μL of RPMI complete medium supplemented with IL-2 (100 units/mL), and plates were then centrifuged at 1000 $\times$ g for 40 min at room temperature before incubation at 37°C. Transduced T cells were harvested after 3 days and washed with RPMI complete medium. Transduced T cells were cultured and expanded in RPMI complete medium with IL-2, and medium was refreshed every 2 days. For the B7H3 CCR and ALK/B7H3 dual CAR constructs, 293T cells were transfected at 80% confluence with GeneJuice using equimolar concentrations of PGK lentiviral vectors, pCMV-dR8.74 packaging plasmids and pCMV-VSV-G envelope plasmids.

Preparation of T cells for transduction

On the day of isolation, PBMCs were depleted of natural killer cells via CD56 depletion using CD56 depletion negative selection columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. PBMCs were then activated with 0.5 μg/mL anti-CD3 and anti-CD28 functional-grade human antibodies (Miltenyi Biotec) to allow for specific T-cell expansion. T cells were expanded in RPMI complete medium supplemented with 100 IU/mL Proleukin (Novartis AG, Basel, Switzerland) every 2 days in 24-well plates at a concentration of 2×10^6 T cells per well. T cells were typically expanded for 72 h after activation before being used for transduction.

Flow cytometry analysis

All samples were analyzed on a BD LSR II (BD, Franklin Lakes, NJ, USA) or CytoFLEX (Beckman Coulter, Inc, Brea, CA, USA) flow cytometer using BD FACSDiva software (BD). For ALK surface expression, the median fluorescence intensity was calculated and compared with controls. In all cases, OneComp eBeads compensation beads (Thermo Fisher Scientific) were stained with the relevant antibodies as per the manufacturer's instructions and used for instrument compensation to prevent fluorescence spillover. All results were analyzed using FlowJo v10 software (BD). TE9 in ALK/B7H3 dual CARs was detected by primary incubation with recombinant human B7-H3 (4Ig)/B7-H3b protein with a C-terminal 10× His tag (R&D Systems Inc, Minneapolis, MN, USA) and secondary staining with anti-His tag antibody (BioLegend, San Diego, CA, USA). Other antibodies used in flow cytometry are listed in supplementary Table 3.

Cytotoxicity assays

Cytotoxicity was determined by 4-h chromium-51 (^{51}Cr) release assay. Briefly, 1×10^6 target cells were labeled with 20 μL ^{51}Cr (PerkinElmer, Waltham, MA, USA) for 60 min at 37°C. Next, target cells were co-cultured with effector CAR T cells at a range of effector:target (E:T) ratios (10:1, 5:1, 2.5:1 and 1.25:1) for 4 h at 37°C in 96-well V-bottom plates (Greiner Bio-One International GmbH, Frickenhausen, Germany). After incubation, the plates were centrifuged at 1500 RPM for 5 min, and 150 μL of the supernatant was transferred to a 96-well OptiPlate-96 HB (PerkinElmer). A total of 50 μL of scintillation fluid was added per well, and the plates were sealed and incubated overnight. The ^{51}Cr release from lysed target cells was counted on a 1450 MicroBeta TriLux scintillation counter (PerkinElmer). Non-transduced T cells were used as a negative control and target cells lysed with 1% Triton X-100 (Thermo Fisher Scientific) were used as a maximum ^{51}Cr release control. In some assays, flow cytometry was used to evaluate cytotoxicity by labeling targets with CellTrace Violet (Thermo Fisher Scientific).

Co-culture assays for cytokine secretion and proliferation

The presence of IL-2 and interferon gamma (IFN-γ) in co-culture supernatant was detected by enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific) as per the manufacturer's instructions. ALK CAR T cells and target tumor cells were plated at a 1:1 E:T ratio in 48-well plates and co-cultured for 24 h at 37°C. The following day, the plates were centrifuged and 600 μL of supernatant was removed for evaluation by ELISA according to the manufacturer's protocol. During these experiments, non-transduced T cells were used as a negative control.

T-cell proliferation on engagement with target cells was measured in the same co-cultures via detection of T cells loaded with CellTrace Violet dye by flow cytometry. Transduced T cells were labeled with a CellTrace Violet cell proliferation kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Briefly, 1 μL CellTrace Violet stock solution was added to up to 1×10^6 ALK CAR T cells, which were then resuspended in 1 mL of phosphate-buffered saline (5-μM working concentration) and incubated for 20 min at 37°C protected from the light. After washing, these labeled T cells were plated into 48-well plates with target cells at an E:T ratio of 1:1 in 1 mL RPMI complete medium. A total of 500 μL of medium was carefully removed and replaced with fresh RPMI complete medium every 2 days. On day 1, day 4 and day 8 of co-culture, cells were removed from the wells and T-cell proliferation and cell phenotype were determined by flow cytometry using two approaches: (i) absolute counting of total and CAR T cells using calibration beads and (ii) quantification of CellTrace Violet dilution using earth mover's distance estimates as previously described [21]. In these experiments, non-transduced T cells were used as a negative control and ALK CAR T cells stimulated with phorbol 12-myristate 13-acetate and ionomycin were used as a positive control.

For all ALK/B7H3 co-cultures, target cells were labeled with CellTrace Violet and effector cells were labeled with carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific) according to the manufacturer's instructions. Labeled cells were then co-cultured at an E:T ratio of 1:1 in 200 μL RPMI complete medium in 96-well U-bottom plates (Corning) for 24 h or 6 days at 37°C in triplicate. Following a 24-h incubation, plates were centrifuged and 150 μL supernatant was frozen for cytokine quantification by ELISA according to the manufacturer's protocol. Remaining cells were stained with LIVE/DEAD fixable near-infrared cell stain (Thermo Fisher Scientific) and acquired by flow cytometry. Total live cell count was quantified using CountBright absolute counting beads (Thermo Fisher Scientific), with targets and effectors differentiated by CellTrace Violet and carboxyfluorescein succinimidyl ester staining, respectively. Cytotoxicity after 24 h was calculated as the percentage of live target cells remaining and proliferation was quantified as fold change of total live effector cells after 6 days. Where stated, target cells were irradiated at 40 Gy prior to co-culture. Irradiated cells were washed, resuspended in RPMI complete medium and 10% dimethyl sulfoxide and stored at −80°C for later use.

Statistical analysis

Data were analyzed with Prism 7 (GraphPad Software Inc, San Diego, CA, USA). Data are displayed as mean ± standard deviation unless otherwise stated. For normally distributed numerical data, parametric tests were used to determine significance of difference between groups. Analysis of variance was used unless otherwise stated. $P < 0.05$ was considered significant.

Results

Anti-ALK CAR constructs show minimal reactivity to antigen-dim targets

Neuroblastoma is a promising target for CAR T-cell therapy, but similar to many solid tumors, there are concerns that some current antigenic targets (GD2, B7H3) might be prone to on-target off-tumor toxicity. Since ALK has a relatively favorable expression profile for avoidance of off-tumor toxicity but relatively low surface expression in neuroblastoma, the authors were interested in determining whether an anti-ALK antibody with optimal binding properties could translate into effective CAR T-cell therapy. To that end, the authors generated an scFv phage library from immunized mice and identified five lead binders with specific binding to ALK-transduced SupT1 cells (see supplementary Figure 1). Binders were cloned into gammaretroviral CAR backbones containing CD28 and CD3ζ endodomains with either CD8 alpha chain hinge and transmembrane (CD8h/tm) or IgG4 Fc and CD28 transmembrane ectodomain spacers (nine constructs cloned) (Figure 1A). The Fc spacers incorporated mutations previously described to avoid interaction with endogenous Fcγ receptors, limiting off-target activation [22]. To screen for binders in CAR T-cell format with increased sensitivity for targets with the low-level ALK expression typically observed in neuroblastoma, we used a panel of neuroblastoma lines that expressed high levels of GD2 but had low expression of ALK on flow cytometry relative to the high-level expression observed in ALK-negative SupT1 cells transduced to express ALK (Figure 1B,C). The nine CAR constructs showed equivalent transduction efficiency in T cells (see supplementary Figure 2). The nine CARs all demonstrated antigen-dependent cytotoxicity (Figure 2A) and inflammatory cytokine secretion (Figure 2B) in response to SupT1-ALK cells expressing high levels of ALK. Six CARs with the highest reactivity—ALK4-Fc-28ζ, ALK8-Fc-28ζ, ALK9-Fc-28ζ, ALK8-CD8-28ζ, ALK9-CD8-28ζ, ALK11-CD8-28ζ—were selected.

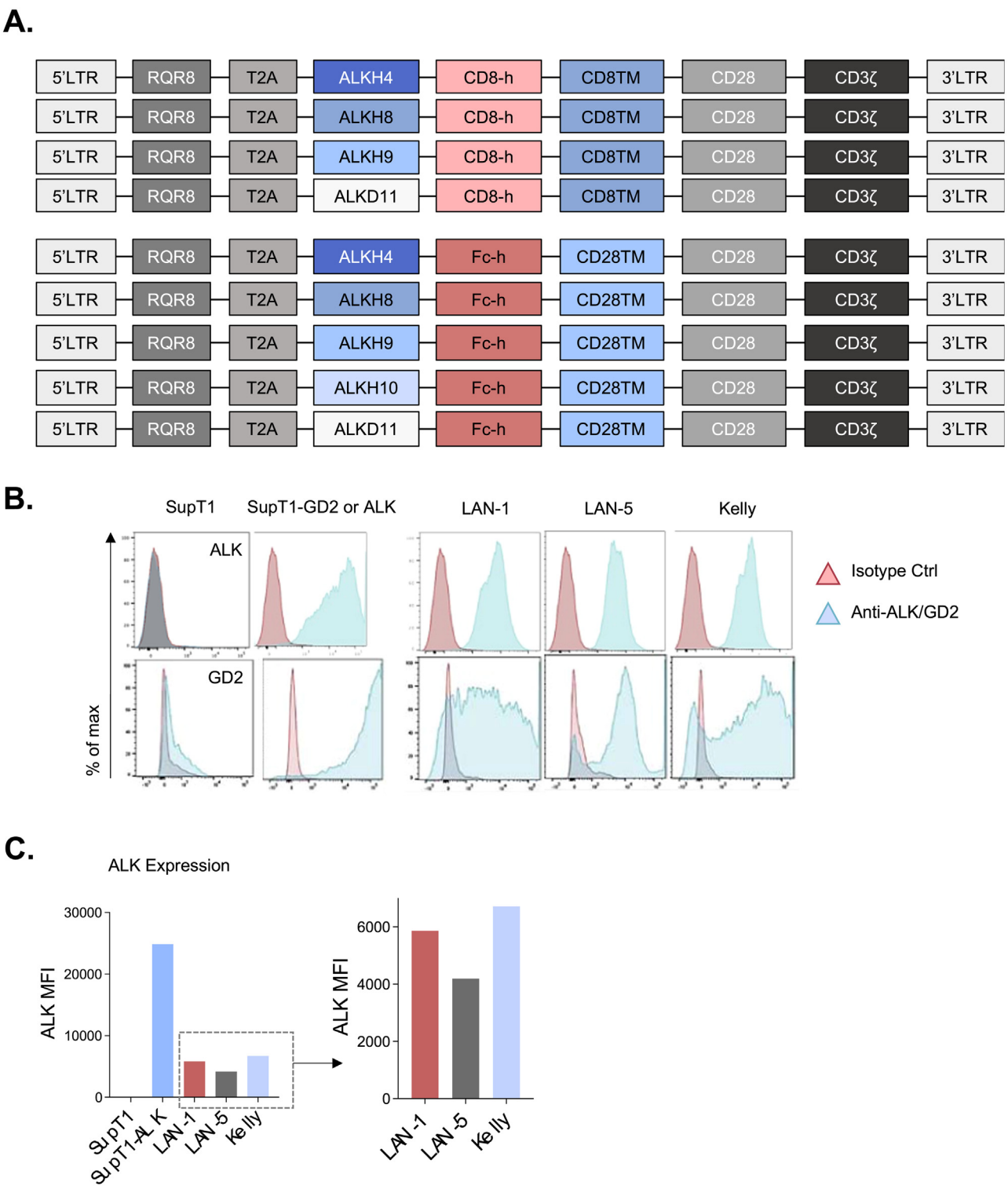

Fig. 1. Anti-ALK CAR constructs and generation of isogenic cell lines. (A) Constructs in SFG gammaretrovirus used for evaluation of novel ALK binders in CAR T cells. *RQR8* is the marker gene expressing human CD34 epitope. T2A is the *Thosea asigna* virus ribosome skip sequence. (B) Expression of cell surface ALK and GD2 in neuroblastoma cell lines and ALK staining with mAb13 monoclonal antibody. (C) Quantification of ALK staining by MFI. Ctrl, control; max, maximum; MFI, median fluorescence intensity.

Short-term co-culture with three ALK-dim neuroblastoma cell lines was used to evaluate CAR functionality against more physiological targets. Although cytotoxicity was significantly higher than that observed with non-transduced controls (Figure 2C), cytokine secretion indicated only very minimal reactivity compared with reactivity against antigen-bright SupT1-ALK cells (Figure 2D). For LAN-1 targets with the lowest level of ALK expression, significant cytotoxicity was observed with only one of the six constructs.

Antigen-dependent proliferative response evaluation identifies a candidate lead CAR lacking tonic signaling

CAR T-cell proliferation response following culture with ALK-positive targets was next used to compare the six lead CARs. Proliferation was quantified using earth mover's distance, which is a measure of distance between two flow cytometry histograms (distributions) and was used here to determine distance between unstimulated and

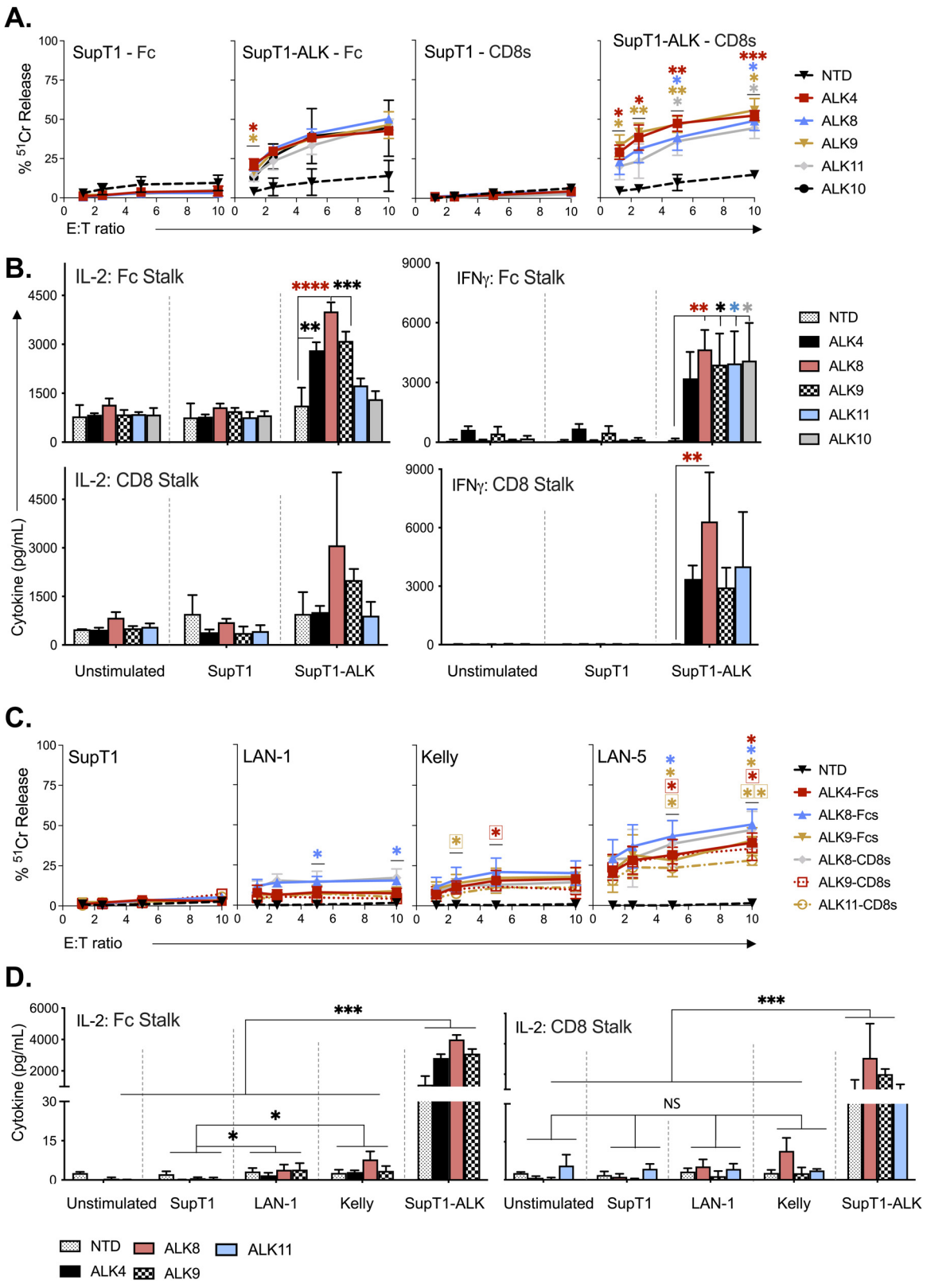

Fig. 2. Anti-ALK CAR T cells show minimal reactivity to antigen-dim targets. (A) Cytotoxicity and (B) cytokine secretion following culture with SupT1-ALK targets of CARs constructed from ALK binders with CD8 and Fc spacers. (C,D) Six selected CARs derived from four ALK binders with either CD8 or Fc spacers were compared for (C) cytotoxicity or (D) cytokine secretion following co-culture with neuroblastoma cell lines LAN-1, Kelly and LAN-5 or ALK-negative SupT1 cells (n = 3 biological replicates). (A–D) Statistical significance was determined with (A,C,D) two-way ANOVA with Greenhouse–Geisser correction, matched values, Dunnett multiple comparisons test and individual variance or (B) Tukey multiple comparisons test. ANOVA, analysis of variance; NTD, non-transduced control T cells.

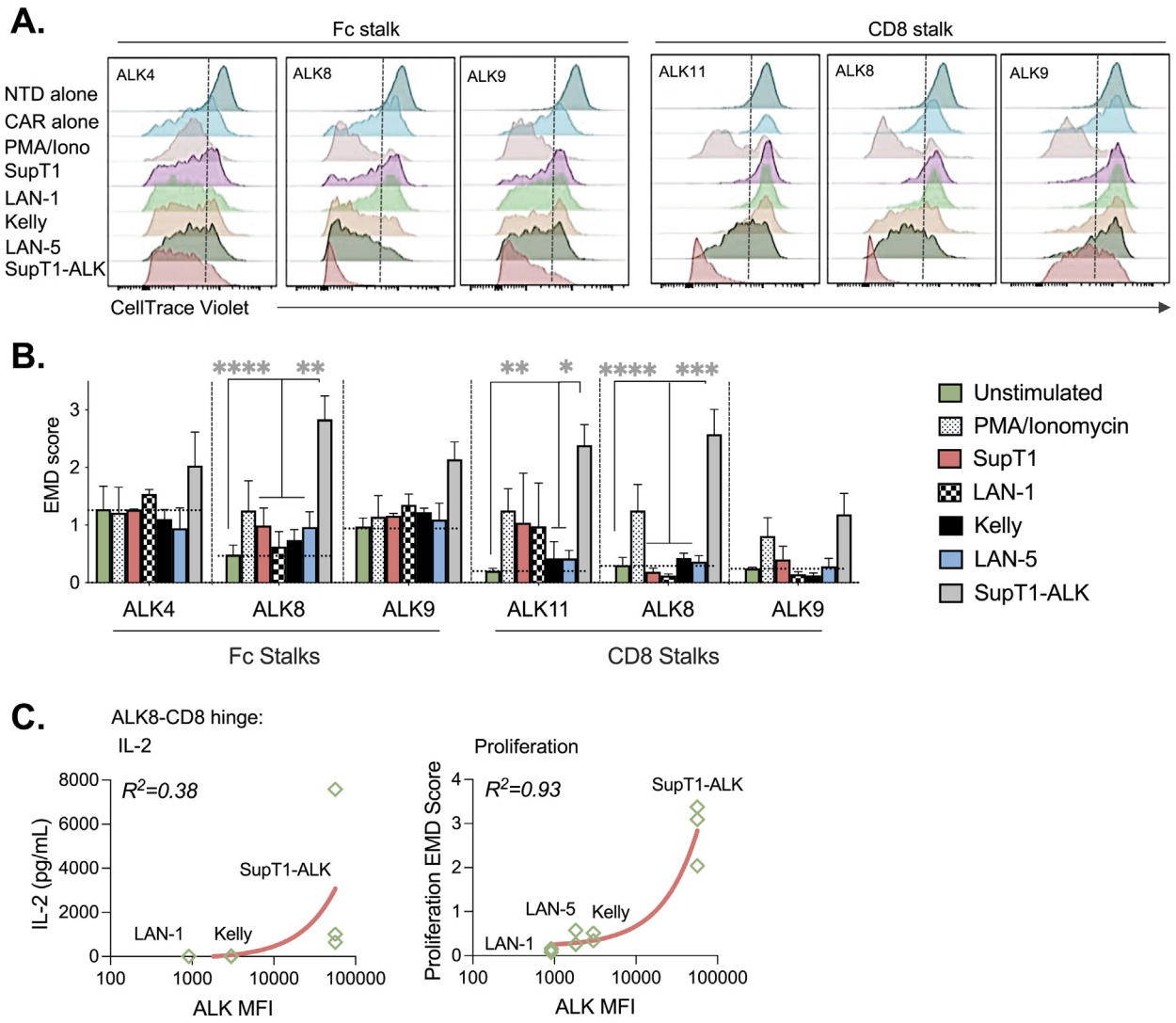

Fig. 3. Antigen-dependent proliferative response evaluation identifies candidate lead CAR lacking tonic signaling. (A) Representative flow cytometry histograms showing dilution of CellTrace Violet as a marker of proliferation. (B) Quantification of dilution shown using EMD. Statistical significance was determined with two-way ANOVA with Tukey multiple comparisons test, with individual variances computed for each comparison (n = 4 independent blood donors). Data are presented as mean ± SEM. Dotted line indicates mean EMD of NTD. (C) Relationship between ALK MFI and IL-2 or proliferation response. ANOVA, analysis of variance; EMD, earth mover's distance; MFI, median fluorescence intensity; NTD, non-transduced control T cells; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; SEM, standard error of the mean.

stimulated conditions. Interestingly, the CAR T-cell constructs with Fc spacer ectodomains showed marked off-target proliferation in the presence of no target or antigen-negative targets (Figure 3A,B), consistent with tonic signaling from the CARs. By contrast, the respective binders with CD8 stalks showed little evidence of antigen-independent proliferation. This phenomenon is best appreciated by comparing the CD8 and Fc spacer-containing domains of the CARs derived from ALK8 and ALK9 binders. The leaky proliferation associated with the Fc stalk was observed in both CD8 and CD4 populations (see supplementary Figure 3). Despite the differences in proliferation associated with the Fc spacer, the authors did not detect differences in memory (see supplementary Figure 4) and exhaustion (see supplementary Figure 5) markers between ALK CARs bearing Fc and CD8 spacers, respectively, although there was a rise in CD8:CD4 ratio in populations with the highest proliferation, consistent with preferential CD8 expansion (see supplementary Figure 6). Of the three CARs with CD8 spacers, none showed a proliferation response that was significantly greater than background in the presence of antigen-low neuroblastoma cells. However, ALK8-CD8-28ζ showed the highest proliferation response to antigen-high SupT1-ALK cells compared with antigen-negative SupT1 cells (Figure 3B) and hence emerged as the lead CAR from the screen. Although the representative donor shown in Figure 3A showed evidence of modest proliferation with LAN-5, this was not significant when evaluated across all donors. Both proliferation and IL-2 production responses of the ALK8-CD8-28ζ CAR were proportionate to the level of expression of ALK on the target cells (Figure 3C). Interestingly, despite leaky proliferation, ALK8 also exhibited the highest Ag-specific reactivity in the lead Fc CARs. A repeat stimulation assay was performed to determine whether any of the CARs with CD8 hinge/transmembrane were capable of significant expansion following weekly stimulation with irradiated targets, and none of the CARs were able to stimulate sustained T-cell expansion (see supplementary Figure 7).

ALK8-CD8-28ζ second-generation CAR shows greater antigen-specific reactivity than a previously described ALK CAR

The ALK CAR previously described by Walker *et al.* [19] afforded the opportunity to benchmark the lead construct (ALK8-CD8-28ζ) against a previously published anti-ALK CAR (designated here as

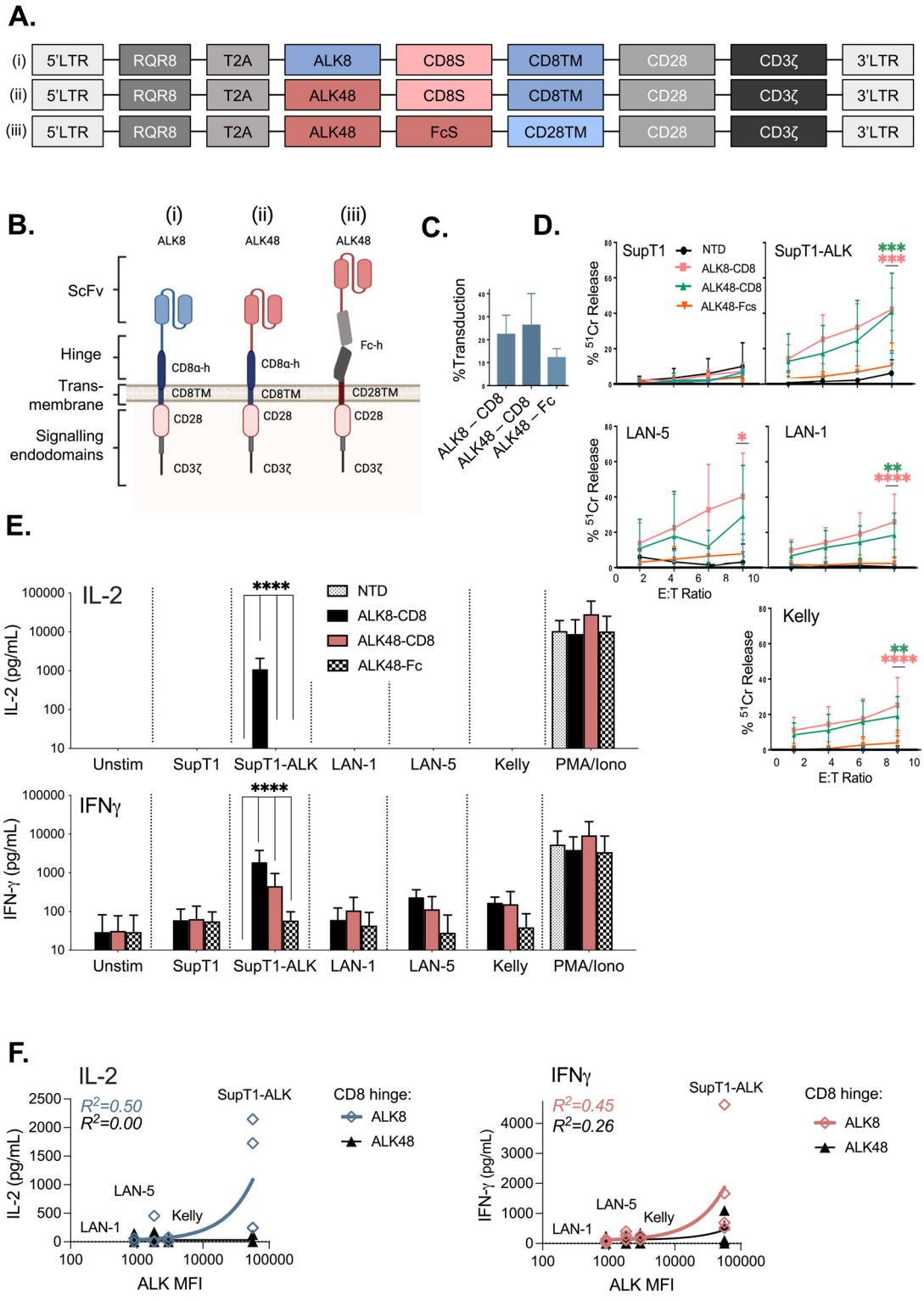

Fig. 4. ALK8-CD8-28ζ second-generation CAR shows greater antigen-specific reactivity than a previously described ALK CAR. (A) Constructs used in comparison. *RQR8* is a marker gene expressing human CD34 epitope. T2A is the *Thosea asigna* virus ribosome skip sequence. (B) Schematic of hinge and transmembrane design. (C) Transduction efficiency as determined by expression of CD34 (n = 3). (D,E) Effector function assays of four ALK CAR constructs evaluated for (D) 4-h cytotoxicity and (E) 24-h assay cytokine secretion (n = 4). (F) Correlation between ALK MFI and IL-2 and IFN-γ secretion. MFI, median fluorescence intensity; NTD, non-transduced control T cells; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; Unstim, unstimulated.

ALK48), which used a CD8 hinge spacer with a CD8 transmembrane. Since epitope position and spacer length and composition can have profound effects on immune synapse formation and CAR T-cell function, the authors cloned the ALK48 binder into second-generation 28ζ format in the same SFG virus, incorporating either CD8 hinge or CH2/CH3 Fc spacers and CD8 or CD28 transmembranes (Figure 4A,B). All three constructs showed transduction efficiency of approximately 15–25% (Figure 4C).

When evaluating ALK CAR T-cell function using cytotoxicity in response to high-level ALK expression by co-culture with SupT1-ALK

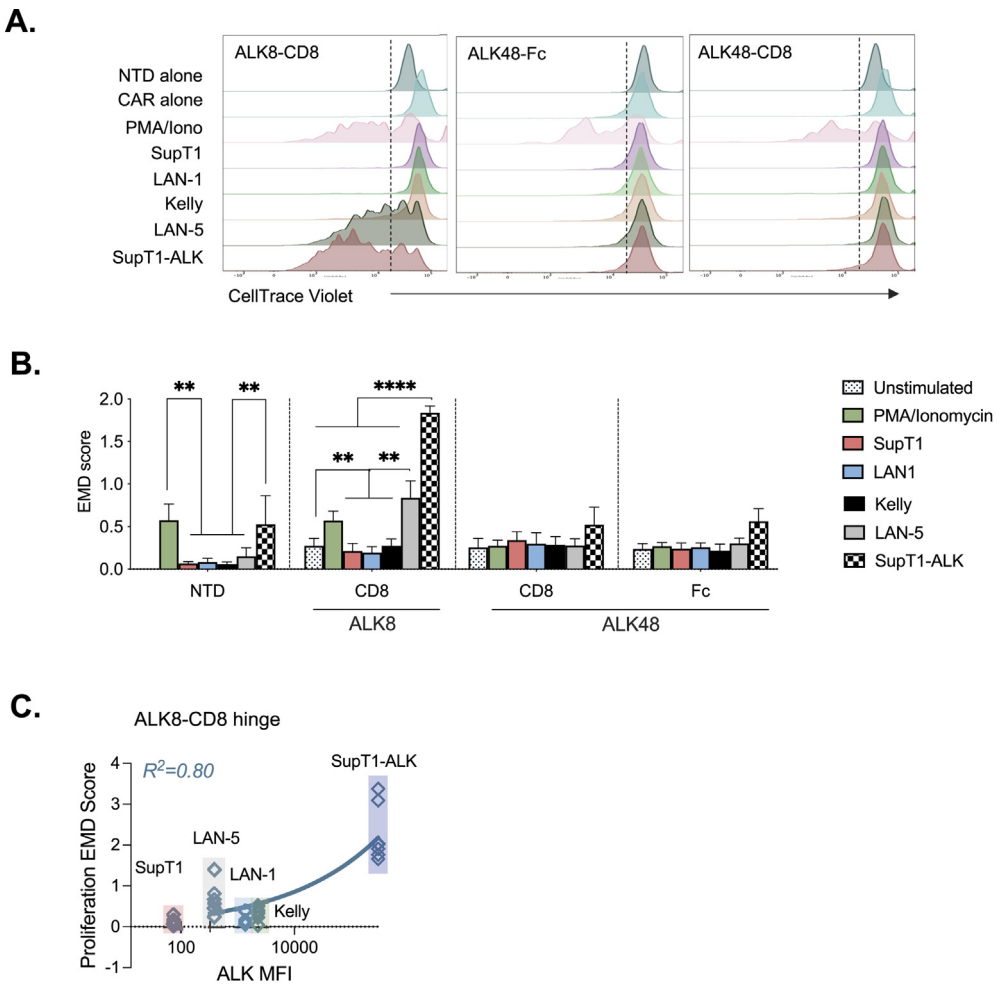

Fig. 5. ALK8-CD8-28ζ second-generation CAR shows greater antigen-specific proliferation than a previously described ALK CAR. (A) Proliferation after 6 days as demonstrated by dilution of CellTrace Violet following co-culture of CAR T cells with neuroblastoma cells or SupT1-ALK cells. PMA/iono was used as a positive control for proliferation. (B) Quantification of proliferation shown using EMD of end proliferation compared with NTD alone (n = 4 independent donors). Error bars represent mean ± SEM. Statistical significance was determined with two-way ANOVA with Tukey multiple comparisons test. (C) Pooled data from independent experiments (n = 7 donors) showing correlation between antigen density and proliferation response. ANOVA, analysis of variance; EMD, earth mover's distance; NTD, non-transduced control T cells; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; SEM, standard error of the mean.

cells or neuroblastoma cells, only ALK8-CD8 and ALK48-CD8 showed specific killing, but there was no significant difference between these binders (Figure 4D). However, cytokine and proliferation assays revealed greater discrimination between constructs. ALK8-CD8 was significantly better than the other constructs in terms of IL-2 response against SupT1-ALK cells (Figure 4E). In co-culture with neuroblastoma lines expressing ALK at a low level, although ALK8-CD8 and ALK48-CD8 showed significant cytotoxicity in short-term killing assays, levels of cytokine response were not significantly above background. In terms of IL-2 response, ALK8-CD8-28ζ was correlated with ALK surface expression, whereas ALK48-CD8-28ζ did not show a correlative relationship (Figure 4F). In contrast to the ALK48 CARs, which did not demonstrate antigen-specific proliferation against ALK-high targets, the ALK8-CD8-28ζ CAR demonstrated enhanced proliferative response to antigen-high targets compared with unstimulated conditions (Figure 5A,B). When evaluating antigen-dim neuroblastoma targets, however, neither ALK8 nor ALK48 CARs induced significant proliferation against ALK-dim LAN1 and Kelly cells (Figure 5B), although the degree of proliferation of ALK8-CD8-28ζ T cells was proportionate to the level of expression of ALK (Figure 5C). Taken together, the data identify ALK-CD8-28ζ as the lead CAR for antigen-specific activity, but its reactivity to neuroblastoma cells naturally expressing low levels of ALK antigen appears subtherapeutic.

An AND gate co-CAR approach with ALK CAR is a strategy for avoiding on-target off-tumor reactivity

ALK is considered a "clean" antigen for neuroblastoma because of the absence of expression in healthy tissues, but it appears limiting in terms of antigen density. Previous studies had identified treatment of neuroblastoma cells with the ALK inhibitor crizotinib as a strategy for increasing its surface expression in ALK-mutated neuroblastoma tumors, thereby increasing sensitivity to immunotherapy [23]. However, in the authors' study, the third-generation ALK inhibitor lorlatinib did not lead to sustained ALK expression in three ALK-mutated neuroblastoma lines (see supplementary Figure 8). Combination studies of lortalininib with ALK CAR T cells were therefore not prioritized.

In contrast to ALK, B7H3 is a neuroblastoma target antigen with high expression, but for which on-target off-tumor toxicity is a theoretical risk - particularly with anti-B7H3 CARs capable of recognising low-level B7H3 on healthy tissues. The authors designed a co-CAR structure based on a first-generation CAR expressing the ALK8 binder and the TE9 anti-B7H3 binder in CCR format (Figure 6A,B). The B7H3 CCR has a CD28 hinge/transmembrane that is not predicted to lead to heterodimerization with the ALK CAR. T cells expressing both receptors are referred to henceforth as ALK/B7H3 co-CAR. Co-expression of

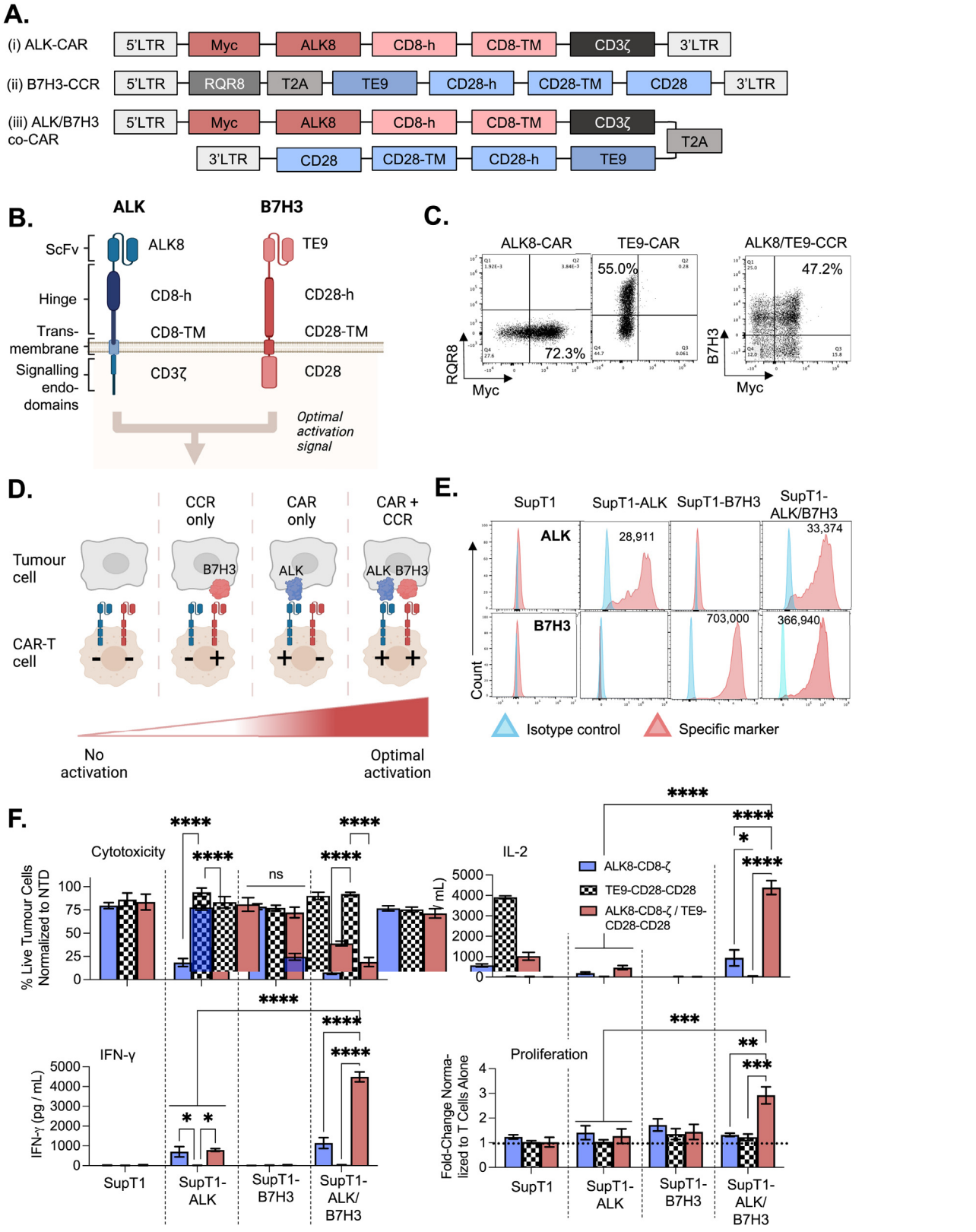

Fig. 6. An AND gate dual receptor approach with ALK CAR and B7H3 CCR is a strategy for enhancing responses to double-antigen-positive cells. (A,B) Schematic of receptors used in the study and structure of expressed proteins. (C) Representative flow cytometry dot plot showing transduction efficiency of doubly transduced cells. (D) Schematic of co-CAR strategy and AND gate concept. (E) Artificial cell line clones derived from SupT1 cells sorted via FACS for high expression of ALK and/or B7H3. (F) Effector function determined after 24-h co-culture with indicated target cells by flow cytometry (cytotoxicity) or cytokine secretion (ELISA) or after 7 days by flow cytometry (proliferation) (n = 3 independent donors). Error bars indicate mean ± SEM. Statistical significance was determined with two-way ANOVA with Tukey multiple comparisons test. ANOVA, analysis of variance; FACS, fluorescence-activated cell sorting; SEM, standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

both components was achieved via expression from a lentiviral vector encoding a single transcript and protein components separated by a self-cleaving T2A sequence (Figure 6C). The arrangement of signaling domains is such that full T-cell activation (signals 1 and 2) should be achieved only if both antigenic targets are present on a target cell (Figure 6D). To effect controllable activation via one or both receptors in dual CAR-positive T cells, the authors constructed SupT1 cells that expressed B7H3 or ALK or both (Figure 6E). Co-culture of the dual CAR constructs against engineered T cells showed short-term cytotoxicity that was restricted to the presence of ALK on target cells since signal 1 of T-cell activation is necessary and sufficient for short-term killing [24]. Production of inflammatory cytokines IL-2 and IFN-γ and proliferation response over 7 days were measured as a readout of effective co-stimulation. Maximal T-cell activity was observed only if both receptors were present in T cells and both antigens were present in target cells, consistent with a Boolean AND gate (Figure 6F).

To determine whether this dual CAR strategy could amplify ALK CAR signaling using a different model, the authors evaluated a CCR incorporating the GD2 antibody huk666 [20] separated from the CD28 co-stimulation domain by an Fc domain spacer and CD28 transmembrane. Here co-expression of both receptors was achieved by co-transduction with two separate gammaretroviral vectors (Figure 7A,B), and differential stimulation was attained through SupT1 cells expressing ALK or GD2 or both (Figure 7C). Transduction efficiencies of both single and double CAR constructs ranged from 55% to 75% (Figure 7B). The authors first evaluated ALK/GD2 dual CAR cytotoxic capacity by CD107a staining via co-culture with SupT1 cells expressing both target antigens. Following 18-h stimulation in the presence of both target antigens, T cells expressing the ALK/GD2 co-CAR showed the highest levels of CD69 upregulation (2- to 3-fold greater than in the single CARs), although some CD69 increase was observed through engagement of the GD2 CCR, possibly associated with signaling from this receptor (Figure 7D). IFN-γ and IL-2 responses of ALK/GD2 co-CAR T cells to ALK and GD2 double-positive targets were significantly higher compared with single-antigen-positive targets and higher than that observed with single CAR T cells cultured with the same targets, indicating augmented activity of ALK CAR T cells induced by the GD2 CCR.

The authors next investigated whether the dual CAR approach was capable of inducing proliferation and cytokine responses when cultured with neuroblastoma cells expressing low levels of cell surface ALK. The ALK/GD2 co-CAR T cells generated detectable IL-2 on culturing with the three neuroblastoma lines (Figure 7E), whereas insignificant amounts had been generated by ALK8-CD8-28ζ CAR T cells with the same targets (Figure 2D). Proliferative responses of the ALK/GD2 co-CAR T cells following culture with irradiated targets were similarly greater than responses with the single-antigen-recognizing constructs (Figure 3B).

Taken together, the authors show that augmentation of ALK CAR signal 1 through co-expression of a CCR targeting alternate neuroblastoma antigens (B7H3 or GD2) increases responses. Moreover, this co-CAR approach is effective at distinguishing single- and double-antigen target cells for optimal recognition, provides greater cytokine and proliferative response to antigen-dim targets than conventional second-generation ALK CAR and lays a foundation for the addition of future engineering modules to enhance sensitivity to antigen-low targets.

Discussion

CAR T-cell signaling follows the formation of an immune synapse between T cell and target cell. The speed of formation and signal strength arising from the synapse are dependent on a number of factors, not least of which is receptor–ligand binding kinetics [25]. Binding kinetics are determined by both affinity of the ligand–receptor interface and avidity, which is a function of target antigen density and CAR expression. Fine-tuning of CAR T-cell construct designs to provide optimal signal from the immune synapse is an important research question since overactivation can lead to exhaustion/dysfunction or on-target off-tumor reactivity to antigen-low healthy tissues, whereas too high an antigen density threshold for signaling may lead to inadequate effector function against tumor cells. Previous studies evaluating ALK as a CAR T-cell target have concluded that ALK target antigen expression in neuroblastoma is below the threshold for adequate CAR T-cell signal and effector function [19]. The authors reasoned that an scFv targeting ALK with favorable binding kinetics for immune synapse stabilization might be one solution to allow signal strength to achieve a threshold for full effector function of ALK CAR T cells against antigen-low neuroblastoma. Recent studies into the relationship of antibody affinity with CAR T-cell function have led to some generalizable findings, such as the observation that fast off-rate can lead to short dwell time and potentially diminish T-cell exhaustion [26,27]. However, the precise rules governing the relationship between binding kinetics and quality and quantity of T-cell signal have yet to be elucidated, and the authors therefore followed an empirical approach of screening a panel of CAR T-cell constructs by effector function.

The authors' strategy was to generate and directly screen an scFv library of novel binders rather than generate single-chain antibodies from existing full-length whole antibodies. An scFv library was generated following immunization of mice with the whole ALK ectodomain, and five scFv sequences showed convincing ALK binding by flow cytometry. The authors empirically evaluated the five binders with two spacer designs, both of which provided flexibility and length to maximize the chance of interaction with the respective ALK epitopes. Interestingly, of the four binders where it was possible to compare CARs with CD8 and Fc spacers, the authors observed similar maximal reactivity (cytokine secretion and proliferation), but the Fc spacer-containing CARs showed marked target-independent proliferation and less cytokine secretion compared with the equivalent construct with a CD8 spacer. Previous studies have described off-target effects of Fc spacer-containing CARs through interaction with Fcγ receptors, and the authors' constructs incorporated mutations to mitigate these effects [22]. The authors' experiments indicated a number of possible mechanisms for this leaky behavior of Fc-containing CARs. The authors speculate that it may be related to clustering of CARs in the absence of antigen through interactions between Fc regions or interaction with Fcγ receptor-expressing cells in the culture conditions.

To the authors' knowledge, this study is one of only two to have described targeting ALK through the use of CAR. Both the authors and Walker *et al.* [19] employed an antibody scFv fragment to engage the ligand. Although the natural ALK ligands have now been identified [28,29] and could be evaluated as an alternate approach, this might not overcome the inherent limitation of low-level ALK antigen expression in neuroblastoma. One strategy the authors explored to solve the inherent limitation was upregulation of surface ALK through the addition of an ALK inhibitor, as has been previously described with the ALK inhibitor crizotinib [23]. However, in the authors' study, lorlatinib, a third-generation ALK inhibitor, did not consistently increase surface expression in the three target cell lines with known ALK mutations, and the role of drug combinations is unproven for ALK CAR.

An alternate strategy for increasing T-cell activation in the presence of low levels of ALK is through further modification of the CAR to increase signal strength; for example, use of a CD28 hinge and transmembrane or additional immunoreceptor tyrosine-based activation motifs in the CD3ζ component of the CAR endodomain [30]. However, the authors' approach was to attempt to boost weak signal 1 through a strong co-stimulatory signal (signal 2) derived via a CCR. The authors have previously shown the use of a CCR to be a useful

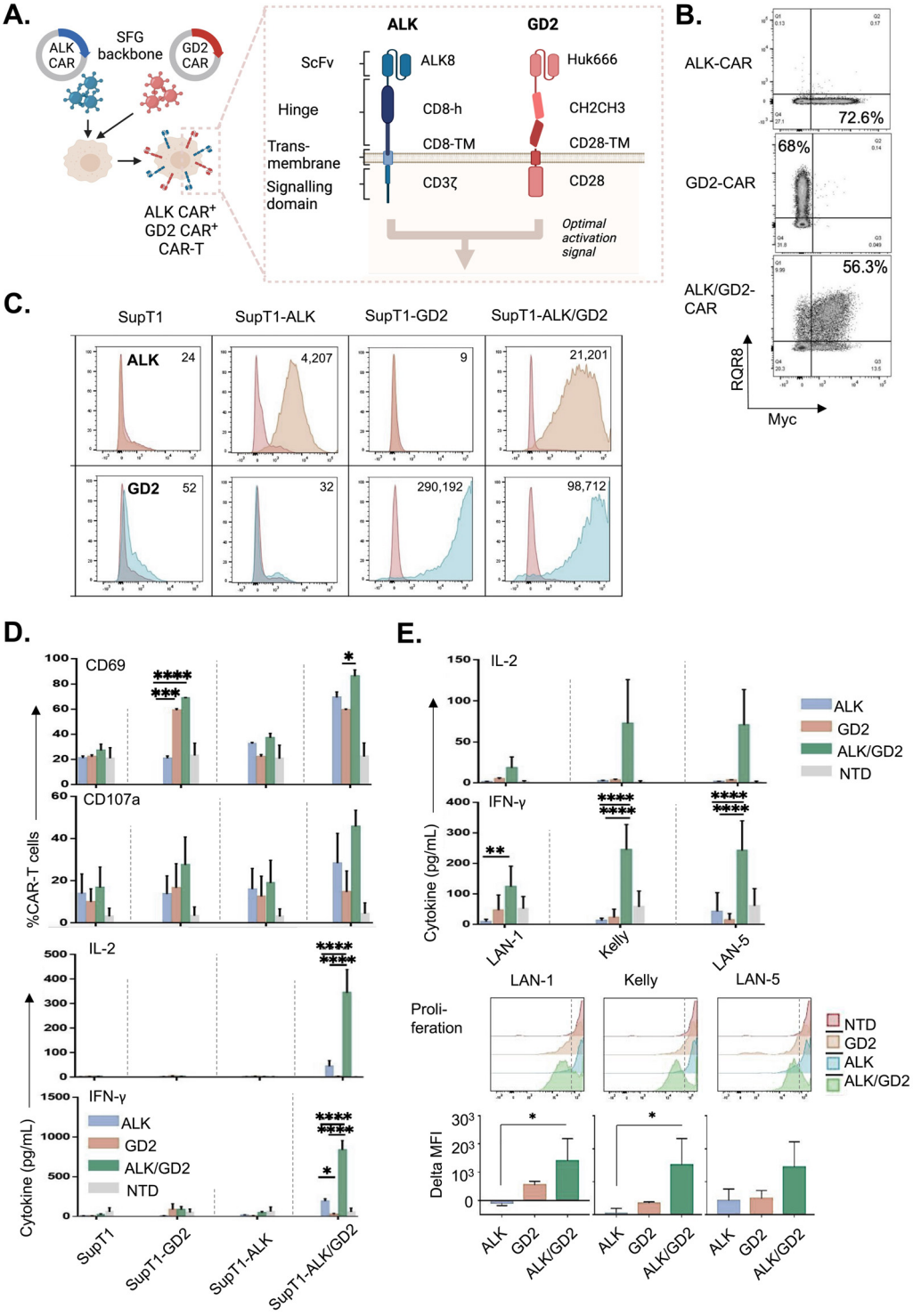

Fig. 7. An AND gate dual receptor approach with ALK CAR and GD2 CCR boosts reactivity against antigen-dim targets. (A) Schematic of co-CAR strategy and AND gate concept. (B) Representative flow cytometry dot plot showing efficiency of doubly transduced cells. (C) Artificial cell line clones derived from SupT1 cells sorted via FACS for high expression of ALK and/or GD2. (D) Activation marker detection by flow cytometry and cytokine secretion in response to engineered SupT1 cell lines. (E) Responses following co-culture with neuroblastoma cell lines showing cytokine secretion (top) and proliferation (bottom). Representative CellTrace Violet dilution in proliferation studies after 4 days is shown. Proliferation was quantified by change in MFI between baseline (NTD, no target) and indicated target cell and CAR combinations. Data show mean ± SD (n = 3 independent biological replicates). Two-way ANOVA was performed followed by Tukey post-hoc analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ANOVA, analysis of variance; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; NTD, non-transduced control T cells; SD, standard deviation.

approach for boosting signal 1 since it avoids tonic signaling and exhaustion [21]. In the current study, the authors show that cytokine and proliferation responses are enhanced through a CCR. In the short-term killing assays evaluated in this study, the first-generation CARs were capable of cytotoxicity without co-stimulation, and longer-term assays might be needed to demonstrate reliance on co-stimulation for sustained killing of single-antigen targets. Moreover, the authors used a configuration of dual CAR incapable of heterodimerization since the authors wished to demonstrate absence of reactivity against cells expressing GD2 and not ALK (such as on

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peripheral nerves), and a recent study has shown that a dual CAR and CCR system capable of heterodimerization leads to *cis*-activation, such that single-antigen-positive targets impart full stimulation to T cells [31]. This latter approach is effective at targeting tumor heterogeneity and limiting antigen loss variants, but its downside is the potential for toxicity against single-antigen-positive targets.

The data the authors present herein contribute to a growing body of literature describing an array of approaches for dual targeting of antigens to fine-tune CAR T-cell responses. Significant interest lies in overcoming tumor heterogeneity and antigen loss variants by use of "OR gates," whereby two single-antigen-positive cells are effectively eliminated. This approach in leukemia has translated to promising clinical results for dual targeting of CD19 and CD22. In neuroblastoma, there is emerging evidence of GD2 antigen loss leading to heterogeneity but also awareness of potential toxicities against healthy tissues expressing neuroblastoma target antigens such as GD2 and B7H3. ALK, including GPC2 [32], appears to be a relatively clean antigen in terms of absence of expression in healthy cells but a weak antigen in terms of levels of expression in tumors. Future developments of ALK CARs may focus on further boosting ALK-induced signal as well as approaches for targeting heterogeneity. Incorporation into dual targeting may prove optimal.

Conclusions

Using two different co-CAR models, the authors show that neuroblastoma reactivity to ALK-dim neuroblastoma cells can be attained by harnessing co-stimulation *in trans* using a second antigenic target. A limitation of the authors' study is that only two representative neuroblastoma target cell lines have been evaluated, both of which have classical ALK mutations. Follow-up studies should evaluate cases lacking mutations or with ALK amplification. Evaluation of fully optimized ALK CAR fine-tuned for reactivity to low antigen density will require a broad range of neuroblastoma targets as well as *in vivo* models.

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Author Contributions

Conception and design of the study: JA, KC and LC. Acquisition of data: EH, AV, MA, AG, CL, TG, JY, TP and LT. Analysis and interpretation of data: EH, AV, MA, AG, CL, TG, JY, TP, LT, MB, JF, JA and AP. Drafting or revising the manuscript: EH, JA, AG and MB. All authors have approved the final article.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2022.10.007.

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