Tuning the Antigen Density Requirement for CAR T-cell Activity

Robbie G. Majzner1,2, Skyler P. Rietberg2, Elena Sotillo2, Rui Dong3, Vipul T. Vachharajani4, Louai Labanieh5, June H. Myklebust6,7, Meena Kadapakkam1, Evan W. Weber2, Aidan M. Tousley1, Rebecca M. Richards3, Sabine Heitzeneder2, Sang M. Nguyen1, Volker Wiebking1, Johanna Theruvath2, Rachel C. Lynn2, Peng Xu2, Alexander R. Dunn8,9, Ronald D. Vale3,10, and Crystal L. Mackall1,2,11
ABSTRACT

Insufficient reactivity against cells with low antigen density has emerged as an important cause of chimeric antigen receptor (CAR) T-cell resistance. Little is known about factors that modulate the threshold for antigen recognition. We demonstrate that CD19 CAR activity is dependent upon antigen density and that the CAR construct in axicabtagene ciloleucel (CD19-CD28ζ) outperforms that in tisagenlecleucel (CD19-4-1BBζ) against antigen-low tumors. Enhancing signal strength by including additional immunoreceptor tyrosine-based activation motifs (ITAM) in the CAR enables recognition of low-antigen-density cells, whereas ITAM deletions blunt signal and increase the antigen density threshold. Furthermore, replacement of the CD8 hinge-transmembrane (H/T) region of a 4-1BBζ CAR with a CD28-H/T lowers the threshold for CAR reactivity despite identical signaling molecules. CARs incorporating a CD28-H/T demonstrate a more stable and efficient immunologic synapse. Precise design of CARs can tune the threshold for antigen recognition and endow 4-1BBζ-CARs with enhanced capacity to recognize antigen-low targets while retaining a superior capacity for persistence.

SIGNIFICANCE: Optimal CAR T-cell activity is dependent on antigen density, which is variable in many cancers, including lymphoma and solid tumors. CD28ζ-CARs outperform 4-1BBζ-CARs when antigen density is low. However, 4-1BBζ-CARs can be reengineered to enhance activity against low-antigen-density tumors while maintaining their unique capacity for persistence.

INTRODUCTION

CD19 chimeric antigen receptor (CAR) T-cell therapy has dramatically altered the landscape for patients with relapsed and refractory B-cell malignancies, with two FDA-approved agents (tisagenlecleucel and axicabtagene ciloleucel) for the treatment of diffuse large B-cell lymphoma (DLBCL) and B-cell acute lymphoblastic leukemia (B-ALL; refs. 1, 2). Remarkable responses following one dose of CD19-CAR T cells in patients with relapsed and refractory disease have surpassed all expectations (3–11). However, emerging follow-up data demonstrates that only 30% to 50% of patients experience long-term disease control following CD19-CAR therapy (5, 7, 12). Furthermore, reproducible clinical activity in other malignancies such as myeloid leukemias and solid tumors has not yet been observed. To diminish the relapse rate in B-ALL, improve the response rate in DLBCL, and translate the success of CAR T cells to diseases outside of B-cell malignancies, a deeper understanding of factors associated with primary and acquired resistance to this class of therapeutics is required (13–15).

Antigen density has emerged as a major factor influencing the activity of CAR T cells (12, 14, 16–22). Across antigens and studies, CAR T-cell potency is highly dependent on target antigen expression, and CARs often fail to exert meaningful antitumor activity when antigen expression falls below a certain threshold, an attribute that differentiates CARs from native T-cell receptors (TCR; refs. 23, 24). When antigens are shared between tumors and vital tissues, such as those expressed by solid tumors, the requirement for high antigen density may open a therapeutic window that allows for targeting of normal tissue antigens (17, 19, 20, 25–29). However, escape with antigen-low variants also provides a pathway for resistance to therapy, as evidenced in a recent clinical trial of CD22 CAR T cells for patients with relapsed and refractory B-ALL, where high complete response rates were tempered by frequent relapses driven by selection of variants that expressed CD22 at levels below the threshold required for CAR T-cell efficacy (21).

CD19 expression is high in a majority of B-ALL cases, but here we present data demonstrating high inter- and intra-patient heterogeneity of CD19 and other surface protein expression in B-cell lymphomas. We further demonstrate that efficacy of CAR T cells targeting CD19 or HER2 is proportional to target antigen density, but that CD28 endodomain-containing CARs outperform 4-1BB endodomain-containing CARs in response to targets with low antigen density. Recent work has focused on reducing CAR signal strength and cytokine production to reduce toxicity (30–32) and enhance CAR T-cell persistence (33), but we demonstrate that such alterations result in a greater likelihood of resistance due to
selection of antigen-low variants, because strength of signal is a major factor driving the antigen density threshold needed for CAR T-cell activity. We further demonstrate that seemingly minor structural changes in CAR design can tune the threshold of antigen density required for optimal CAR T-cell activity. These insights provide new opportunities for more precise engineering of CAR T-cell receptors designed for optimal recognition of target antigens on cancer while avoiding reactivity toward the same antigens expressed at lower levels on nonmalignant tissues.

RESULTS

B-cell Malignancies Exhibit a Wide Range of Expression Levels of Pan-B Cell Antigens, Including CD19, and Low CD19 Expression Limits CD19 CAR Reactivity

With few exceptions, CD19 expression is high in newly diagnosed B-ALL (34), but CD19 expression in other B-cell malignancies is not as well characterized. Using flow cytometry, we measured CD19 expression levels on a panel of diagnostic samples obtained from patients with DLBCL, mantle cell lymphoma (MCL), follicular lymphoma, and chronic lymphocytic leukemia (CLL). Whereas CLL samples consistently demonstrated CD19 expression levels that approximated those seen on normal B cells, DLBCL, MCL, and follicular lymphoma samples demonstrated significantly lower median CD19 levels, with the greatest interpatient variability observed for DLBCL (Fig. 1A). Furthermore, lymphoma cells from individual patients with DLBCL at the time of initial diagnosis displayed significant heterogeneity in CD19 expression, with some cases even containing lymphoma cells with undetectable levels (Fig. 1B). We also found significant inter- and intrapatient heterogeneity in expression of other pan-B cell targets for which CAR T cells have been developed, including CD22 (21, 35), CD20 (18, 36, 37), CD79b (38), Igκ (39, 40), and Igλ (ref. 41; Supplementary Fig. S1A and S1B). We also semiquantitatively measured the number of CD19, CD22, CD20, CD79b, and Igκ molecules on a panel of B cells from healthy donors (Supplementary Fig. S1C). Together, these results raise the prospect that limiting antigen density could be an important mechanism of primary and/or acquired resistance to CAR therapeutics for B-cell lymphomas.

To explore how CD19 antigen density influences CD19 CAR efficacy, we used CRISPR/Cas9 to knock out CD19 from the well-described NALM6 B-cell leukemia model (16), transduced those cells to express a truncated CD19 protein, and used FACS sorting and single-cell cloning to establish a library of NALM6 lines expressing different amounts of surface CD19 (Fig. 1C). To exclude any contribution of CD19 signaling in these assays, only the transmembrane and extracellular portions of CD19 were expressed. CD19 signaling is not required for CAR-mediated in vitro activity in B-ALL because similar antitumor effects were observed in mice inoculated with a NALM6 clone expressing a comparable amount of truncated CD19 to the wild-type parental cell line (NALM6-CD1945,851; Supplementary Fig. S2A and S2B). We tested the CD19-4-1BBζ CAR construct contained in this transgeneleucel in an array of in vitro assays against NALM6 clones expressing different amounts of CD19 on their surface. CD19-4-1BBζ CAR T cells demonstrated reduced killing capacity (Fig. 1D), reduced proliferation (Fig. 1E), and reduced cytokine production (Fig. 1F) in response to lines expressing low levels of CD19 compared with those expressing high levels.

CARs with CD28 Costimulatory Domains Demonstrate Enhanced Activity against Low-Antigen-Density Targets

To test whether the potency of the CD19-28ζ-CAR T-cell construct employed in axicabtagene ciloleucel is limited at lower antigen densities, as is the construct in tisagenlecleucel, we compared their function in an array of in vitro assays (Fig. 2A; Supplementary Fig. 3A–S3C). Both constructs killed and proliferated in response to the high-antigen-density clone (NALM6-CD1945,851) equally well, but only the CD19-28ζ construct was able to kill (Fig. 2B) and robustly proliferate (Fig. 2C) in response to tumor cells expressing low levels of CD19 (NALM6-CD192,053). At all antigen densities tested, CD19-28ζ CAR T cells produced more IL2 in response to antigen encounter than CD19-4-1BBζ CAR T cells, and at low antigen densities only CD19-CD28ζ produced measurable amounts of IL2 (Fig. 2D). Importantly, cytokine production was reduced even by CD19-CD28ζ CAR T cells when antigen density was low.

To test whether the improved in vitro activity of the CD19-CD28ζ CAR against low-antigen-density tumors translates in vivo, we treated mice with CD19-CD28ζ, CD19-4-1BBζ, or untransduced MOCK CAR T cells 4 days after inoculation of CD19-low leukemia (NALM6-CD19100). In line with our in vitro findings, CD19-4-1BBζ CAR T cells demonstrated minimal antitumor activity against CD19-low leukemia and did not increase survival compared with MOCK CAR T cells. Conversely, CD19-CD28ζ CAR T cells demonstrated robust antitumor activity and significantly extended survival compared with CD19-4-1BBζ CAR T cells (Fig. 2E–G). Ultimately, mice treated with CD19-28ζ CAR T cells developed recurrent leukemia which had significantly downregulated CD19 expression. Conversely, leukemia recovered from mice treated with CD19-4-1BBζ CAR T cells expressed CD19 at levels similar to mice treated with control T cells, consistent with a lack of immune pressure from the CD19-4-1BBζ CAR. CD81, an accessory molecule known to traffic with CD19, was not reduced (Fig. 2H and I).

To probe whether the differential antigen density requirement observed between CD19-28ζ and CD19-4-1BBζ CAR T cells was generalizable to other targets, we generated comparable HER2-targeting CARs containing 4-1BB or CD28 costimulatory domains (Supplementary Figs. S3A–S3C and S4A) and NALM6 clones expressing differing amounts of surface HER2 (Supplementary Fig. S4B). Similar to CD19 CAR constructs, IL2 production by HER2 targeting CAR T cells was proportional to HER2 antigen density on target cells, and HER2-28ζ CARs outperformed HER2-4-1BBζ CARs at low antigen density (Supplementary Fig. S4C). Furthermore, in an in vivo xenograft model of HER2-low 143b osteosarcoma (Supplementary Fig. S4B), mice treated with HER2-4-1BBζ CAR T cells demonstrated tumor growth kinetics similar to mice treated with untransduced MOCK T cells, whereas...
Figure 1. CD19 antigen density influences CD19 CAR activity. A, Primary diagnostic samples of DLBCL, MCL, follicular lymphoma (FL), and CLL were analyzed by flow cytometry for expression of CD19 compared with normal B cells from healthy donors. Shown is CD19 protein expression, relative to healthy donor PBMC B cells on a log 2 scale. DLBCL: n = 8, FL: n = 27, CLL: n = 13, MCL: n = 35. Statistical differences between groups were analyzed by one-way ANOVA nonparametric test with Dunn post-test correction. B, Representative contour plots illustrating expression levels of CD19 and CD20 in three DLBCL cases as compared with PBMC B cells from healthy donors. C, Flow cytometric analysis of the expression levels of truncated CD19 on the surface of a library of NALM6 clones. Number of molecules of CD19 for each clone were semiquantitatively determined by the BD Quantibrite Kit. D, NALM6 clones expressing indicated densities of surface CD19 molecules were cocultured at a 1:1 ratio with CD19–4-1BBζ CAR T cells, and tumor cell killing was measured in an Incucyte assay. Representative of six experiments with different T-cell donors. Statistical analysis performed with repeated measures ANOVA. E, CD19–4-1BBζ CAR T cells were labeled with cell trace violet (CTV) and then cocultured at a 1:2 ratio with NALM6 clones expressing either 963 or 45,851 molecules of surface CD19. T-cell proliferation was measured by flow cytometry four days later. Representative of three experiments with different T-cell donors. F, CD19–4-1BBζ CAR T cells were cocultured with NALM6 clones expressing various amounts of CD19 for 24 hours, and secreted IL2 was measured by ELISA. Shown is the concentration of cytokine measured as compared with log of the CD19 molecule number for that specific clone and curve fitting was done using a four-parameter variable slope dose–response curve. Representative of six experiments with different T-cell donors. For all experiments, error bars represent SD. P < 0.05 was considered statistically significant, and P values are denoted with asterisks as follows: P > 0.05, not significant, NS; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
NALM6-CD19-4-1BB-ζ

Cytotoxicity index

CD19-CD28-ζ

CD19-4-1BB-ζ

NALM6-CD192,053

CD28

MOCK CD19-4-1BB-ζ

MOCK CD19-CD28-ζ

CD19KO CD19-4-1BB-ζ

CD19KO CD19-CD28-ζ

Day 0

Day 7

Percent survival

Days post tumor inoculation

Mean fluorescence index

MOCK CD19KO CD19-4-1BB-ζ

MOCK CD19KO CD19-CD28-ζ

MOCK CD19KO
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mice treated with HER2-CD28ζ CAR T cells demonstrated clear antitumor efficacy, as evidenced by significantly delayed tumor growth and prolonged survival (Supplementary Fig. S4D and S4E), mirroring our findings with CD19 CARs.

To test whether recognition of high-antigen-density target cells remained within a heterogeneous tumor could spur increased reactivity against low-antigen-density cells *in vivo*, we inoculated mice with both CD19-low leukemia (NALM6-CD19<sup>2,053</sup> expressing luciferase and CD19-high NALM6-wild-type that does not express luciferase. The bioluminescence captured from these mice came only from the CD19-low clones. After treatment with CD19-4-1BBζ CAR T cells, there was no difference in bioluminescence or survival between mice inoculated with both CD19-low and CD19-high leukemia versus those with CD19-low leukemia only (Supplementary Fig. S5A and S5B), indicating that CD19-4-1BBζ CAR T-cell reactivity against low antigen density did not benefit from activation by high-antigen-density cells.

**Modulating CAR Signaling Strength Tunes the Antigen Density Threshold for CAR T Cells**

On the basis of previous work demonstrating higher signal strength in CD28ζ versus 4-1BBζ CAR T cells (42), we hypothesized that differential signaling strength could explain the greater capacity for CD28ζ CARs to recognize targets with low antigen density. We conducted single-cell analysis of calcium influx in CD19 CAR T cells following CAR cross-linkage which demonstrated that CD19-CD28ζ CAR T cells manifest more rapid and robust calcium influx compared with CD19-4-1BBζ CAR T cells (Fig. 3A). Next, we analyzed phosphorylation of proximal (pCD3ζζ-CAR) and distal (pERK) signaling proteins in CAR T cells stimulated with varying concentrations of soluble idiotype and cross-linking antibodies as a proxy for variable antigen density. We observed higher levels of CD3ζζ-CAR and ERK phosphorylation at all idiotype concentrations in CD19-CD28ζζ CAR T cells compared with CD19-4-1BBζζ CAR T cells. There were even greater distinctions at low idiotype concentrations, wherein only the CD19-CD28ζζ CAR T cells demonstrated a response (Fig. 3B). Thus, increased signal strength in T cells expressing CD28ζζ CARs compared with 4-1BBζζ CARs provides a plausible mechanism to explain the enhanced activity of CD28ζζ CAR T cells in response to targets expressed at low antigen densities.

To test the hypothesis that the antigen density threshold for CAR T-cell reactivity could be lowered by enhancing proximal signaling, we engineered a CD19-4-1BBζζ CAR that incorporated two copies of the zeta chain (CD19-4-1BBζζζζ, “double zeta,” Fig. 3C). This CAR expressed similarly on the surface of T cells to the CD19-4-1BBζζ “single zeta” CAR, and despite the addition of three additional immunoreceptor tyrosine-based activation motifs (ITAM), there was no increase in the baseline expression of canonical exhaustion markers PD-1, TIM3, or LAG3 or production of baseline IFNγ in the absence of antigen (Supplementary Fig. S3A–S3C). Following stimulation with idiotype antibody, pERK and CD3ζζζζ-CAR were higher in “double zeta” compared with “single zeta” CAR T cells, indicating that the increased signal generated in “double zeta” CARs is propagated distally (Fig. 3D).

We next tested the *in vitro* and *in vivo* functionality of “double zeta” versus “single zeta” CAR T cells. Both killing and proliferation in response to target cells with low CD19 density were increased in “double zeta” CARs, with functionality of CD19-4-1BBζζζζ CAR T cells closely approximating a CD19-28ζζζζ CAR (Fig. 3E and F). More IL2 was generated in response to lower antigen densities in “double zeta” versus “single zeta” CARs (Fig. 3G), although IL2 production was still less than CD28ζζ CARs (Fig. 3D). In our *in vivo* model of CD19-low leukemia, “double zeta” CAR T cells demonstrated greater antitumor activity than “single zeta” CAR T cells (Fig. 3H), resulting in significantly prolonged survival (Fig. 3I) and immune pressure resulting in CD19 downregulation (Fig. 3J and K). Although *in vitro* control by CD19-4-1BBζζζζ CAR T cells remained slightly inferior to CD28ζζ CARs, CD19-4-1BBζζζζ CAR T cells exhibited significantly increased persistence in animals compared with CD19-CD28ζζ CAR T cells (Fig. 3L).

Figure 2. CD19-CD28ζζ CAR T cells display superior activity compared with CD19-4-1BBζζ CAR T cells against low-antigen-density target cells. A, Schema of CARs employed in these experiments. The CD19-CD28ζζ CAR molecule is identical to the CAR construct contained in tisagenlecleucel whereas the CD19-CAR molecule is identical to the CAR construct contained in axicabtagene ciloleucel. B, NALM6 clones expressing either 963 or 45,851 molecules of surface CD19 were cocultured at a 1:1 ratio with either CD19-CD28ζζ or CD19-4-1BBζζ CAR T cells, and tumor cell killing was measured in an luciferase assay. Representative of six experiments with different T-cell donors. Statistical analysis performed with repeated measures ANOVA. C, CD19-CD28ζζ and CD19-4-1BBζζ CAR T cells were cocultured with cell trace violet (CTV) and then cocultured with NALM6 clones expressing either 963 or 45,851 molecules of surface CD19. T-cell proliferation was measured by flow cytometry four days later. Representative of three experiments with different T-cell donors. D, CD19-CD28ζζ and CD19-4-1BBζζ CAR T cells were cocultured with NALM6 clones expressing various amounts of CD19 for 24 hours, and secreted IL2 was measured in the supernatant by ELISA. Shown is the concentration of cytokine measured as compared with log of the CD19 molecule number for that specific clone, and curve fitting was done using a four-parameter variable slope dose-response curve. Representative of six experiments with different T-cell donors. E, One million NALM6-CD19<sup>2,053</sup> cells were engrafted into NSG mice by tail-vein injection. Four days later, mice were injected with 3 × 10<sup>6</sup> CD19-CD28ζζ CAR T cells, CD19-4-1BBζζ CAR T cells, or unlabeled control T cells (MOCK). Tumor progression was measured by bioluminescence photometry and flux values (photons per second) were calculated using Living Image software. Representative images are shown. F, Quantified tumor flux values for individual mice treated as in E. The MOCK group on day +15 were either found dead prior to imaging or sick with limited perfusion such that imaging results were unreliable and were thus excluded. Statistical analysis performed with repeated measures ANOVA. G, Survival curves shown for mice treated as in E. Statistical analysis performed with the log-rank test. H–K are representative of six experiments with different T-cell donors (n = 5 mice per group). H and I, Leukemia cells from the bone marrow of treated mice (n = 5) were phenotyped by flow cytometry for expression of CD19 and CD81. The CD19 knockout (KO) cell line from cell culture was used as reference control. Shown are representative flow plots (H) and quantified mean fluorescence intensity data (I). Representative of three different experiments with different T-cell donors. Statistical comparisons performed by Mann–Whitney between the indicated groups. For *in vitro* experiments, error bars represent SD and for *in vivo* experiments, error bars represent SEM. *P < 0.05 was considered statistically significant, and p values are denoted with asterisks as follows: P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
**RESEARCH ARTICLE**

**A**

[Graph showing cross-linking and ionomycin effects on Car2 influx and MFI normalized.]

**B**

<table>
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**C**

[Diagram showing CD19-4-1BBζζ and FMC63 stimulation.]

**D**

[Graph showing no stimulation and 0.5, 1, and 5 µg/mL of CD3ζY142-CAR and CD3ζ-CAR effects on pERK1/2Y202/Y204 and ERK1/2.]

**E**

[Graph showing NALM6-CD19963 toxicity index over hours.]

**F**

[NALM6-CD19963 MFI values for CD19-28ζ and CD19-1BBζ.]

**G**

[Graph showing IL2 (pg/mL) vs Log [CD19 molecule number].]

**H**

[Graph showing NALM6-CD19963 Flux (p/s) vs Day post-treatment.]

**I**

[Graph showing percent survival vs Days post tumor inoculation.]

**J**

[Graph showing MFI vs Log [CD19 molecule number].]

**K**

[Graph showing Absolute CAR T cells vs Log [CD19 molecule number].]

**L**

[Graph showing absolute CAR T cells vs Log [CD19 molecule number].]
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Previous work has demonstrated that decreasing the number of functional ITAMs in a CAR molecule reduces T-cell signal strength and diminishes downstream effector functions (33, 43, 44). A recent study demonstrated increased persistence and decreased T-cell exhaustion of CD19-CD28ζζ CAR T cells that were engineered through either truncation or mutation to contain only one of three active CD3ζ ITAMs (33). Given our findings demonstrating the importance of signal strength in CAR T eradication of low-antigen-density target cells, we hypothesized that decreased signal in single ITAM CARs would reduce functionality against low-antigen-density targets. Consistent with this, CD19-CD28ζζ and CD19-4-1BBζζ CAR T cells, which each express only one (the most membrane-proximal) CD3ζ ITAM (Fig. 4A and B; Supplementary Fig. S6A, S6B, and S6F), demonstrated no difference from their wild-type counterparts in their ability to control tumors with high CD19 antigen density. However, they demonstrated reduced killing of CD19-low NALM6 clones (Fig. 4C and D) and reduced ability to produce IL2 in response to tumor cells expressing low levels of CD19 (Fig. 4E–F) compared with identical CARs with intact ITAMs. Similarly, a recent publication described a CD19-CD28ζζ CAR containing the hinge-transmembrane (H/T) region from CD8ζζ (CD19-CD8H/T-CD28ζζ; Fig. 4G; Supplementary Fig. S6C and S6G) that demonstrated decreased signaling and cytokine production while maintaining activity against the wild-type NALM6 CD19-high cell line (30). Similar to results with ITAM-deleted CARs, CD19-CD8H/T-CD28ζζ CARs demonstrated similar cytolytic capacity against CD19-high cell lines as CD19-CD28ζζ, but diminished activity against CD19-low lines (Fig. 4H–I).

Together, these results are consistent with a model wherein modulating CAR signaling strength can tune the antigen density threshold for CAR T-cell reactivity. Modifications that enhance signaling strength result in a lower antigen density threshold for CAR reactivity, whereas alterations that diminish the signaling strength increase the antigen density threshold for CAR T-cell reactivity. These results further demonstrate that augmentation of signal strength in 4-1BB containing CARs endows them with a near-equivalent capacity to recognize antigen-low targets compared with CD28-containing CARs, while retaining the hallmark property of increased persistence compared with CD28 CARs (3–7, 45).

The CD28 H/T Domain Confers Enhanced Reactivity against Low-Antigen-Density Targets

The CD19 CAR constructs contained in axicabtagene ciloleucel and tisagenlecleucel differ in their costimulatory domains, but they also differ in the hinge and transmembrane regions that link the extracellular scFv to the intracellular signaling endodomains. The CAR construct in axicabtagene ciloleucel contains an H/T from CD28, which is continuous with the costimulatory molecule, whereas tisagenlecleucel contains a CD8 H/T. These regions are nearly identical in size (70 amino acids for CD8 and 67 amino acids for CD28). Given our finding that substitution of a CD8 H/T region for the CD28 H/T reduces CD19-CD28ζζ CAR T-cell activity at low antigen density, we hypothesized that incorporation of a CD28 H/T would enhance the efficacy of a CD19-4-1BBζζ CAR (Fig. 5A; Supplementary Fig. S3A–S3C). Indeed, CD19-4-1BBζζ CARs that incorporated a CD28 H/T (CD19-28H/T-4-1BBζζ CAR) demonstrated superior killing of CD19-low leukemia cells compared with those incorporating a CD8 H/T (Fig. 5B), and cytokine production of CD28 H/T containing CD19-4-1BBζζ CARs approached that seen with CD19-CD28ζζ CARs (Fig. 5C), especially at low CD19 antigen densities. This increased activity translated in vivo, with the CD19-28H/T-4-1BBζζ CAR outperforming the CD19-4-1BBζζ CAR against CD19-low leukemia, performing similarly to the
**A**

CD19-CD28-ζ**

FMC63

CD28

CD3ζ**

**C**

NALM6-CD1945,851

CD19-CD28ζ

CD19-CD28ζ**

NALM6-CD19963

**E**

IL2 (pg/mL)

CD19-CD28ζ

CD19-CD28ζ**

**B**

CD19-4-1BB-ζ**

FMC63

CD8

4-1BB

CD3ζ**

**D**

NALM6-CD1945,851

CD19-4-1BBζ

CD19-4-1BBζ**

NALM6-CD19963

**F**

IL2 (pg/mL)

CD19-4-1BBζ

CD19-4-1BBζ**

**G**

CD19-CD8H/T-CD28-ζ

FMC63

CD8

CD28

CD3ζ

**H**

NALM6-CD1945,851

CD19-CD8ζ

CD19-CD8H/T-CD8ζ

NALM6-CD19963

**I**

IL2 (pg/mL)

CD19-CD28ζ

CD19-CD8H/T-CD28ζ

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*Figure legends*

**A** and **B** show the expression of CD19-CD28ζ and CD19-4-1BBζ, respectively, in NALM6 cells under different conditions. **C** and **D** illustrate the cytotoxicity index (CD19-CD28ζ and CD19-4-1BBζ) over time (0-60 hours). **E** and **F** depict the IL2 levels (pg/mL) for CD19-CD28ζ and CD19-4-1BBζ, respectively, across various conditions. **G** through **I** display the expression of CD19-CD8H/T-CD28ζ and its impact on IL2 production.
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CD19-CD28 CAR (Fig. 5D and E). Furthermore, although the CD19-4-1BBζ CAR failed to control tumors in a NALM6 CD19 wild-type stress test model (46), the CD19-CD28H/T-4-1BBζ CAR demonstrated clear antitumor activity, similar to the CD19-CD28ζ CAR (Fig. 5F and G). Importantly, the CD19-CD28H/T-4-1BBζ CAR also demonstrated superior in vivo persistence in both the spleen and bone marrow compared with CD19-CD28ζ CARs, and similar in vivo persistence in CD19-4-1BBζ CARs (Fig. 5H and I; Supplementary Fig. S7A–S7D) in a standard NALM6 model at curative doses. No major differences in CAR T-cell exhaustion marker expression were observed between any of the constructs when cells were obtained from in vivo experiments (Supplementary Fig. S7E and S7F).

Given the profound increase in efficacy gained by modifying the H/T region while maintaining the 4-1BB costimulatory molecule, we wondered whether the CD28 H/T domain could rescue CAR function even in the absence of a costimulatory domain. Previous studies of first-generation CAR T cells containing only a CD3ζ domain primarily used a CD8 H/T domain (47) or hybrids of an IgG hinge or spacer with a CD4 or CD3 transmembrane domain (36, 37, 48–50). Previously published comparisons between first- and second-generation CARs were often confounded by different hinge and/or transmembrane domains employed in the constructs (51–54). We compared a first-generation CAR with either a CD28 or CD8 H/T domain (Fig. 6A; Supplementary Fig. S6D and S6H) and found that the CD28 H/T domain conferred reactivity against low-antigen-density and higher levels of cytokine production in response to all antigen densities (Fig. 6B–C). In fact, the first-generation CD19-CD28H/T-ζ CAR compared favorably with second-generation constructs, generating as much IL2 as a traditional CD19-4-1BBζ construct against CD19-high lines (Fig. 6C).

**Incorporation of a CD28 H/T Region Rescues Function of Solid-Tumor CARs in In Vivo Models of Low Antigen Density**

To assess the generalizability of the results observed with CD19-CD28H/T-ζ CAR T cells, we tested CARs targeting other cell-surface proteins in solid-tumor models in which antigen density was limiting. Mice bearing HER2-low orthotopic 143b osteosarcoma xenografts (Supplementary Fig. S4B) received HER2-CD28H/T-4-1BBζ (Fig. 6D; Supplementary Fig. S4E) or HER2-CAR T cells. Similar to the results described above, 4-1BBζ CARs incorporating a CD28 H/T domain outperformed those with a CD8 H/T domain, demonstrating improved tumor control and significantly extending survival (Fig. 6E and F). In addition, we recently published results using a B7-H3 targeting CAR with activity in several models of pediatric solid tumors (17). Although this CAR demonstrated clear efficacy in xenograft models where B7-H3 expression was high, we found that it was less effective when target antigen density was low. Expression of B7-H3 on neuroblastoma cell lines is lower than many of the tumor types we have previously studied (Supplementary Fig. S8A). We found that the published B7-H3-4-1BBζ CAR demonstrated intermediate killing of neuroblastoma cell lines, whereas activity of the B7-H3-CD28H/T-4-1BBζ CAR (Fig. 6G; Supplementary Fig. S6E and S6I) against this low-antigen-density tumor was enhanced both in vitro (Supplementary Fig. S8B) and in vivo (Fig. 6H–J). Thus, a CD28 H/T region imparts superior function as compared with a CD8 H/T region in second-generation 4-1BB containing CARs against a variety of targets in a range of in vivo models, establishing a strong rationale for adopting this structure to increase the clinical efficacy of CAR T cells in settings where induction of responses toward antigen-low targets would not induce unacceptable toxicity.

The CD28 H/T Domain Results in Faster Tumor-Cell Killing and a More Efficient Immune Synapse

Given the enhancement we observed in CAR T-cell activity using a CD28 H/T region, we hypothesized that pERK may be higher for the CD19-CD28H/T-4-1BBζ CAR than the traditional CD19-4-1BBζ CAR after stimulation. However, after a 5-minute stimulation, we found decreased levels of pERK in CD19-CD28H/T-4-1BBζ compared with CD19-4-1BBζ (Supplementary Fig. S9A). In addition, despite clearly improved function in both low CD19 density and stress test models (Fig. 5D–G), the CD19-CD28H/T-4-1BBζ CAR did not mediate rapid calcium influx as was seen for the CD19-CD28ζ CAR (Supplementary Fig. S9B); neither did the HER2 CAR with similar architecture (Supplementary Fig. S9C). To
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NALM6-CD192,053 cells were engrafted into NSG mice by tail-vein injection. Three days later, mice were injected with $5 \times 10^6$ CD19-CD28 cells. These results are representative of two experiments with different T-cell donors ($n = 5$ mice per group).

Figure 5. Altering the H/T region dramatically affects CD19 CAR activity against low-antigen-density tumors. A, Schema of a CD19 CAR containing the CD28 H/T region and the 4-1BB and CD3ζ endodomains (CD19-CD28/NALM6). B, NALM6 clones expressing 963 molecules of surface CD19 were cocultured at a 1:1 ratio with either CD19-CD28ζ, CD19-4-1BBζ, or CD19-CD28H/T-4-1BBζ CAR T cells, and tumor cell killing was measured in an Incucyte assay. Representative of three experiments with different T-cell donors. Statistical analysis performed with repeated measures ANOVA.

C, CD19-CD28ζ, CD19-4-1BBζ, and CD19-CD28H/T-4-1BBζ CAR T cells were cocultured with NALM6 clones expressing various amounts of CD19 for 24 hours, and IL2 was measured in the supernatant by ELISA. Representative of three experiments with different T-cell donors. Statistical comparisons performed by the Student t-test (two sided) between CD19-4-1BBζ and CD19-CD28ζ CAR T cells. One million NALM6-CD19ζ CAR T cells. Tumor progression was measured by bioluminescence photometry, and flux values (photons per second) were calculated using Living Image software. Quantified tumor flux values for individual mice are shown. Statistical analysis performed with repeated measures ANOVA. E, Mouse survival curves for mice as treated in D. Statistical analysis performed with the log-rank test. D and E are representative of three experiments with different T-cell donors ($n = 5$ mice per group). F, One million NALM6-wild-type (WT) cells were engrafted into NSG mice by tail-vein injection. Four days later, mice were injected with $3 \times 10^9$ CD19-CD28ζ, CD19-4-1BBζ, or CD19-CD28H/T-4-1BBζ CAR T cells. Tumor progression was measured by bioluminescence photometry and flux values (photons per second) were calculated using Living Image software. Quantified tumor flux values for individual mice are shown. Statistical analysis performed with repeated measures ANOVA.

G, Mouse survival curves for mice as treated in F. Statistical analysis performed with repeated measures ANOVA. H and I are representative of two experiments with different T-cell donors ($n = 5$ mice per group). H and I, One million NALM6-wild-type cells were engrafted into NSG mice by tail-vein injection. Three days later, mice were injected with $5 \times 10^9$ CD19-CD28ζ, CD19-4-1BBζ, or CD19-CD28H/T-4-1BBζ CAR T cells. The spleens (H) and bone marrow (I) of treated mice were obtained at day +16 ($n = 5$ per group) as well as day +9 and day +29 (Supplementary Fig. 5) post CAR T-cell treatment. Presence of CAR-positive T cells was assessed by flow cytometry. Performed one time ($n = 5$ per CAR construct per timepoint). Statistical comparisons performed by Mann-Whitney between the indicated groups. For in vitro experiments, error bars represent SD, and for in vivo experiments, error bars represent SEM. $P < 0.05$ was considered statistically significant, and $P$ values are denoted with asterisks as follows: $P > 0.05$, not significant, NS; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.
better understand the kinetics of T-cell activation, we performed a longer time course, which demonstrated that stimulation of CD19-CD28H/T-4-1BBζ CAR results in slower activation kinetics than either traditional CAR construct, with phosphorylation of ERK evident only after 30 minutes, continuing to rise at 45 minutes (Supplementary Fig. S9D). Phosphorylation of CAR CD3ζ-CAR was also more moderate, requiring longer film exposures for visualization (Supplementary Fig. S9D). In addition, we observed phosphorylation of endogenous CD3ζ only in the CD19-CD28H/T-4-1BBζ CAR, raising the prospect that co-opting of the endogenous cellular machinery could contribute to superior function of CAR constructs as previously reported (55). To test whether endogenous TCR/CD3ζ contributes to CAR efficacy, we used CRISPR/Cas9 to disrupt the TRAC locus in CD19-CD28H/T-4-1BBζ CAR T cells (Supplementary Fig. S9E). Despite near-complete knockdown of the TCR, we observed no differences in the ability of this CAR to kill or generate cytokines in response to either CD19-low or CD19-high target cells (Supplementary Fig. S9F–S9G). Therefore, we conclude that endogenous CD3ζ phosphorylation is not required for the superior function seen with CD19-CD28H/T-4-1BBζ CAR T cells.

To further explore the basis for the increased functionality of the CD28 H/T region, we performed live-cell imaging of single CAR T-cell and tumor cell interactions in microwells.

**Figure 6.** The CD28 H/T region enhances activity in a variety of tumor models and CAR architectures. A, Schema of first-generation CD19 CARs with either a CD8 or CD28 H/T region (CD19-CD8H/T-ζ and CD19-CD28H/T-ζ). B, NALM6 clones expressing either 963 or 45.851 molecules of surface CD19 were cocultured at a 1:1 ratio with either CD19-CD28ζ, CD19-4-1BBζ, CD19-CD28ζ CAR T cells, and tumor cell killing was measured in an Incucyte assay. Representative of three experiments with different T-cell donors. Statistical analysis performed with repeated measures ANOVA between CD19-CD28ζ and CD19-4-1BBζ, CD19-CD28ζ, CD19-CD28ζ, CD19-4-1BBζ, CD19-CD28ζ, and CD19-CD28ζ CAR T cells were cocultured with NALM6 clones expressing various amounts of CD19 for 24 hours, and secreted IL2 was measured in the supernatant by ELISA. Representative of three experiments with different T-cell donors. Statistical comparisons performed with the Student t test (two sided) between CD19-CD28ζ and CD19-4-1BBζ. D, Schema of a HER2 CAR containing a CD28 H/T region and 4-1BB costimulatory domain (HER2-CD28ζ). E, One million 143b osteosarcoma cells were orthotopically implanted in the hind leg of NGS mice. After 7 days, mice were treated with 1 x 10^6 HER2-4-1BBζ CAR T cells, HER2-CD28ζ, or untransduced control T cells (MOCK). Leg measurements were obtained twice weekly with digital calipers. Measurements for individual mice are shown. Statistical analysis performed with repeated measures ANOVA. F, Survival curves for mice treated as in E. Statistical analysis performed with the log-rank test. A–F are representative of two experiments with different T-cell donors (n = 5 mice per group). (Continued on next page)
injected with 10 million B7-H3-4-1BBζ million CHLA255 neuroblastoma cells were engrafted into NSG mice by tail-vein injection in a metastatic neuroblastoma model. Six days later, mice were

CAR compared with the CD19-CD28H/T-4-1BBζ

because the H/T domain appeared to affect the interaction of the CAR T cell and tumor, we next imaged the CAR synapse, to test the hypothesis that differences in the H/T region affect receptor clustering and recruitment of ZAP70, which propagates CAR signaling (57). To do this, we generated CD19-4-1BBζ and CD19-CD28H/T-4-1BBζ-mCherry fusion constructs that expressed similarly in primary human T cells (Supplementary Fig. S10D). We imaged these two constructs using confocal microscopy and saw no differences in their distribution on the T-cell membrane or localization to the intracellular vesicular compartments (Supplementary Fig. S10E). In addition, to examine the distribution of the CAR in the plane of the plasma membrane, we exposed the T cells to supported lipid bilayers containing only ICAM-1 to increase adhesion, and no differences were observed in the CAR distribution by total internal reflection fluorescence (TIRF) microscopy (Supplementary Fig. S10E).

To compare both synapse formation and T-cell activation at the immune synapse, we transduced T cells with a ZAP70-GFP fusion construct and either the CD19-4-1BBζ-mCherry or CD19-CD28H/T-4-1BBζ-mCherry constructs. ZAP70-GFP expression was identical between the two CAR constructs (Supplementary Fig. S10D). We then seeded the supported lipid bilayer with increasing amounts of CD19 to simulate interaction with cells of different antigen densities for use in TIRF microscopy (Fig. 7C). We found significantly increased recruitment of ZAP70 by the CD28 H/T CAR T cell compared with the CD8 H/T CAR, a difference that was especially pronounced at low CD19 density (Fig. 7D–G). CD28 H/T CARs also demonstrated an increase in the formation of microclusters and then later a supramolecular activation cluster at the center of the cell. These data indicate that the CD28 H/T imparts the CAR with greater ability to organize into clusters that have been associated with T-cell activation (Fig. 7D and H–J). Importantly, there were no differences between the two constructs in the number of CAR molecules observed at the synapse between the T cell and the supported lipid bilayer (Supplementary Fig. S10F). This demonstrates that the differences observed between the two constructs, for any given CD19 density, are not due to differences

Figure 6. (Continued)  G, Schema of a B7-H3 CAR containing a CD28 H/T region and 4-1BB costimulatory domain [B7-H3-CD28H/T-4-1BBζ]. H, One million CHLA255 neuroblastoma cells were engrafted into NSG mice by tail-vein injection in a metastatic neuroblastoma model. Six days later, mice were measured by bioluminescence photometry, and flux values (photons per second) were calculated using Living Image software. Representative bioluminescence images are shown. I, Quantified tumor flux values for individual mice treated as in H. Statistical analysis performed with repeated measures ANOVA. J, Survival curves for mice treated as in H. Statistical analysis performed with the log-rank test. H–J are representative of two experiments with different T-cell donors. For in vitro experiments, error bars represent SD, and for in vivo experiments, error bars represent SEM. P < 0.05 was considered statistically significant, and P values are denoted with asterisks as follows: P < 0.05, not significant, NS; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
in CAR density, but rather are due to differences in functionality in spatial organization. Overall, these data reveal that CARs containing a CD28 H/T domain can, at low antigen density, stimulate enhanced receptor clustering and recruitment of proximal signaling molecules.

**DISCUSSION**

CD19 CAR T cells are revolutionizing the treatment of relapsed and refractory B-cell malignancies, with complete response rates ranging from 70% to 90% in B-ALL and 40% to 50% in non-Hodgkin lymphoma (NHL; refs. 1, 3–11). Thus
far, mechanisms of resistance have fallen into two categories, either loss of T cells (due to CAR T-cell dysfunction and lack of persistence; refs. 58, 59) or antigen remodeling on tumor cells (12). In a trial of CD22 CAR T cells, we found that antigen remodeling can be driven by target antigen downregulation below a threshold required for CAR T-cell activity (21), and the same has been seen in clinical trials of BCMA CAR T cells (22). As CARs are translated to solid tumors, it is expected that tumor heterogeneity, resulting in selection of both antigen-negative as well as antigen-low cells, will emerge as a major issue affecting the efficacy of CAR T cells.

Consistent with this, we demonstrate that primary NHL samples display a remarkable amount of heterogeneity in CD19 expression, which could account for the lower reported response rates in this disease compared with B-ALL (1, 5, 7, 9, 10, 60). Using the well-described NALM6 preclinical model, we demonstrate the limited activity of CD19 CARs against low-antigen-density tumor cells, in line with previously reported data for various CARs (16–20, 56). Although some studies have shown that some tumor cell killing can be maintained by CAR T cells even when antigen density is very low (18, 61, 62), our data demonstrate that evidence of cytotoxicity in vitro is not sufficient for in vivo activity and that antigen density must be above thresholds required for cytokine production and proliferation in order for CARs to be effective in murine models (16, 17, 21). These data also clearly differentiate CARs from native TCRs, which are known to target much lower levels of antigen, as low as one to ten molecules of peptide as presented in the MHC (23, 24).

We found that the CD19-28ζ CAR construct employed in axicabtagene ciloleucel is more active against CD19-low tumor cells than the CD19-4-1BBζ CAR construct employed in tisagenlecleucel. Of note, neither of the constructs employed here completely matches the manufacturing processes for the FDA-approved clinical products; tisagenlecleucel uses a lentivirus, whereas our methods employ retrovirus, and manufacturing of both products is different from that employed in our laboratory. In addition, the regulation of CD19 in response to CAR pressure seen in our model does not reflect natural physiology and human clinical experience, as the CD19 protein is artificially expressed under the control of a lentiviral promoter. Nevertheless, our findings are consistent with a recent study that found that CD19-CD28ζ CAR T cells are less susceptible to trogocytosis-mediated antigen downregulation than CD19-4-1BBζ CAR T cells (56) and other studies demonstrating enhanced signal strength for CD28 CAR T cells (42). We confirmed the generalizability of this finding by using a CAR targeting HER2 in a xenograft model of HER2-low osteosarcoma. We posit that insufficient CAR signaling by 4-1BBζ CARs is responsible for their attenuated response to low-antigen-density tumors, which is in agreement with findings in which CARs with 4-1BBζ domains were shown to have lower signal strength (42, 63, 64). Although this difference would not account for the rate of CD19-negative relapse in B-ALL, which is often associated with complete loss of the surface epitope recognized by the CAR (65, 66) and is more frequently observed with CD19-4-1BBζ CARs due to sustained immune pressure (12), it could explain differences in response rates in NHL, where CD19 antigen density could potentially be limiting CAR T-cell responses in some cases (9, 60). Prospective studies of the role of CD19 antigen density, as studied by flow cytometry on patient samples, in driving responses in NHL are required to explore this question.
We have identified a fundamental principle relating to strength of signal and CAR efficacy against tumors with low antigen density. Whereas CD19-4-1BBζ CAR T cells are generally preferred for their persistence in patients with B-ALL (5, 6), engineering a long-term persistent CAR T cell that can recognize antigen-low targets would be a significant advance. We hypothesized that signaling could be augmented in CAR T cells by adding additional ITAMs to the zeta chain in the CAR. We generated a CD19-4-1BBζ CAR and found increased signaling in response to antigen that translated to improved in vivo activity. Importantly, despite the enhanced signal strength, these CAR T cells maintained the persistence that has been the hallmark of CARs containing 4-1BB costimulatory domains (4, 6). Therefore, enhancing the strength of cell signaling can result in enhancement of CAR activity against low-antigen-density tumors, extending their clinical reach. This approach would not, however, overcome mechanisms of resistance such as antigen splice variants (65) or complete silencing of genes encoding the target antigen. In addition, increased signal strength could potentially contribute to overactivation and T-cell exhaustion, especially when T cells encounter high-antigen-density target cells. Therefore, mechanisms to decrease T-cell exhaustion while enhancing CAR activity against low-antigen-density cells are particularly important (67).

Several recent articles have suggested that altering CAR design to reduce signal strength could be advantageous to either increase T-cell persistence or reduce cytokine-mediated toxicity (30–33). However, our results demonstrate that reducing signal intensity through any of these methods may decrease CAR T-cell efficacy when antigen density is low. Thus, although limiting CAR T-cell signal strength could successfully reduce toxicity or improve persistence, it may also result in a liability against low-antigen-density-expressing tumor cells, presenting an additional opportunity for tumor immune escape.

In addition to differences in the costimulatory domain, the CAR constructs contain in tisagenlecleucel and axicabtagene ciloleucel also differ in their H/T domains. Previous work has implicated the hinge domain in CAR T-cell efficacy but has focused largely on hinge length (68, 69), whereas the two hinges compared here were of comparable sizes. We generated a new CAR that contains the CD28 H/T region with 4-1BB and CD3ζ endodomains (CD19-CD28H/T-4-1BBζ). Compared with a CD19-4-1BBζ CAR containing a CD8 H/T domain, this CAR regained function against low-antigen-density tumors and performed similarly to CD19-28ζ CAR T cells in an in vivo model of CD19 low-expressing leukemia. This new CAR also demonstrated improved activity in a NALM6 wild-type stress test, while maintaining increased T-cell persistence associated with 4-1BB, indicating that it may represent a superior backbone for further clinical development. Intriguingly, this CD28 H/T domain enhanced the cytolytic capacity and cytokine production of even first-generation CAR T cells that contain no costimulatory domain. This finding calls into question some of the assumptions that have been made around CAR design and brings into focus the need for carefully controlling all constructs when comparing different CARs in preclinical studies.

To understand the mechanism of enhanced antigen-low recognition by CARs containing a CD28 H/T region, we used live-cell imaging and TIRF microscopy. We demonstrate that, similar to CD19-CD28ζ CAR T cells (56), CD19-CD28 H/T-4-1BBζ CAR T cells kill their target cells more quickly post-engagement than CD19-4-1BBζ CAR T cells. In addition, compared with CD19-CD28ζ CAR T cells, CD19-CD28 H/T-4-1BBζ CAR T cells are less likely to die themselves during interaction with a tumor cell. Imaging the immune synapse for 4-1BBζ CAR T cells with either a CD28 or a CD8 H/T domain, we have found that the CD28 H/T domain results in a more organized and stable synapse that is able to recruit both more CAR–ligand complexes and downstream ZAP70, resulting in superior antitumor activity.

We employed the CD28H/T-4-1BBζ CAR architecture using scFV’s targeting two additional tumor antigens, B7-H3 and HER2, and found that it rescued the function of these CARs in clinically relevant models of solid tumors expressing low levels of target antigen. Clinical application of constructs with enhanced reactivity against targets with low-antigen-density would need to be weighed against the possibility of normal tissue toxicity to essential organs that express the targets at low levels.

Next-generation CARs will require precise engineering to “thread the therapeutic window” between differential antigen expression on tumor versus normal tissues. We have found that changes in the signaling domains or the H/T region can alter activity against low- versus high-antigen tumors, deepening our understanding of how CAR architecture can be manipulated to tune CAR function. With targets such as CD19, where normal tissue expression does not represent a major concern for toxicity, CARs can be designed to recognize very low levels of antigen density to increase their efficacy and decrease antigen escape. However, when designing CARs to target shared self-antigens that are expressed at lower levels on normal tissues, one could alter CAR structure to open a therapeutic window that could prevent possible on-target, off-tumor toxicity. This work has implications for CAR design as investigators begin to harmonize the competing interests of enhancing CAR T-cell efficacy and minimizing toxicity.

**METHODS**

**Generation of NALM6 Clones**

The NALM6 cell line expressing GFP and luciferase was obtained from S. Grupp (University of Pennsylvania, Philadelphia, PA; ref. 70). A NALM6 CD19 knockout clone was generated as described previously (16). NALM6 CD19 knockout cells were transduced with truncated CD19 and then FACS sorted to different antigen densities. Cells went through one to two rounds of single-cell cloning to obtain clones expressing variable and distinct amounts of CD19. CD19 antigen density was estimated using the BD Quantitrue Kit per the manufacturer’s protocol. NALM6 cells were also transduced with a lentiviral construct encoding full-length HER2 (Origene) and then FACS sorted to different antigen densities. Cells went through one to two rounds of single-cell cloning to obtain clones expressing variable and distinct amounts of HER2.
**Generation of CAR Constructs**

All CAR constructs were generated using codon optimization (GeneArt, Invitrogen) for the amino acid sequences listed in Supplementary Table S1. Retroviral vectors for CD19-CD28, and CD19-4-1BB CARs were described previously (45). To generate CAR constructs with multiple CD3ζ domains, DNA fragments were codon optimized to differ in DNA sequence from the domains already contained in the CAR constructs. CARs with only one CD3ζ domain were created by truncating the construct just after the first ITAM (33). New CAR constructs were directly ordered from GeneArt (Invitrogen) and cloned into existing CAR vectors or cloned using In-Fusion techniques.

**Production of Retroviral Supernatant, CAR T-cell Transduction, and In Vitro Assays**

Retroviral supernatant was produced as described previously (45). For CAR T-cell transduction, we followed previously published protocols (45) with the exception of using isolated T cells rather than bulk peripheral blood mononuclear cells (PBMC). CAR T-cell cytotoxicity assays were performed by coculturing 50,000 GFP-positive tumor cell targets with CAR+ T cells at the indicated ratios in RPMI 1640 in a 96-well plate and acquiring images every 2 to 3 hours using an Incucyte (Sartorius). The cytotoxicity index was calculated by dividing the total green fluorescence intensity at every time point by the same measurement at the first time point. For CHLA-225 killing experiments, cells were lentivirally transduced with Incucyte Nuclight Red (Eisen BioScience) and flow sorted to a purely transduced population. Killing experiments were performed as above measuring total red fluorescence. Cytokine release was assayed by coculturing 100,000 CAR+ T cells with 100,000 tumor cell targets in complete RPMI 1640. At 24 hours, culture media were collected and cytokines were measured by ELISA (BioLegend). All CAR T-cell cytotoxicity and cytokine in vitro assays were performed on day 10 after activation. T-cell proliferation was measured with Cell Trace Violet (Thermo Fisher Scientific) per manufacturer’s recommendations for a coculture period of four days with indicated tumor cells at a 1:2 ratio performed on day 14 after T-cell activation.

**CAR T-cell Stimulation Experiments**

On day 10 post-T-cell activation, CAR T cells were resuspended at a concentration of 2.5 × 10^6 CAR+ T cells per mL in complete RPMI 1640. T-cell transduction efficiencies were assessed by flow cytometry to ensure that they were comparable in all groups. T cells were stimulated by adding CD19 idiotypic antibody (kindly provided by L. Cooper, MD Anderson Cancer Center; ref. 71) as well as a goat anti-mouse cross-linking antibody (Jackson ImmunoResearch) to the indicated concentrations and incubated at 37°C for the indicated time periods. At the end of the period of stimulation, cells were quenched with cold PBS and cell pellets were collected and flash-frozen.

**Immunoblotting**

Whole-cell protein lysates were obtained in non-denaturing buffer (150 mmol/L NaCl, 50 mmol/L Tris-pH 8, 1% NP-10, 0.25% sodium deoxycholate). Protein concentrations were estimated by using with DC Protein colorimetric assay (Bio-Rad, 5000116). Per sample, 20 μg of protein was mixed with 5× reducing loading buffer (Pierce, 39000), boiled at 95°C for 5 minutes, and loaded onto 10% PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Signals were detected by enhanced chemiluminescence (Pierce) or with the Odyssey imaging system. Representative blots are shown. The following primary antibodies used were purchased from Cell Signaling Technology: ERK1/2 (no. 9101) and pERK1/2 (no. 9101). The CD3ζ (A142-F6) and phospho-CD3ζ (EP265[2Y]) antibodies were purchased from Abcam. Densitometric analysis of the phosphorylation-specific antibodies was performed using the ImageJ v1.51j (NIH). Phosphorylation levels were determined by calculating the ratio of the intensity of the signal obtained with phospho-specific antibodies relative to the total. Relative values were normalized to one of the untreated controls in every gel.

**Calcium Flux**

T cells were first barcoded with FTTC, PE-Cy7, or PerCP Cy5.5-conjugated anti-CD45 (BioLegend) in PBS no Ca2+/Mg2+ with 2% FBS for 30 minutes at 4°C. Cells were then pooled together and loaded with 5 μmol/L Indo-1 ratiometric dye (Thermo Fisher Scientific) for 45 minutes at 37°C. Cells were washed twice, resuspended in RPMI Ca2+/Mg2+ without phenol red, and incubated for an additional 15 minutes at 37°C. Ca2+ measurements were acquired on a BD Fortessa flow cytometer. CD19 CAR cross-linking was induced via 5 μg/mL anti-idiotype antibody plus 5 μg/mL goat anti-mouse Fab’2 (Jackson ImmunoResearch). As a positive control, T cells were treated with 1 μg/mL ionomycin (Thermo Fisher Scientific) at the conclusion of the assay.

**CRISPR/Cas9 Editing of TRAC Locus**

Activated T cells were electroporated after removal of activation beads. Electroporation and gene targeting were performed as described previously (72). High-performance liquid chromatography-purified sgRNA targeting the sequence GAGAATCAAAATCG GTGAAT in the TRAC gene with chemical modifications at the three terminal nucleotides on both ends (ref. 73; Synthego) was complexed with high-fidelity spCas9 protein (ref. 74; IDT) at a molar ratio of 2.5:1 (sgRNA:protein) to form ribonucleoprotein. The complex was electroporated into activated T cells using a 4D-Nucleofector (Lonza) in buffer P3 (Lonza) using program E0-115. One million cells were treated per reaction per cuvette in 16-cuvette strips. The cells were resuspended in media [X-Vivo 15 (Lonza) supplemented with 5% human serum (Sigma-Aldrich) and 100 IU/mL IL2 (PeproTech)] after electroporation and diluted to the target density.

**Single-Cell Microwell Killing Assay**

This assay was adopted from previously published experiments (36). CAR T cells were labeled with CellTrace Far Red (Thermo Fisher Scientific) 12 to 24 hours before experiments and resuspended in Phenol Red–free RPMI media. Thin-walled 50-μm square PDM5 micro-grid arrays (MicroSurfaces) were adhered to 24-well glass-bottomed imaging dishes (CellVios). NALM6-GFP and CAR T cells were seeded at low density (32,000 cells per large well). Propidium iodide (PI) 1× stock solution (eBioscience) was added to each well to a final dilution of 1:10,000 from stock. Each experimental run contained one well of each CAR construct, enabling paired comparison to account for experiment-to-experiment variation. Six experimental replicates were performed across two distinct T-cell donors.

Microwells were imaged for 10 to 12 hours every 10 minutes using a Nikon Ti-E inverted microscope at 10× magnification at 37°C and 5% CO2. Images were acquired using Differential Interference Contrast and Epifluorescence with 488nm (GFP), 555 (PI), and 647 (Far Red) excitation wavelengths. Acquisition was controlled using Micro-Manager software (75).

T-cell and NALM6 images were analyzed using a custom Python script using the TrackPy library (https://doi.org/10.5281/zenodo.3492186). Briefly, T cell–NALM6 conjugates were defined as instances in which the centroids of a T cell and NALM6 cell were located within a threshold distance (approximately 1.5 cell radii) for at least six consecutive frames. The PI fluorescence was tracked for each cell in the conjugate and used to classify conjugates as lytic (NALM6 PI spikes first), abortive (cells dissociate without a PI fluorescence spike for either cell), or T-cell death (T-cell PI spikes...
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first). For lytic and T-cell death conjugates, the time to PI influx was measured by fitting a sigmoid to the plot of PI fluorescence over time and taking the time to half-maximal PI fluorescence.

Time to PI influx was pooled across experiments and compared using an unpaired two-sample t test. The fraction of conjugates per experiment that were lytic and the fraction of nonlytic conjugates per experiment that resulted in T-cell death were compared using a paired Wilcoxon signed-rank test, because these values were constrained to the range (0–1) and thus cannot be approximated as normally distributed.

Supported Lipid Bilayer Experiments

Preparation of Lipid Bilayer. All the following lipids were purchased from Avanti Polar Lipids: 16:0-18:1 POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; catalog no. 850457), 180 PEG5000 PE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt) (PEG5000-PE; catalog no. 880220), 18:1 DGS-NTA (Ni2+) 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Ni2+-NTA-DOGS; catalog no. 790404), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(caprotilong) (sodium salt) (Biotin-Cap-PE; catalog no. 870277). Two types of lipid mixtures were prepared: (i) “DGS-NGA-Ni,” which contains 97.5% POPC, 0.5% PEG5000-PE, 2.0% Ni2+-NTA-DOGS; (ii) “Biotin-Cap-PE,” which contains 97.5% POPC, 0.5% PEG5000-PE, 2% Biotin-Cap-PE. Lipids were dissolved in chloroform in glass tubes, and dried under a stream of argon gas followed by further drying in the vacuum for 2 hours. The dried lipid films were then hydrated with PBS pH 7.4 (Invitrogen). The small unilamellar vesicles (SUV) were produced by 20 freeze–thaw cycles (~80°C and 37°C) and collected as the supernatant after centrifugation at 53,000 × g for 45 minutes at 4°C. SUVs were stored at 4°C and 37°C and 1°C and used within 2 weeks. Glass coverslips (Ibidi, catalog no.10812) were RCA-cleaned followed by extensive washing with pure water, and dried with nitrogen. PDMS (Dow Corning) wells were made by preparing PDMS substrate mixtures according to the manufacturer’s instructions and casting the PDMS mixtures into laser-cut acrylic molds. To build supported lipid bilayers (SLB), PDMS wells and glass coverslips were cleaned with plasma in a Harrick Plasma cleaner before assembling them into glass-bottomed PDMS chambers. SUV suspensions were mixed with various volumetric ratio of the “biotin-PE” SUVs to “DGS-NGA-Ni” SUVs (“biotin-PE” concentration of 0.8%, 0.2%, 0.05%, 0.0125%), and then deposited in each chamber and allowed to form for 1 hour. After 1 hour, wells were washed extensively with PBS to remove excessive SUVs. SLBs were then functionalized by incubation for 10 minutes with streptavidin–Alexa 647 followed by incubation for 20 minutes with biotin-CD19. Before imaging, wells were washed with the imaging buffer containing 20 mmol/L HEPES pH 7.4, 1 mmol/L CaCl2, 135 mmol/L NaCl, 0.5 mmol/L MgCl2, 4 mmol/L KCl, and 10 mmol/L glucose.

Imaging. Imaging was performed on an inverted microscope (Nikon TiE) equipped with a Yokogawa spinning disk confocal and TIRF combined system (Spectral Diskover), a Nikon 100 × Plan Apo 1.49 NA oil immersion objective, and four laser lines (405, 488, 561, and 640 nm), a Hamamatsu Flash 4.0, and µManager software to run the microscope and capture the images. Confocal images were captured using an Andor iXon electron-multiplying charge-coupled device camera. For TIRF imaging, a polarization filter was placed in the excitation laser path to polarize the light perpendicular to the plane of incidence. The angle of illumination was controlled with either a standard Nikon TIRF motorized positioner or a mirror moved by a motorized actuator (CMA-25CCCL; Newport). Data collection was performed at 37°C. Before imaging, cells were pelleted, washed, and resuspended with the imaging buffer. The experiments were performed two times with different T-cell donors. Each experiment consisted of each SLB condition with each CAR construct in triplicate.

Image Analysis. Images were analyzed using Fiji. To quantify the recruitment/clustering levels, a uniform cell-sized circular region of interest (ROI) that is 10 μm in diameter was manually placed over the region of cell fluorescence. The average and the SD of fluorescence intensity inside the ROI was measured respectively, and the index of dispersion/normalized variance, that is, the ratio of the SD fluorescence intensity to the average fluorescence intensity, was used to indicate the dispersive distribution of fluorescence intensity for each cell. The threshold for ZAP70-GFP recruitment was set at the index-of-dispersion = 0.7, above which the ZAP70 fluorescence at the plasma membrane reached an intensity level higher than that of the cytosolic ZAP70 fluorescence, and formed clusters that could be appreciated from the TIRF images. The threshold for the ligand–receptor complexes was set at the index-of-dispersion = 0.15, above which the fluorescent-labeled ligand formed clusters that could be identified on the otherwise evenly diffuse planar lipid bilayer.

Flow Cytometry

Other than for human primary tumor and PBMC samples (see below), flow cytometry was performed using a FACScan Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star). CD19 expression was measured using either PE- or APC-conjugated antibody (clone SJ25C1, BD Biosciences). For CD19 CAR detection, CD19 CAR idotype antibody (71) was directly conjugated to DyLight 650 with an Antibody Labeling Kit (Thermo Fisher Scientific). For HER2 CAR detection, recombinant human E8B2/HER2 Fc chimera protein (R&D Systems) was directly conjugated to DyLight 650 with an Antibody Labeling Kit (Thermo Fisher Scientific). For B7-H3 CAR detection, recombinant human B7-H3 Fc chimera protein (R&D Systems) was directly conjugated to DyLight 650 with an Antibody Labeling Kit (Thermo Fisher Scientific). In cell trace violet experiments, CAR T cells were distinguished from tumor cells by staining for CD45 (eBioscience, clone HI30, PerCP-Cy5.5) and anti-CD19 CAR idotype antibody-DyLight 650. For the T-cell exhaustion panel, T cells were stained with anti-human LAG3 (eBioscience, clone 3DS223H, PE), PD-1 (eBioscience, clone J105, PE-Cy7), and TIM3 (BioLegend, clone F38-2E2, BV510). In mouse experiments, leukemia cells obtained from mouse bone marrow were identified by high GFP expression and stained for CD19 (clone SJ25C1, BD Biosciences, PE) and, in some experiments, CD81 (BioLegend, clone 5A6, PE/Cy7). CAR T cells (bone marrow and spleen) were identified by staining for CD45 (eBioscience, clone HI30, PerCP-Cy5.5), CD4 (BD Biosciences, clone SK3, BUV395), CD8 (BD Biosciences, clone SK1, BUV805), and CD19 CAR (DyLight-650 conjugated CD19 idioype). TCR expression was measured using either PE- or APC-conjugated mouse anti-human B7-H3 antibody (R&D Systems, clone MAB1027) and HER2 with PE or APC conjugated anti-human CD340 antibody (BioLegend, clone 24D2). Viability dye was used in all coculture and m rm experiments (eBioscience Fixable Viability Dye) to gate out dead cells.

Immunophenotyping of Human Samples by Flow Cytometry

All specimens were obtained with written informed consent in accordance with the Declaration of Helsinki from either Stanford University Medical Center (Stanford, CA) or the Norwegian Radium Hospital (Oslo, Norway), with approval from Stanford University’s Administrative Panels on Human Subjects in Medical Research and the regional ethical committee in Norway (2.2007.2949). The patient cohorts have been described previously (76). Samples were
pretreatment specimens from DLBCL (n = 18), follicular lymphoma (n = 27), CLL (n = 13), and MCL (n = 42). PBMCs were from healthy volunteers at Stanford Hospital. The lymphoma samples and samples from healthy donors were thawed as described and stained with antibodies to CD3, CD19, CD20, CD22, CD79B (BD Biosciences, clones UCHT1, SJ25C1, L27, S-HCL-1, 3A1-2E7 respectively), Igκ (Thermo Fisher Scientific, polyclonal), and Igκ (Thermo Fisher Scientific, clone HP6062), and acquired on a LSR II flow cytometer. Data was analyzed using Cytobank Software (www.Cytobank.org). Lymphoma cells were identified as CD3-negative cells and healthy donor B cells as CD3-CD20+ cells. Relative protein expression was calculated using log-transformed median fluorescence intensity data and normalized to healthy donor PBMC B cells run in the same experiment. Antigen densities in B cells from 3 healthy donor PBMCs were estimated by staining with PE-conjugated antibodies (clones as above) and using the BD Quantibrite Kit per the manufacturer’s protocol.

In Vivo Experiments

Immunodeficient NSG mice (NOD.Cg-PrkdcscidIl2rgtm1Wjl/Sjcl) were purchased from The Jackson Laboratory or bred in house. Mice used for in vivo experiments were between 6 and 10 weeks old, and the ratio of male to female mice was matched in experimental and control groups. All animal studies were carried out according to Stanford University Animal Care and Use Committee-approved protocols.

NALM6 In Vivo Models

In the CD19-low model, NSG mice were injected with 1 × 106 NALM6-CD192,053 cells and then treated with 3 × 106 CAR+ T cells or an equivalent number of untransduced MOCK control CAR T cells four days later. NALM6-CD192,053 was used in all CD19-low in vivo models, as it best illustrated differences in disease control between CARs of different structures. To test the effects of activation of CAR T cells by CD19-high tumor cells on their activity against CD19-low tumor cells, two groups of 5 mice were injected with 1 × 106 NALM6-CD192,053 (expressing GFP-luciferase) and then one day later one group was injected with 0.5 × 106 NALM6–wild-type (CD19-high and no GFP-luciferase). Both groups of mice were then treated with 3 × 106 CAR+ T cells four days after inoculation of the CD19-low tumor cells. In the NALM6 stress test model, mice were injected with 1 × 106 NALM6–wild-type cells and then treated with 2.5 × 106 CAR+ T cells or an equivalent number of total untransduced MOCK control T cells three days later. In the NALM6-CD191,987 experiments, mice were injected with 1 million NALM6-CD191,987 cells and then treated with 3 million CAR+ T cells or an equivalent number of untransduced MOCK control T cells three days later. Mice were sacrificed when they began displaying signs of clinical leukemia. In the T-cell persistence experiments, mice were injected with the parental NALM6-GFP-luciferase line and then treated with 5 × 106 CAR+ T cells or an equivalent number of untransduced MOCK control T cells three days later. Mice were then sacrificed at indicated timepoints to harvest spleens and bone marrow for cell counting and phenotyping. In all experiments, leukemia burden was evaluated using the Xenogen IVIS Lumina (Caliper Life Science). Mice were injected intraperitoneally with 3 mg d-luciferin (PerkinElmer) and then imaged 4 minutes later with an exposure time of 30 seconds. Saturated images were then reimagined with auto exposure. Luminescence images were analyzed using Living Image software (PerkinElmer).

143b Osteosarcoma In Vivo Model

One million 143b cells were injected periosteal to the tibia in NSG mice. Three days later, mice were treated with 1 × 106 of the indicated CAR+ T cells or an equivalent number of total untransduced MOCK control T cells. Tumor growth was measured with digital calipers once to twice weekly, and the tumor area was calculated by multiplying the lengths of the major and minor axes. Mice were euthanized when the tumor exceeded a size set by institutional protocols.

CHLA-255 In Vivo Metastatic Experiment

CHLA-255 was kindly provided by R. Seeger (Children’s Hospital Los Angeles, Los Angeles, CA). CHLA-255 cells were transduced with GFP-luciferase and flow sorted to a pure population. On day 0, NSG mice were intravenously injected with 1 × 107 CHLA-255 cells. On day 7, mice were intravenously treated with 1 × 105 of the indicated CAR+ T cells or an equivalent number of total untransduced MOCK control T cells. Disease burden was evaluated using the Xenogen IVIS Lumina (Caliper Life Science) as described above.

Statistical Analysis

Data were visualized and analyzed using GraphPad Prism software. Graphs represent either individual values or group mean values ± SEM for in vivo experiments and group mean values ± SD for in vitro experiments. The P values were calculated with the statistical test described in the relevant figure legend. P < 0.05 was considered statistically significant, and P values are denoted with asterisks as follows (P > 0.05, not significant; NS, *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001).

Disclosure of Potential Conflicts of Interest

R.G. Majzner is a consultant at Xyphos Inc./Astellas Pharma and Lyell Immunopharma and is a scientific advisory board member at GammaDelta Therapeutics. S.P. Rietberg is an employee at Lyell Immunopharma. E. Sotillo is a consultant at Lyell Immunopharma. L. Labanieh is a consultant at Lyell Immunopharma, and has ownership interest (including patents) in Stanford University. E.W. Weber is a consultant at Lyell Immunopharma. J. Theruvath is a consultant at Dorrant Therapeutics. R.C. Lynn is an associate director at Lyell Immunopharma. R.D. Vale is vice president and executive director, Janella Research Campus, Howard Hughes Medical Institute. C.L. Mackall is an advisory board member at Vor, Servier, and Allogene, and is a founder/consultant at Lyell Immunopharma, reports receiving commercial research grants from Obsidian and Bluebird Bio, and has ownership interest (including patents) in Lyell Immunopharma, Allogene, and Vor. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: R.G. Majzner, A.R. Dunn, R.D. Vale, C.L. Mackall
Writing, review, and/or revision of the manuscript: R.G. Majzner, E. Sotillo, R. Dong, L. Labanieh, E.W. Weber, R.C. Lynn, A.R. Dunn, R.D. Vale, C.L. Mackall
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.G. Majzner, P. Xu, C.L. Mackall
Study supervision: R.G. Majzner, C.L. Mackall
Tuning the Antigen Density Requirement for CAR T Cells

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Robbie G. Majzner, Skyler P. Rietberg, Elena Sotillo, et al.


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