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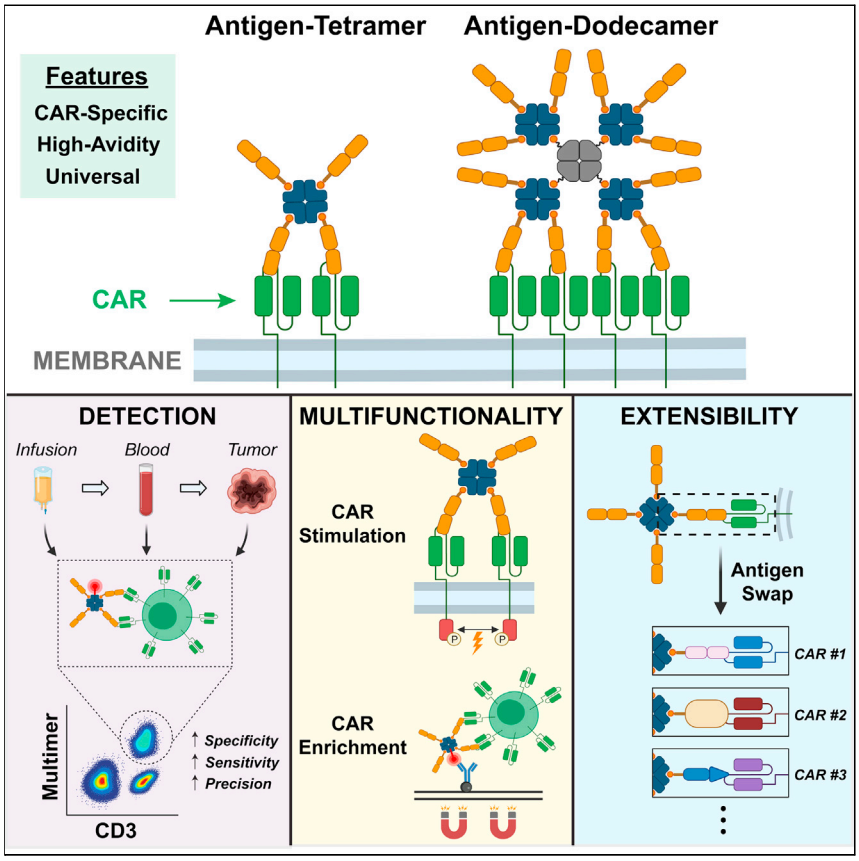
Antigen multimers: Specific, sensitive, precise, and multifunctional high-avidity CAR-staining reagents



More effective reagents for staining chimeric antigen receptors (CARs) will catalyze applications of CARs in the clinic. Here, we engineered antigen multimers, reagents that specifically stain CARs with high avidity. In addition to high CAR-detection sensitivity and precision, antigen multimers enable ≥100-fold enrichment of rare CAR T cells, CAR T cell stimulation for phenotyping, and high-purity isolation of CAR T cells from patient biospecimens. Since they can be readily extended to new CAR systems, antigen multimers will support the fast development of next-generation CARs.

Development
Practical, real world, technological considerations and constraints

Yifei Hu, Guoshuai Cao, Xiufen Chen, ..., Hans Schreiber, Justin P. Kline, Jun Huang

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Highlights

Antigen multimers detect CARs with high specificity, sensitivity, and precision

Antigen multimers enable magnetic enrichment and stimulation of CAR T cells

Antigen multimers can isolate and phenotype CAR T cells from patient biospecimens

Antigen multimers are readily extensible to existing and new CARs

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Antigen multimers: Specific, sensitive, precise, and multifunctional high-avidity CAR-staining reagents

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SUMMARY

Although chimeric antigen receptor (CAR) T cell therapy has transformed cancer treatment, high-quality and universal CAR-staining reagents are urgently required to manufacture CAR T cells, predict therapy response, decipher CAR biology, and engineer new CARs. Here, we developed tetrameric and dodecameric forms of a multifunctional and extensible category of high-avidity CAR-staining reagents: antigen multimers. Antigen multimers detected CARs against CD19, HER2, and Tn-glycoside with significantly higher specificity, sensitivity, and precision than existing reagents. In addition to accurate CAR T cell detection by flow cytometry, antigen multimers also enabled ≥100-fold magnetic enrichment of rare CAR T cells, selective CAR T cell stimulation, and high-dimensional CAR T cell profiling by single-cell multi-omics analyses. Finally, antigen multimers accurately captured clinical anti-CD19 CAR T cells from patients' cellular infusion products, post-infusion peripheral blood, and tumor biopsies. Antigen multimers can be readily extended to other CAR systems by switching the antigen ligand. As such, antigen multimers have broad clinical and research applications.

INTRODUCTION

Chimeric antigen receptor (CAR) T cell therapy combines immunoengineering, gene therapy, and synthetic biology to treat cancer. A CAR is a chimeric membrane protein that generally consists of an extracellular single-chain antibody fragment (scFv), an extracellular hinge, a transmembrane region, and intracellular signaling domains. CARs can specifically recognize tumor-associated surface antigens, initiate T cell activation, promote T cell proliferation and differentiation, and trigger cytotoxicity against cancer cells.[1,2] To treat cancer, CARs have been specifically engineered to target various tumor-associated surface antigens, including CD19,[3] human epidermal growth factor receptor 2 (HER2),[4] Tn-glycoside,[5] epidermal growth factor receptor variant III,[6] and mesothelin.[7]

Over the course of autologous CAR T cell therapy, a patient's lymphocytes are collected through apheresis, activated, retrovirally transduced with the CAR, expanded *ex vivo*, and infused back into the patient for cancer treatment. Upon recognition of antigen on cancer cells, CAR T cells proliferate, differentiate, kill antigen-expressing cancer cells, and produce cytokines *in vivo*. To date, the US Food and Drug Administration (FDA) has approved four autologous CAR T cell formulations to target $CD19^+$ tumor cells in relapsed/refractory B cell malignancies: tisagenlecleucel

Progress and potential

Chimeric antigen receptor (CAR) T cell therapy involves modifying a cancer patient's T cells to kill cancer cells. However, better CAR-staining tools are required to ensure CAR manufacturing quality control, analyze treatment effects, decipher biological mechanisms, and engineer new formulations. To meet this critical need, we engineered antigen multimers, reagents that specifically bind CARs with high avidity. In addition to high CAR-detection sensitivity and precision, antigen multimers broadly support many functions, including magnetic enrichment of rare CAR T cells, selective CAR T cell stimulation for phenotyping, and high-purity isolation of CAR T cells from patient biospecimens for multi-omics profiling. Since antigen multimers are readily extensible to existing and new CAR systems, antigen multimers can keep pace with the fast development of next-generation CARs. Therefore, we expect broad and sustainable applications for antigen multimers in laboratories and clinics.

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(Kymriah), axicabtagene ciloleucel (Yescarta), brexucabtagene autoleucel (Tecartus), and lisocabtagene maraleucel (Breyanzi). Remarkably, anti-CD19 CAR T cell therapy elicited durable complete response rates of 40%–54% in diffuse large B cell lymphoma (with axicabtagene ciloleucel, lisocabtagene maraleucel, and tisagenlecleucel),[8–11] 60% in B cell acute lymphoblastic leukemia (with tisagenlecleucel),[12] and 67% in mantle cell lymphoma (with brexucabtagene autoleucel).[13] Furthermore, many CARs are being actively investigated in clinical trials for multiple myeloma and even solid tumors, including neuroblastoma, prostate cancer, breast cancer, ovarian cancer, non-small cell lung cancer, and melanoma.[14]

Despite success and excitement around CAR therapies, more accurate and universal CAR-staining reagents are required to fulfill the needs of CAR T cell detection in research, industry, and clinics.[15,16] Simultaneously, numerous challenges exist in the CAR T cell therapy field, including clinical non-response/relapse mechanisms, *in vivo* CAR T cell biology, and poor efficacy against solid tumors.[14,15,17,18] To improve CAR T cell therapy, scientists and clinicians require specific, sensitive, precise, and multifunctional CAR-staining reagents to perform biochemical assays, conduct preclinical studies, profile patient biospecimens, and develop new CARs.

However, existing CAR-staining reagents are each associated with limitations.[15] Generic CAR-staining reagents (polyclonal anti-IgG antibodies and Protein L) that bind the CAR scFv are limited by non-specific binding, incompatibility with other antibodies, and requirements for multi-step staining procedures.[19] Specific CAR-staining reagents (target antigen and anti-idiotype antibodies) can be commercially available but often are limited by protein instability and high developmental cost.[20–23] Furthermore, all existing CAR-staining reagents are limited by $\leq$2-valency binding. Although higher-avidity CAR-staining reagents may improve CAR-detection performance, such reagents have not been developed.[15]

To address these limitations, here we designed, constructed, tested, and validated tetrameric and dodecameric forms of a new, multifunctional, and extensible category of high-avidity CAR-staining reagents: antigen multimers. According to results from cell lines and patient biospecimens, antigen multimers enabled highly specific, sensitive, and precise CAR detection, magnetic enrichment for sensitive detection of rare CAR T cells, temperature-controlled and specific CAR T cell stimulation for activation phenotyping, and high-dimensional CAR T cell profiling via single-cell multi-omics analyses. In addition, antigen multimers successfully captured clinical anti-CD19 CAR T cells from patients' cellular infusion products, post-infusion peripheral blood, and tumor biopsies, underscoring their broad utility for clinical and translational research. Finally, antigen multimers can be readily extended to existing and new CAR systems by switching the antigen ligand. We demonstrated this extensibility across three independent CAR systems (anti-CD19, anti-HER2, and anti-Tn). Although we only tested antigen tetramers and antigen dodecamers in these current studies, antigen multimers may also be extended to other valencies, such as dimers, pentamers, octamers, and dextramers.[24] Due to their multifunctional and extensible nature, antigen multimers are a new class of CAR-staining reagents with broad CAR T cell research and clinical applications.

RESULTS

Antigen multimer design and validation

To generate high-avidity CAR-staining reagents for CAR T cell detection, we designed, constructed, and validated two forms of antigen multimers: antigen tetramer

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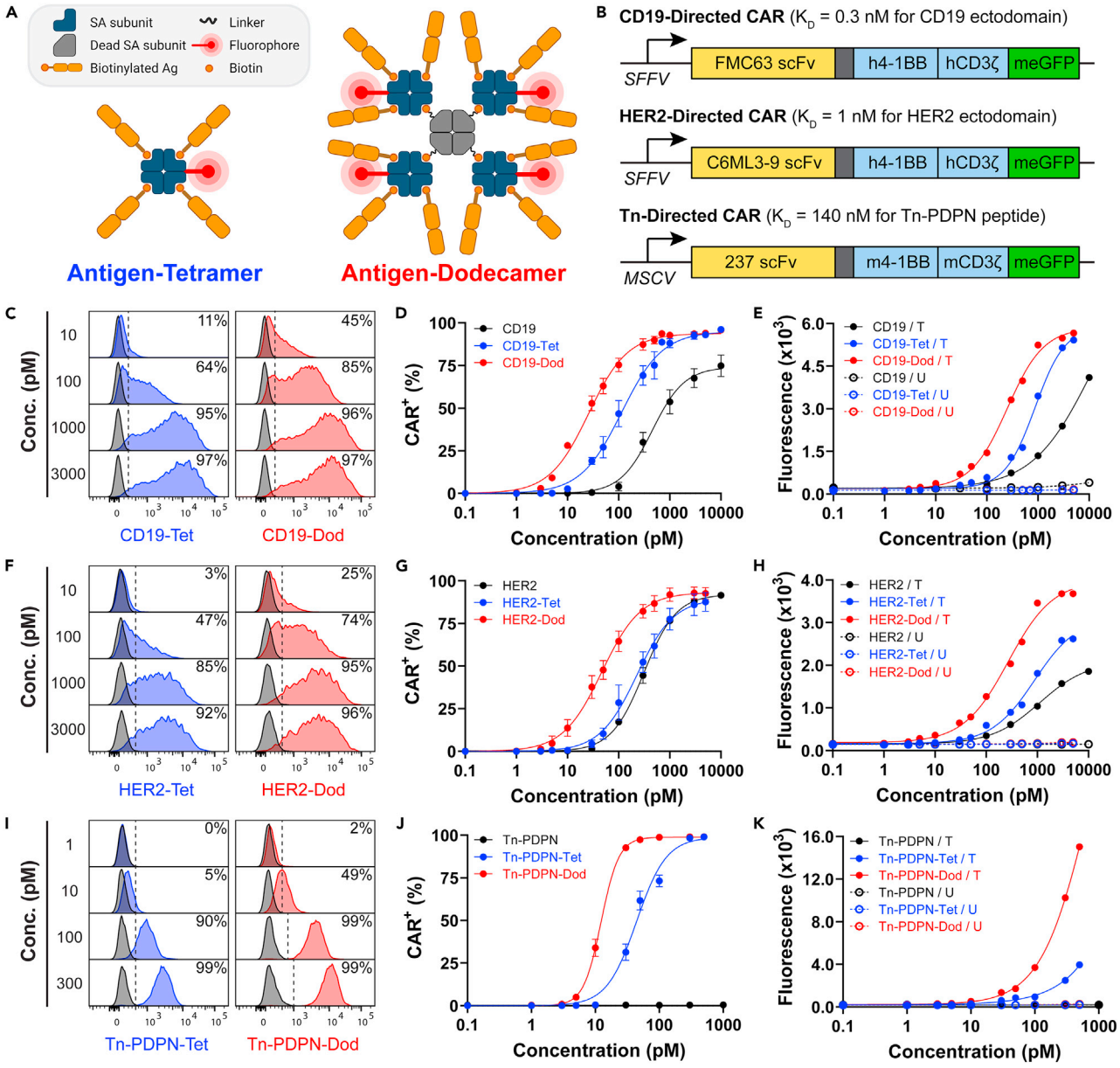

Figure 1. Antigen multimer design and validation in CARs directed against CD19, HER2, and Tn-glycoside

(A) Cartoon depicting antigen tetramer and antigen dodecamer with each major molecular component.

(B) Diagram depicting CD19-, HER2-, and Tn-directed CAR constructs used to validate antigen multimers. Each CAR contains a C-terminal meGFP to track CAR expression. CAR transcription is directed by either a spleen focus-forming virus (SFFV) or a murine embryonic stem cell virus (MSCV) promoter.

(C, F, and I) Staining titration of three types of antigen tetramers (blue) and antigen dodecamers (red) on their matched CAR-transduced cell lines. Staining of the untransduced cell line is shown in gray and used for gating. Histograms are representative of three independent titrations.

(D, G, and J) Triplicate staining titration results for antigen tetramers, antigen dodecamers, and monomeric antigen controls were fitted to dose-response curves. The mean ± standard error of the mean is depicted for each concentration.

(E, H, and K) Plots from representative titrations (fitted to dose-response curves) showing the relationship between geometric mean fluorescence intensity and staining reagent concentration on CAR-transduced (T) and untransduced cell lines (U).

See also Figure S1.

and antigen dodecamer. Analogous to MHC multimers,[25–27] antigen multimers are comprised of the CAR antigen ligand multimerized on a streptavidin scaffold (Figure 1A). The antigen ligand moiety is expected to bind the CAR with antibody-like

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affinity. We designed antigen tetramers and antigen dodecamers with 4-valency and 12-valency binding, respectively, in order to leverage higher-avidity binding for enhanced detection.

To construct antigen tetramers, fluorescent tetrameric streptavidin was associated with four biotinylated antigen ligand molecules. To construct antigen dodecamers, biotinylated tetrameric dodecamer base protein was associated with four fluorescent tetrameric streptavidin, each of which was further associated with three biotinylated antigen ligand molecules. For all experiments, antigen ligand molecules were site-specifically biotinylated instead of randomly biotinylated, in order to curtail higher-order oligomerization and steric obstruction of the CAR binding site.[28]

Next, we validated antigen multimers on three second-generation CARs: anti-CD19 CAR (clone FMC63), anti-HER2 CAR (clone C6ML3-9), and anti-Tn CAR (clone 237) (Figure 1B). From N terminus to C terminus, each validation CAR consists of an extracellular scFv, CD8α hinge and transmembrane region, 4-1BB and CD3ζ intracellular domains, and monomeric enhanced green fluorescent protein (meGFP). The C-terminal meGFP was included to track CAR transduction efficiency and expression. The hinge, transmembrane region, and intracellular domains were designed identically to the clinical CAR construct used in tisagenlecleucel. The anti-CD19 and anti-HER2 CARs utilize human domains, while the anti-Tn CAR utilizes murine domains. Our three validation CARs can test antigen multimers for utility: (1) in clinical research, since our anti-CD19 CAR utilizes the same FMC63-based scFv as in clinical CD19-directed CAR therapies; (2) under CAR binding affinities widely ranging from 0.3 nM to 140 nM;[4,29,30] and (3) under antigen ligand molecular sizes widely ranging from 2.6 kDa to 73 kDa.

Antigen multimers stain CARs directed against CD19, HER2, and Tn-glycoside

Staining capabilities of antigen multimers were first examined on CAR-transduced cell lines, including human Jurkat cells and murine $58^{-/-}$ hybridoma cells. The meGFP-tag on the CAR served as a marker for successful CAR transduction and expression (Figures S1A–S1C). Subsequently, CD19 multimers, HER2 multimers, and Tn-podoplanin peptide multimers (Tn-PDPN multimers) were titrated to stain CAR-meGFP cell lines. The monomeric antigen ligand was included as a control for avidity. Staining reagent and meGFP fluorescence intensities were simultaneously measured by flow cytometry.

Staining titrations demonstrate that CD19 multimers, HER2 multimers, and Tn-PDPN multimers stained their matched CAR-transduced cell lines (Figures 1C, 1F, and 1I). Untransduced cells were negligibly stained. Furthermore, a negative control (BSA multimers) generated minimal non-specific fluorescence (Figures S1D and S1E). Higher antigen multimer concentrations stained more cells (Figures 1D, 1G, and 1J). At the highest concentrations, antigen tetramers and antigen dodecamers equally captured $\geq$92% of their matched target cells. Higher antigen multimer concentrations also stained with greater mean fluorescence intensity (Figures 1E, 1H, and 1K). These findings indicate that high-avidity antigen multimers stain matched CARs in a dose-dependent manner, while generating negligible non-specific fluorescence (with both biological and BSA-multimer controls).

At equimolar concentrations, antigen dodecamers enhanced fluorescence relative to antigen tetramers. Furthermore, both antigen multimers enhanced fluorescence relative to the monomeric antigen ligand. The magnitude of these enhancements positively correlated with the dissociation constant of the CAR for its antigen ligand.

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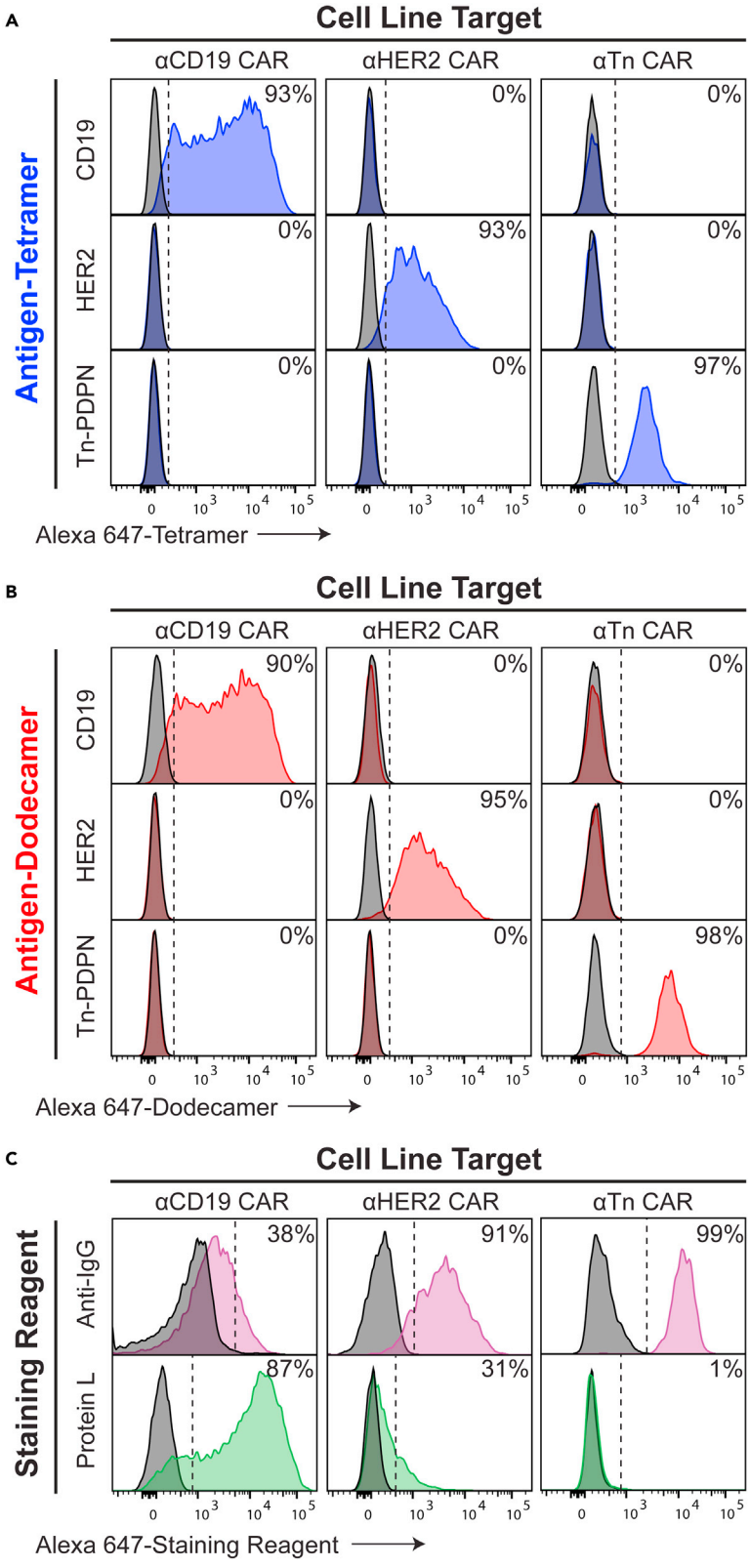

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Figure 2. Antigen multimers are highly specific

(A and B) Antigen tetramers (A) and antigen dodecamers (B) were evaluated for specificity by staining cell lines transduced with CD19-, HER2-, and Tn-directed CARs. Staining of untransduced cell lines is shown in gray and used for gating.

(C) Similar staining assays were conducted with existing CAR-staining reagents, which include polyclonal anti-IgG antibodies and Protein L. Staining of untransduced cell lines is shown in gray and used for gating. Histograms are representative of three independent staining experiments. See also Figure S2.

Fluorescence enhancement by avidity was marginal when staining the anti-CD19 CAR (K_D = 0.3 nM), moderate when staining the anti-HER2 CAR (K_D = 1 nM), and substantial when staining the anti-Tn CAR (K_D = 140 nM). For the Tn-PDPN ligand, Tn-PDPN dodecamers caused up to 4-fold greater fluorescence than Tn-PDPN tetramers, while monomeric Tn-PDPN completely failed to stain at similar concentrations (Figures 1K and S1F). Monomeric Tn-PDPN only stained the anti-Tn CAR at >1,000-fold higher concentrations (Figure S1G). Hence, the binding avidity of an antigen multimer enhances its staining capability, particularly for lower-affinity CARs.

Antigen multimers are highly specific

Having titrated three types of antigen tetramers and antigen dodecamers against their respective matched CARs, we further examined antigen multimers for staining specificity. One source of non-specificity is staining of cells not expressing CARs. Another source of non-specificity is staining of cells expressing unmatched CARs. For example, CD19 multimers should not stain cells expressing anti-HER2 or anti-Tn CARs. Our staining results demonstrate that antigen tetramers (Figure 2A) and antigen dodecamers (Figure 2B) stained $\geq$90% of their matched CAR-transduced cell lines. On the other hand, staining of untransduced cells and non-matched CAR-transduced cell lines were equally negligible ($\leq$1%). Therefore, antigen multimers are highly CAR-specific.

Then, we evaluated the staining specificity of existing generic CAR-staining reagents: polyclonal anti-IgG antibodies and Protein L.[15,19] Since both CAR-staining reagents bind to Fab-like molecules on the cell surface, neither can discriminate between different CARs. Relative to antigen multimers, polyclonal anti-IgG antibodies (Figure 2C, top) provided equivalent staining of anti-HER2 and anti-Tn CARs. However, anti-IgG antibodies were significantly less efficient than CD19 multimers in staining the anti-CD19 CAR, due to substantial non-specific staining of untransduced cells. Protein L (Figure 2C, bottom), which binds to immunoglobulin κ light chains, stained the anti-CD19 CAR, but the anti-HER2 CAR was barely stained, and the anti-Tn CAR was not stained. This inconsistency across different CARs may be due to the differential affinity of Protein L for alternative κ light chains. These observations support the conclusion that antigen multimers are more specific than existing generic CAR-staining reagents.

Finally, we evaluated the staining specificity of a commercially available anti-FMC63 anti-idiotype antibody, which was specifically designed to detect the FMC63-based anti-CD19 CAR. However, this antibody does not stain non-FMC63-based anti-CD19 CARs under clinical or preclinical investigation.[29,31] At the highest concentrations, anti-FMC63 captured 83% of anti-CD19 CAR cells (Figures S2A and S2B), which was lower than CD19 tetramers (93%) and CD19 dodecamers (90%) did at saturation. Non-specific staining of untransduced cells was negligible (Figure S2C). Based on these findings, while anti-FMC63 and CD19 multimers are equally specific, anti-FMC63 is less efficient at generating fluorescence at saturation (Figures S2D

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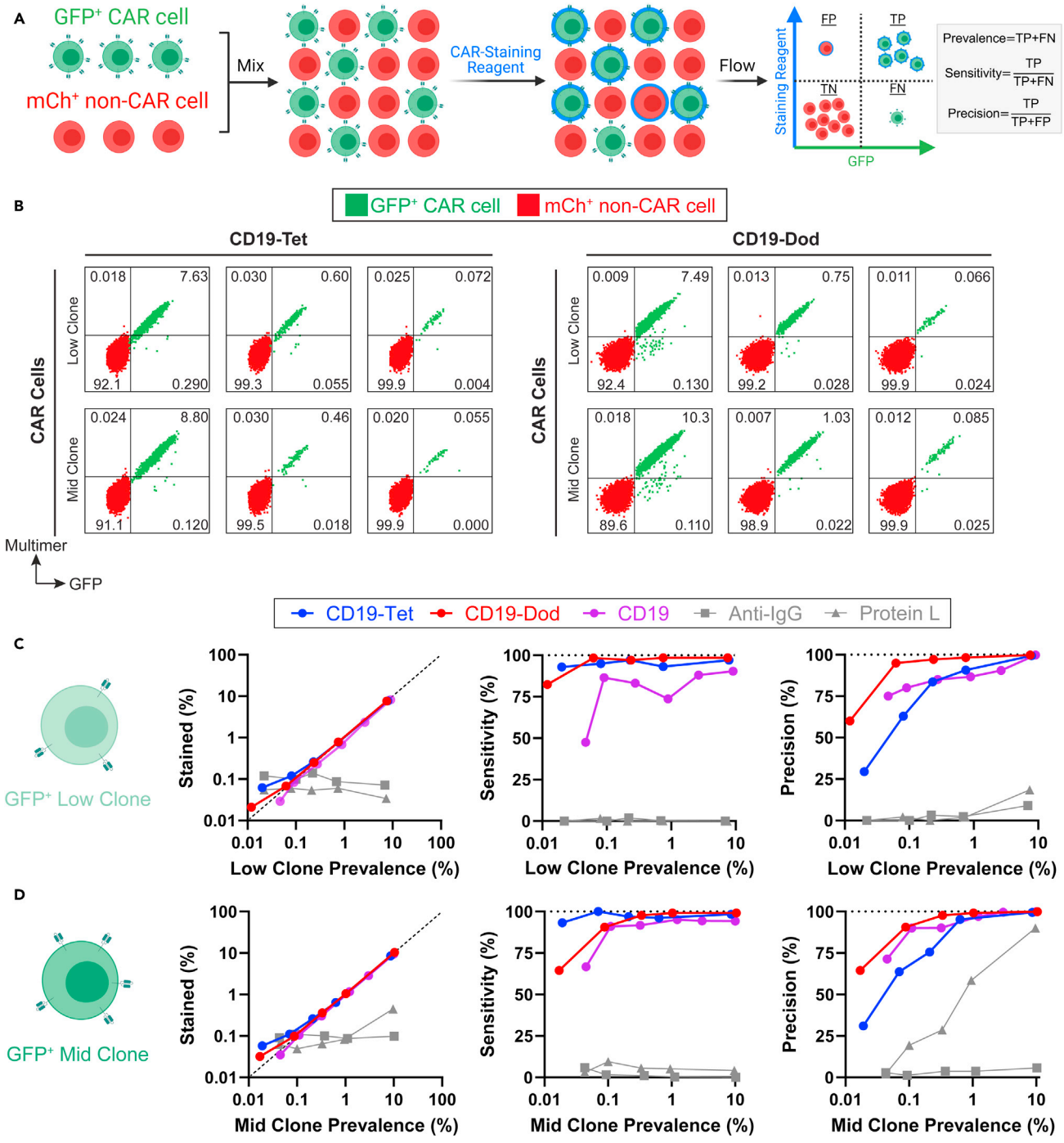

Figure 3. Antigen multimers are highly sensitive and precise

(A) Diagram depicting spike-in assays to measure a CAR-staining reagent's sensitivity and precision. Cell mixtures, constructed from CAR-meGFP-expressing cells and mCherry-expressing non-CAR cells, were stained with a CAR-staining reagent. After analysis by flow cytometry, the cells were placed into four categories: true-positives (TP), true-negatives (TN), false-positives (FP), and false-negatives (FN). Formulas to calculate prevalence, sensitivity, and precision are depicted.

(B) Two anti-CD19 CAR-meGFP-transduced clones (low clone on top row and mid clone on bottom row) with low CAR expression were spiked into mCherry-expressing non-CAR cells. The prevalence of CAR cells was measured by percentage of meGFP$^+$ cells by flow cytometry. Subsequently, CD19 tetramers (left) or CD19 dodecamers (right) were applied. Cells on the flow plots are colored according to fluorescent protein expression.

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Figure 3. *Continued*
(C and D) The prevalence of CAR cells (meGFP$^+$) in cell mixtures plotted against the percentage of cells stained by CAR-staining reagents (left), sensitivity of detection (middle), and precision of detection (right) from spike-in assays with the low clone (C) and mid clone (D). For the stained percentage plot, if a point lies on the dotted line of unity, then the staining reagent accurately captured CAR cell prevalence. For the sensitivity and precision plots, if a point lies on the dotted line (100%), then the staining reagent was completely accurate in discriminating CAR cells from non-CAR cells. See also Figures S3 and S4.

and S2E). The decreased efficiency can be explained by decreased binding avidity for the anti-CD19 CAR.

Antigen multimers are highly sensitive and precise

After examining detection specificity, we evaluated antigen multimers for detection sensitivity and precision. These metrics were measured by applying CD19 multimers to detect meGFP-tagged anti-CD19 CAR cells that are spiked into mCherry-tagged non-CAR cells. All analyses were performed by flow cytometry (Figure 3A). Sensitivity was measured by monitoring for false-negatives. Precision was measured by monitoring for false-positives.

Sensitivity and precision are most critical when CAR cells are rare and CAR expression is low. These conditions are biologically relevant since CAR T cells downmodulate CAR expression after antigen engagement.[32–34] To simulate these conditions, spike-in experiments were performed with two monoclonal cell lines with stably low CAR expression: "low clone" and "mid clone" (Figures S3A–S3D). The low clone (~150,000 CAR molecules per cell) expresses less CAR than the mid clone (~250,000 CAR molecules per cell). Both clones express less CAR than the pre-sorted polyclonal CAR cells (~330,000 CAR molecules per cell) used for previous staining assays. Finally, non-CAR cells were transduced with mCherry to provide an additional distinction between CAR and non-CAR cells.

In spike-in experiments, we targeted cell mixtures with CAR cell prevalence between 10% and 0.01%. However, the true CAR cell prevalence in each cell mixture was measured by percentage of meGFP$^+$ cells. Both CD19 tetramers and CD19 dodecamers specifically stained CAR cells (Figure 3B). As expected, the mid clone was stained more than the low clone (Figure S3C). For both clones, CD19 multimers accurately captured CAR cell prevalence down to 0.1%. Sensitivity remained $\geq$90% down to 0.1% (Figures 3C and 3D). Precision differed between CD19 tetramers and CD19 dodecamers at lower CAR cell prevalence. For the mid clone, CD19 dodecamers captured a purer population than CD19 tetramers did below 1% CAR cell prevalence. For the low clone, CD19 dodecamer captured a purer population than CD19 tetramers did below 10% CAR cell prevalence. Our results demonstrate that CD19 multimers stain with high sensitivity and precision. Importantly, CD19 multimers can sensitively isolate a highly pure ($\geq$90%) CAR cell population from Jurkat cell mixtures, even when CAR cells have low CAR expression and are exceptionally rare ($\leq$1%).

Next, we performed spike-in experiments with existing CAR-staining reagents: monomeric CD19, polyclonal anti-IgG antibodies, and Protein L (Figures S3E–S3G). Monomeric CD19 staining was comparable with CD19-multimer staining for the mid clone. However, detection sensitivity was reduced for the low clone, which suggests that binding avidity increases detection sensitivity for CAR T cells with lower CAR expression. Anti-IgG antibodies failed to detect both clones with low CAR expression. Sensitivity and precision were $\leq$10% at all CAR cell prevalences. Protein L marginally detected some mid clone cells, but failed to detect low clone

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cells. For detection of the mid clone, precision was ≥90% at 10% CAR cell prevalence, but rapidly dropped at lower CAR cell prevalence. Sensitivity never exceeded 10%. These results suggest that neither polyclonal anti-IgG antibodies nor Protein L can capture CAR T cells from cell mixtures when CAR T cells (at any rarity) express fewer CAR molecules per cell.

Finally, we evaluated the detection sensitivity of monomeric CD19 and CD19 multimers under more physiological cell mixtures. Post-infusion CAR T cell therapy patient biospecimens were modeled by spiking the low clone into human peripheral blood lymphocytes at 1% prevalence (Figure S4A). Since the CAR contained an meGFP label, we checked the accuracy of our spike-in via flow cytometry (Figure S4B). This mixture captures both CAR downmodulation[32–34] and potential low prevalence[8] of post-infusion patient CAR T cells. Subsequently, we performed full-dose titrations to compare the abilities of multimers and monomers to detect low clone cells in this mixture. Multimers outperformed monomers across all concentrations (Figure S4C). At saturation, monomers missed ~24% of CAR cells that were detectable by multimers (Figure S4D). Furthermore, compared with monomers, tetramers and dodecamers exhibited 6-fold and 19-fold lower staining half-maximal effective concentration (EC_{50}) respectively (Figure S4E). These findings are consistent with results from cell line mixtures (Figure 3C), and support our conclusion that binding avidity enhances detection sensitivity.

Antigen multimers magnetically enrich for CAR T cells

Having established antigen multimers as highly sensitive CAR-staining reagents even for cells with low CAR expression, we predicted that antigen multimers can enrich rare CAR T cells from cell mixtures. CAR T cell enrichment is expected to facilitate the detection and investigation of rare CAR T cell subsets.[25] Importantly, CAR T cells can be rare *in vivo*, particularly those with a persistent, resting memory phenotype, which are correlated with durable responses in patients.[35] Moreover, CAR T cell enrichment may be useful during CAR T cell infusion product manufacturing if CAR transduction efficiency is low.

To perform CAR T cell enrichment with antigen multimers, we magnetically selected for CAR cells by adapting a method employed with MHC tetramers (Figure 4A).[36] Cells were stained, first with allophycocyanin (APC)-labeled CD19 tetramers or dodecamers, and second with anti-APC antibodies conjugated to magnetic particles (anti-APC microbeads). The stained populations were applied to a magnetic column. Non-CAR cells did not bind the column and were removed (negative selection). CAR cells were subsequently eluted under pressure after removal of the magnet (positive selection).

We employed this magnetic selection strategy to enrich for $meGFP^+$ CAR cells spiked into $mCherry^+$ non-CAR cells. To simulate difficult enrichment conditions, we used CAR cells with stably low CAR expression (low clone and mid clone, previously described in Figures S3A–S3D). CAR cells in initial stained populations were rare (~0.2%, Figure 4B). Based on CAR cell prevalence in the flow-through, ~35% and ~55% of low clone and mid clone CAR cells respectively were retained on the column (Figure 4C). After negative selection, eluted cells exhibited >100-fold higher CAR cell prevalence (Figure 4D). Moreover, APC fluorescence in eluted cells was substantially higher than in initial stained populations (Figure S5). Relative to tetramers, dodecamers enriched more efficiently. This observation is expected because dodecamer staining results in higher fluorescence than tetramer staining. These results demonstrate that antigen multimers can magnetically enrich for rare CAR T cells with low CAR expression.

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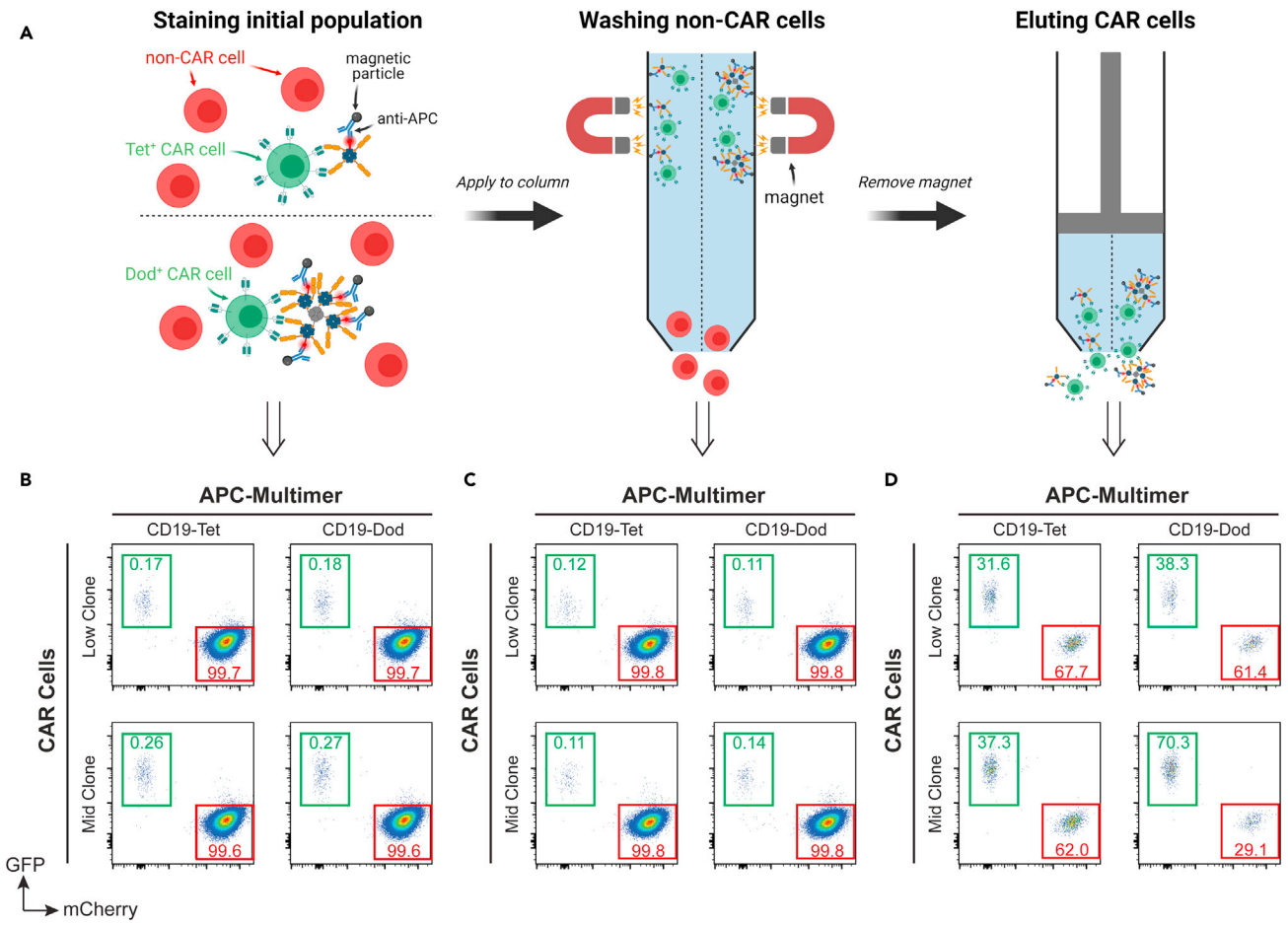

Figure 4. Antigen multimers magnetically enrich for CAR T cells

(A) Cartoon depicting magnetic enrichment procedure. Mixtures of meGFP$^+$ CAR cells and mCherry$^+$ non-CAR cells were stained with either APC-labeled antigen tetramers or antigen dodecamers, followed by staining with anti-APC magnetic microbeads. The stained cell mixture (left) is applied to a magnetic column for negative (middle) and positive (right) selection.

(B–D) Flow plots showing prevalence of meGFP$^+$ CAR cells and mCherry$^+$ non-CAR cells (low clone on top row and mid clone on bottom row) under magnetic enrichment with antigen tetramers or antigen dodecamers. The initial stained mixture (B), column wash (C), and column elution (D) are shown. See also Figure S5.

Antigen multimers specifically stimulate CAR T cells in a temperature-controlled manner

Next, we determined if antigen multimers can oligomerize CARs and stimulate CAR T cells.[37] CAR-specific stimulation would characterize a CAR T cell's activation phenotype, which can be associated with *in vivo* CAR T cell efficacy.[38,39] Traditionally, a T cell's activation phenotype can be characterized with T cell mitogens, such as anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate (PMA)/ionomycin treatment, or phytohemagglutinin (PHA) treatment. However, these mitogens are neither CAR-specific nor mimic biologically relevant CAR stimulation. Alternatively, CAR-specific stimulation can be performed by incubating CAR T cells on plates pre-coated with CAR-staining reagents.[33,40,41] The surface enhances the CAR-staining reagent's binding avidity. Since antigen multimers already bind the CAR with higher avidity, we predicted that they could activate CAR T cells directly as soluble reagents.

Incubation of second-generation anti-CD19 CAR cells with CD19 tetramers or CD19 dodecamers at 37°C upregulated both CD69 (early activation marker; Figure 5A)

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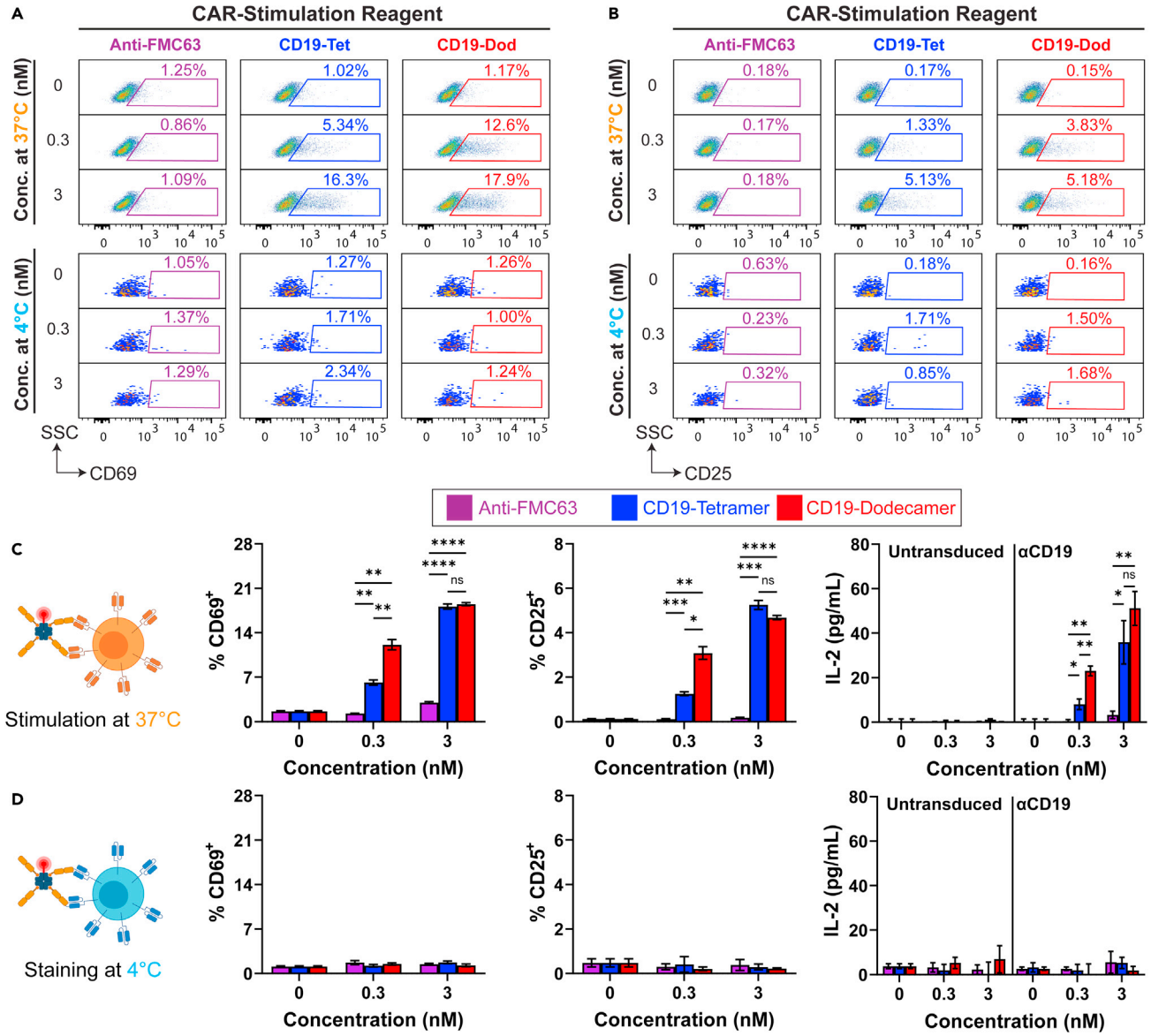

Figure 5. Antigen multimers specifically stimulate CAR T cells in a temperature-controlled manner

(A and B) Representative flow plots showing expression of CD69 (left) and CD25 (right) on anti-CD19 CAR-transduced cells after incubation with anti-FMC63, CD19 tetramers, or CD19 dodecamers at various concentrations at 37°C (top row) and 4°C (bottom row).

(C and D) Bar graphs (mean ± standard error of the mean) depicting CD69 expression (left), CD25 expression (middle), and IL-2 secretion (right) of quadruplicate data from staining of anti-CD19 CAR-transduced cells at 37°C (C) or 4°C (D). The effects of CAR-staining reagents were compared by two-way ANOVA and post-hoc t tests; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

See also Figures S6 and S7.

and CD25 (later activation marker; Figure 5B). Higher multimer concentrations caused greater percentages of $CD69^+$ and $CD25^+$ cells (Figure 5C). On the other hand, although the anti-FMC63 antibody can stain the CAR, it did not stimulate at any concentration in its soluble form. Multimer-stimulated CAR cells also secreted interleukin (IL)-2. IL-2 secretion correlated with CD69 and CD25 upregulation. At 0.3 nM, dodecamers stimulated more effectively than tetramers did. At 3 nM, both multimers stimulated equally well. Similar results were obtained with first-generation anti-CD19 CAR cells (Figures S6A–S6C). Stimulation with either tetramers or

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dodecamers did not influence cell viability (Figure S6D). These results demonstrate that antigen multimers stimulate CAR T cells as high-avidity, soluble staining reagents at 37°C. On the contrary, the anti-FMC63 antibody did not stimulate CAR T cells, presumably due to lower binding avidity or affinity. Attachment of the anti-FMC63 antibody to solid surfaces will likely increase its CAR stimulation capabilities.

Next, we similarly incubated CAR cells with CD19 multimers at 4°C to simulate staining incubations (Figure 5D). The lower temperature is predicted to slow metabolism and minimize CAR signaling. As predicted, no CD69 or CD25 upregulation was observed with either multimer at any concentration. Similarly, no IL-2 was secreted. Cell viability was not affected (Figure S6D). Therefore, stimulation by antigen multimers is abrogated at lower temperatures. These results suggest that when antigen multimers are used for CAR detection, the staining incubation can be performed at 4°C to avoid unwanted stimulation, as we did for all staining experiments in this study.

Finally, we probed two possible mechanisms for CAR T cell activation by antigen multimers (Figure S7A). Antigen multimers can bind CARs in *cis*, causing CAR cross-linking and oligomerization. Alternatively, antigen multimers can bind CARs in *trans*, causing cell-cell conjugation and kinetic segregation.[42] After stimulating CAR cells with antigen multimers at 37°C, we found that antigen multimers do not cause cell-cell conjugation (Figure S7B). Therefore, *cis* CAR oligomerization, rather than *trans* cell-cell conjugation, is the likelier mechanism for multimer-induced CAR T cell stimulation.

Antigen multimers detect CAR T cells from patient infusion product, peripheral blood, and tumor biopsies

After demonstrating usage of antigen multimers on CAR-transduced cell lines, we further applied CD19 multimers on clinical biospecimens from patients with diffuse large B cell lymphoma or B cell acute lymphoblastic leukemia who were treated with anti-CD19 CAR T cell therapies. Over the course of therapy, CAR T cells originate from the infusion product, circulate through peripheral blood, and home in to the tumor (Figure 6A). Therefore, we tested CD19 multimers on infusion products, peripheral blood, and tumor biopsies.

First, we titrated CD19 multimers on axicabtagene ciloleucel and tisagenlecleucel infusion products. For both $CD4^+$ and $CD8^+$ T cells from either infusion product formulations, higher CD19-multimer concentrations stained more cells. On the other hand, CD19 multimers did not stain T cells from a healthy donor (Figures S8A and S8B). At staining saturation, both CD19 tetramers and CD19 dodecamers detected equal percentages of CAR^+ cells across six patients (Figure 6B). Next, we fitted the relative stained percentage of patient cells to dose-response curves to quantify staining EC_{50} values. Staining EC_{50} values for CD19 tetramers were consistently greater than those for CD19 dodecamers (Figure 6C), which was consistent with previous findings on cell lines (Figure 1D). In addition, staining EC_{50} values were equivalent between the two infusion products. Finally, staining EC_{50} values for $CD8^+$ CAR T cells were consistently greater than those for $CD4^+$ CAR T cells. $CD8^+$ CAR T cells may express fewer CAR molecules per cell, making these cells harder to capture at intermediate CD19-multimer concentrations.

Next, we applied CD19 tetramers upon a longitudinal set of post-infusion peripheral blood mononuclear cells from a patient treated with axicabtagene ciloleucel (Figure 6D). Our data showed that CAR T cell percentages rose and reached peak

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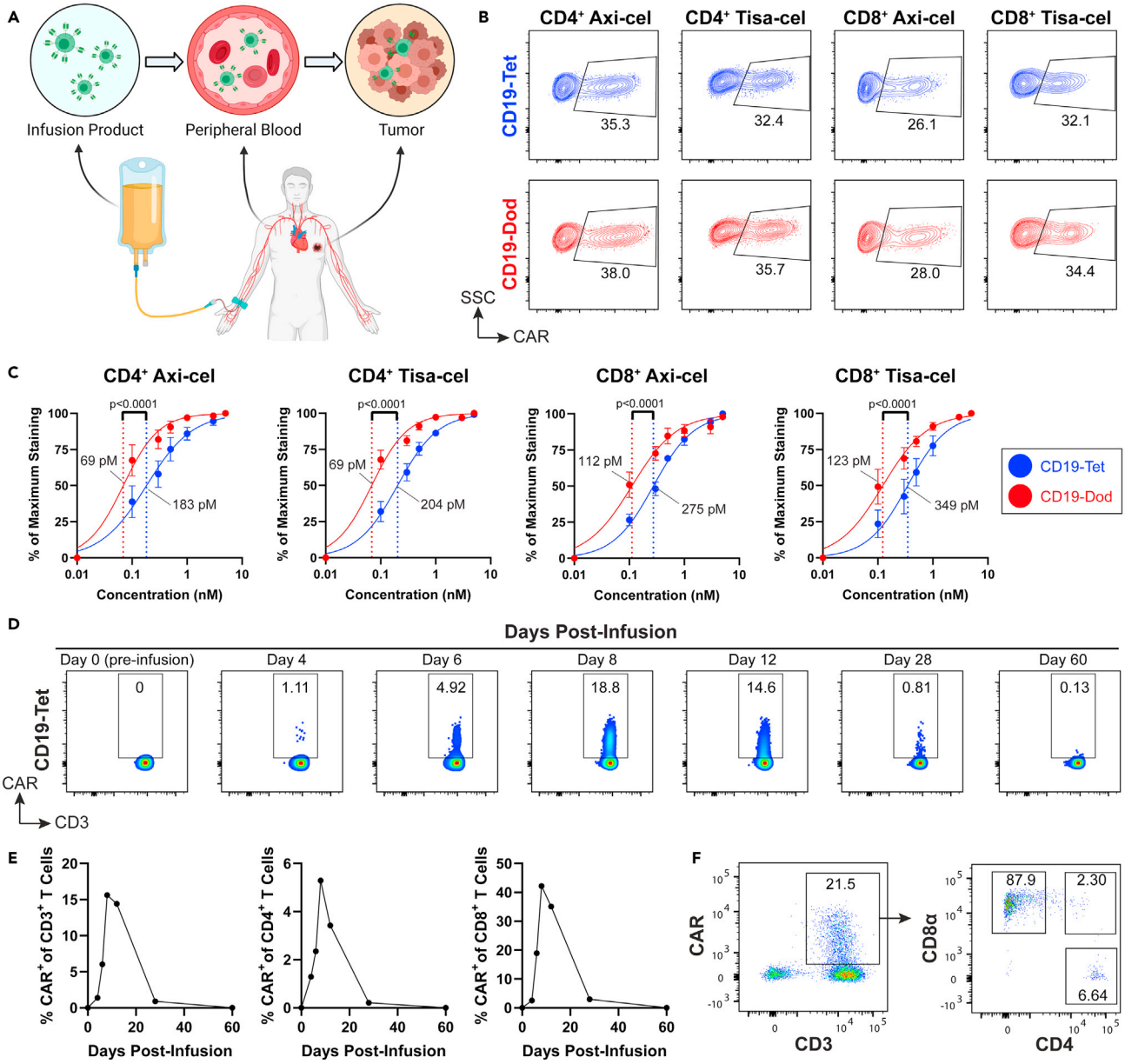

Figure 6. Antigen multimers detect CAR T cells from patient infusion product, peripheral blood, and tumor biopsies

(A) Diagram depicting the different patient biospecimens that may contain CAR T cells throughout their *ex vivo* and *in vivo* life cycles. CAR T cells are manufactured in the infusion product, circulate through peripheral blood, and home in to the tumor.

(B) Representative flow plots showing that CD19 tetramers (5 nM, blue, top row) and CD19 dodecamers (5 nM, red, bottom row) can detect both $CD4^+$ and $CD8^+$ CAR T cells from axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel) infusion products. Flow plots are representative of six independent infusion products (three axi-cel and three tisa-cel). Gates were drawn off CD19-multimer fluorescence-minus-one (FMO) controls.

(C) Relative percentage of maximum staining from infusion product titrations were fitted to dose-response curves. Since each infusion product contains a different prevalence of CAR T cells, percentage stained was normalized to that of maximum staining for each infusion product. For each concentration, the point displays mean ± standard error of the mean. The fitted staining EC_{50} values were compared with a sum-of-squares *F*-test.

(D and E) Flow plots showing CD19-tetramer staining of a longitudinal set of peripheral blood samples from a patient with diffuse large B-cell lymphoma infused with an axi-cel infusion product. CAR T cells were analyzed for CD4 and CD8α expression. Expansion and contraction of CAR T cells are summarized in (E).

(F) Staining of a dissociated lymphoma tumor biopsy from a patient treated with an axi-cel infusion product. $CD3^+CAR^+$ cells were analyzed for CD4 and CD8α expression.

See also Figure S8.

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expansion within 8 days post infusion. Subsequently, CAR T cell percentages decreased, and were virtually undetectable at 60 days post infusion (Figure 6E). Most post-infusion CAR T cells expressed CD8. These observations match expected patterns of CAR T cell expansion and contraction. As further validation, the anti-FMC63 antibody was used to quantify CAR T cell percentages in the same biospecimens (Figure S8C). Percentages from CD19 tetramers and the anti-FMC63 antibody were highly correlated (Figure S8D). These findings suggest that CD19 tetramers accurately detected CAR T cells from patient biospecimens. Finally, we applied CD19 dodecamers upon a lymphoma tumor cell suspension from a patient treated with axicabtagene ciloleucel 14 days prior (Figure 6F). CAR T cells represented 22% of leukocytes. Eighty-eight percent of CAR T cells were $CD8^+$ T cells, and 7% were $CD4^+$ T cells. A small population (2%) of apparent double-positive $CD4^+CD8^+$ CAR T cells was also observed. Collectively, our results demonstrate that CD19 multimers can accurately capture both $CD4^+$ and $CD8^+$ CAR T cells from patient infusion products, post-infusion blood, and tumor biopsies.

Antigen multimers isolate CAR T cells from a patient biospecimen for single-cell omics analyses

Having established the utility of CD19 multimers for identifying CAR T cells from patient biospecimens, we subsequently applied CD19 tetramers for the isolation of anti-CD19 CAR T cells from a patient's post-infusion peripheral blood biospecimen for single-cell omics assays. The chosen sample was derived from a patient with diffuse large B cell lymphoma who was treated with axicabtagene ciloleucel. The patient achieved clinical criteria for a complete response by day 30. The blood sample was taken at 21 days post infusion.

At day 21, 10.7% of $CD3^+$ T cells were also CAR^+ by CD19-tetramer staining (Figure 7A). Using CD19 tetramers, CAR^+ T cells ("CAR-T") and endogenous CAR^- T cells ("Endo-T") were isolated by fluorescence-activated cell sorting (FACS) for analysis by paired single-cell RNA sequencing (RNA-seq) and T cell receptor sequencing (TCR-seq). Single-cell RNA-seq demonstrated that expression of the axicabtagene ciloleucel CAR transgene was highly specific to CAR T cells (Figures 7B, S9A, and S9B). This observation was consistent with the high specificity, sensitivity, and precision of the CD19 tetramer, and suggested that antigen multimers can sort a highly pure and true CAR T cell population from patient biospecimens. Moreover, although multiple diverse T cell receptor (TCR) clonotypes were discovered in both CAR-T and Endo-T cells, there was minimal TCR clonotype overlap ($\leq$1%) between the populations (Figures 7C and S9H). Altogether, our results show that CD19 tetramers captured a clonally distinct population of CAR transgene-expressing T cells from the patient biospecimen.

Through uniform manifold approximation and projection (UMAP) and unsupervised Louvain clustering, we identified 12 T cell clusters based on known markers (including *FOXP3*, *CCR7*, *TCF7*, *GZMB*, *KLRB1*, and *TRDC*), including proliferating, effector $CD8^+$, cytotoxic $CD4^+$, central memory, effector memory, $\gamma\delta$, and regulatory T cells, after filtering out three non-T-cell clusters (Figures 7D and S9C–S9F). Relative to Endo-T cells, CAR-T cells were enriched for $CD8^+$ T cells (Figure S9G). Furthermore, CAR-T cells were enriched in proliferating and effector T cell clusters, while Endo-T cells were enriched in memory and regulatory T cell clusters (Figure 7E). Notably, CAR-T cells were discovered within both the $\gamma\delta$ and regulatory T cell clusters. Differential gene analysis revealed that CAR-T cells have higher expression of activation genes (*CXCR3*, *LAG3*, *HAVCR2*), cytotoxicity-associated genes (*GNLY*, *KLRD1*, *GZMB*, *PRF1*, *GZMA*), and a T cell senescence-associated gene (*KLRG1*)

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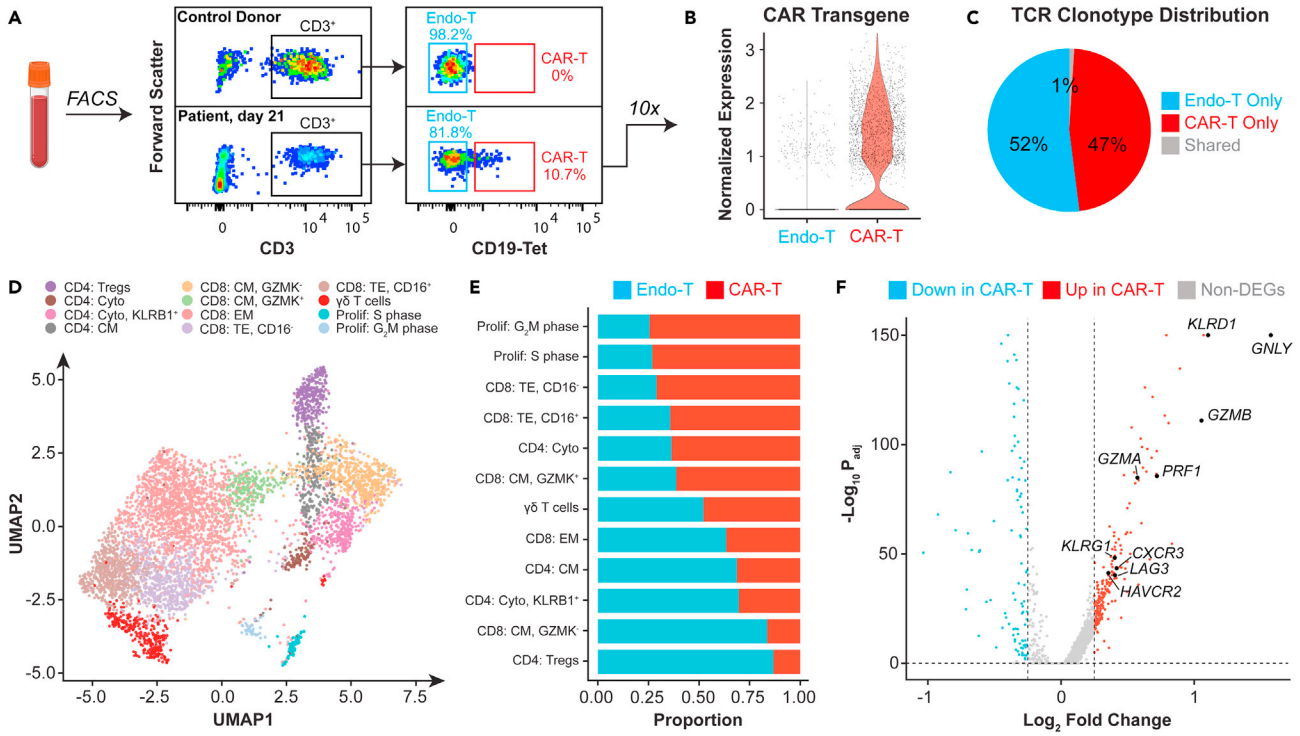

Figure 7. Antigen multimers isolate CAR T cells from a patient biospecimen for single-cell omics analyses

(A) Flow plots demonstrating use of CD19 tetramers (3 nM) for CAR T cell sorting and single-cell omics assays. After gating for $CD3^+$ T cells, PBMCs from a healthy donor (biological control) were used to draw CAR^- and CAR^+ gates. Gates were used to sort for Endo-T and therapeutic CAR-T cells from a patient biospecimen. Sorted Endo-T and CAR-T cells were used for single-cell omics (RNA-seq and TCR-seq).

(B) Violin plot depicting normalized CAR transgene mRNA expression in Endo-T and CAR-T samples.

(C) Pie chart depicting distribution of TCR clonotypes between Endo-T and CAR-T cells.

(D) Single-cell data from Endo-T and CAR-T were combined and visualized by UMAP and unsupervised Louvain clustering. Twelve T cell clusters were annotated based on known markers. Abbreviations: cyto, cytotoxic; CM, central memory; EM, effector memory; TE, terminal effector.

(E) Stacked bar graph depicting proportions of Endo-T and CAR-T cells represented in each T cell cluster.

(F) Volcano plot depicting differentially expressed genes (DEGs) between Endo-T and CAR-T cells, with a cut-off based on $\log_2$ fold change. DEGs of interest are labeled.

See also Figure S9.

(Figure 7F). These signatures are consistent with CAR-specific T cell activation, cytotoxicity, and differentiation. Altogether, these observations are consistent with CD19 tetramers capturing a distinct and clinically active T cell population from the patient biospecimen. These experiments demonstrate that antigen multimers can be reliably used for CAR T-cell isolation and profiling.

DISCUSSION

With our goal of generating an extensible and universal high-avidity CAR-staining reagent, we have designed, constructed, tested, and validated antigen multimers. Through staining titrations and spike-in experiments, we showed that antigen multimers detect CARs with high specificity, sensitivity, and precision, even when target cells had low CAR expression. In addition to CAR detection, we demonstrated that antigen multimers enable magnetic enrichment of CAR T cells for rare populations, temperature-controlled CAR T cell stimulation for activation phenotyping, and high-purity CAR T cell sorting for high-dimensional CAR T cell profiling by single-cell multi-omics analyses. For clinical and translational applications, we demonstrated that CD19 multimers can identify CAR T cells in infusion products, peripheral blood

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samples, and tumor biopsies from patients undergoing CD19-directed CAR T cell therapy. Finally, through staining titrations from three independent CAR systems (anti-CD19, anti-HER2, and anti-Tn), we demonstrated that antigen multimers are readily extensible to existing and new CAR systems.

Antigen multimers offer critical advantages over existing CAR-staining reagents.[15] Relative to monomeric antigen ligand, polyclonal anti-IgG antibodies, and Protein L, antigen multimers exhibited greater specificity, sensitivity, and precision during CAR detection. In particular, antigen multimers demonstrated consistent performance between different CARs, as well as high sensitivity and precision for detecting low-frequency CAR T cells with low CAR expression. This is a critical advantage since CAR T cells downmodulate CAR expression *in vivo* after antigen engagement.[32–34] In contrast, anti-IgG antibodies and Protein L showed inconsistent performance between different CARs and may not even detect CAR T cells with low CAR expression. Furthermore, unlike with antigen multimers, neither anti-IgG antibodies nor Protein L can stain CAR T cells concurrently with exogenous antibodies, which significantly restricts their utility for multiparameter flow cytometry. Compared with monomeric antigen ligand, antigen multimers stained with more fluorescence at saturation. Notably, only antigen multimers, but not monomers, can stain the low-affinity anti-Tn CAR at reasonable concentrations. Finally, for the detection of CAR T cells with low CAR expression, CD19 multimers exhibited greater sensitivity than monomeric CD19 while simultaneously reducing the number of staining steps. These observations regarding antigen multimers' critical advantages can be explained by multivalent interactions between antigen multimers and CARs. Multivalent interactions can significantly decrease the multimer dissociation rate and increase the multimer residence time on the CARs. Therefore, in addition to increasing staining fluorescence, multivalent interactions are expected to increase the stability of the fluorescence signal over time, leading to more reproducible CAR detection.

Importantly, CD19 multimers caused greater fluorescence at saturation than an anti-FMC63 anti-idiotype antibody, which is considered the gold standard for CAR detection. Furthermore, unlike with antigen multimers, anti-idiotype antibodies are fundamentally restricted to particular antibody clones. For instance, the anti-FMC63 anti-idiotype antibody can stain FMC63-based CARs but not newer anti-CD19 CARs, including the Hu19-CAR engineered with fully human scFv domains, and the CAT-CAR engineered for lowered binding affinity.[29,31] Moreover, since monoclonal antibodies can be time consuming or expensive to develop, anti-idiotype antibodies may not be scalable or sustainable to keep pace with the fast research progress in the CAR field. On the other hand, antigen multimers are not restricted to particular clones, and can be readily generalized for use in existing and new CAR systems.

In addition to CAR detection, antigen multimers enable magnetic enrichment of CAR T cells, which is expected to facilitate the detection and investigation of rare CAR T cell subsets. Historically, magnetic enrichment made it possible to investigate rare naive T cells of a given antigen specificity by achieving 50–100-fold enrichment.[24] In our study, CAR T cells were enriched by >100-fold with either antigen tetramers or antigen dodecamers. Since our enrichments were performed on CAR T cells with low CAR expression, the potential benefits of antigen multimers for magnetic enrichment may be even higher in real-world scenarios. This increase in sensitivity through enrichment may facilitate studies on rarer CAR T cell subsets, such as persistent memory CAR T cells, which are correlated with durable responses in patients.[35] In addition, CAR T cell enrichment can be critical for manufacturing

CAR T cell infusion products. During infusion product manufacturing, if CAR transgene delivery efficiency is low, there may be fewer CAR T cells available for therapy. This may be a particular problem with non-viral transgene delivery methods (e.g., through transposons or CRISPR-Cas9), which are less efficient than retroviral methods.[43,44] Even if adequate CAR T cell numbers are reached through longer *ex vivo* expansion, the resulting cells may have progressed toward a terminally differentiated effector phenotype, which reduces therapy efficacy.[45] CAR T cell enrichment may potentially salvage inefficient CAR transgene delivery.

In addition to serving as CAR-staining reagents at 4°C, antigen multimers can also specifically stimulate CAR T cells at 37°C. This helps to characterize a CAR T cell's activation phenotype, which is associated with *in vivo* CAR T cell efficacy.[38,39] Unlike generic T cell mitogens (neither CAR specific nor mimic biologically relevant CAR stimulation) or plates pre-coated with CAR-stimulating reagents,[33,40,41] antigen multimers directly stimulate CAR T cells through its antigen ligand in soluble form. Our data suggest that stimulation through antigen multimers likely occurs through CAR oligomerization. We further anticipate that antigen multimers may be useful in stimulating CAR T cells during *ex vivo* production and expansion. *Ex vivo* CAR stimulation would also enrich lymphocytes for CAR T cells in lieu of positive selection for CAR^+ cells. Currently, this process is performed by adding irradiated antigen-positive feeder cells.[46] However, antigen-positive feeder cells may adversely influence CAR T cell phenotype. Feeder cells may also risk introducing unwanted ligands or proliferation-competent cells through incomplete irradiation. Controlled, chemically defined antigen multimers may circumvent these limitations.

Finally, for clinical and translational applications, we demonstrated that CD19 multimers can identify CAR T cells from multiple patient biospecimen sources, including infusion products, peripheral blood samples, and tumor biopsies. We then employed single-cell omics to high dimensionally profile CD19-tetramer-sorted adoptively transferred CAR^+ T cells and endogenous CAR^- T cells from a CAR T cell therapy patient's post-infusion blood sample. Analysis of CAR transgene expression, TCR clonotype usage, and transcriptomic patterns between CAR^- and CAR^+ cells revealed that CD19 tetramers captured a pure, distinct, and clinically active CAR T cell population. Since multi-omics profiling has become increasingly utilized on CAR T cell patient biospecimens to interrogate clinically relevant CAR T cell phenotypes,[47–49] we expect antigen multimers to be broadly applicable for translational researchers and cell therapists involved in CAR research.

Two types of antigen multimers were engineered in our study: tetramers and dodecamers. Our staining titrations showed that dodecamers required lower concentrations to saturate CAR staining than the corresponding tetramers did. These findings indicate that target antigen avidity influences the interaction of a given antigen multimer for its matched CAR. In general, dodecamers stained with greater fluorescence than tetramers did. However, in our titrations, the two multimers were equally proficient at capturing almost all CAR-expressing cell lines at saturating concentrations. Since antigen multimers would be expected to be used at saturating concentrations in real-world scenarios, the benefits of using dodecamers over tetramers are modest. Since streptavidin is commercially available, tetramers are also easier to construct. On the other hand, dodecamers may be ideal for use when the staining reagent is paired with a dim fluorophore or when the CAR's affinity for target antigen is low. Although we specifically tested tetramers and dodecamers, antigen multimers of additional valencies (such as dimers, pentamers, octamers, or dextramers) could be generated.[24] These alternative

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binding valencies may offer differential advantages for CAR detection by modulating binding avidity.

Despite their numerous advantages, antigen multimers are associated with three important limitations. First, antigen multimers are dependent on the accessibility and stability of the biotinylated antigen ligand. Eukaryotic proteins can sometimes be difficult to express or site-specifically biotinylate. Second, antigen multimers should not be used with an antibody that also binds to the antigen ligand at an overlapped epitope. Third, it is possible that preparation of the streptavidin-based scaffold for antigen dodecamers results in spurious heterogeneous and higher-order oligomeric forms. To reduce these side reactions, the concentrations of the biotinylated dodecamer scaffold protein and streptavidin during the incubation can be modified to control the frequency of molecular collisions and decrease higher-order oligomerization.

In conclusion, we have developed antigen multimers as extensible and multifunctional high-avidity CAR T cell detection, enrichment, and stimulation reagents. We anticipate that antigen multimers will be a versatile tool to probe CAR T cells for clinical and research applications.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Huang (huangjun@uchicago.edu).

Data and code availability

- All single-cell RNA-seq and TCR-seq data have been deposited in the Gene Expression Omnibus (GEO) and are available with the accession code GEO: GSE169086 as of the date of publication.
- All custom codes used in this study are available upon reasonable request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

MATERIALS AVAILABILITY

The materials generated in this study are available upon reasonable request.

Lentiviral production and transduction

Plasmids carrying a human CAR or mCherry were cloned onto the pHR vector backbone.[50] Plasmids carrying a mouse CAR were cloned onto the pMP71 vector backbone. Transfer plasmids were transfected into the Lenti-X 293T packaging cell line (Takara, 632180) or Platinum-E retroviral packaging cell line (Cell Biolabs, RV-101) for lentiviral or ecotropic retroviral production. Both packaging cell lines were routinely cultured in DMEM and 10% fetal bovine serum (X&Y Cell Culture, FBS-500).

In brief, packaging cells were grown overnight and transfected the following day via Lipofectamine 3000 (Invitrogen, L3000001). For lentiviral production, transfer plasmids (0.95 μg, containing the CAR on the pHR vector backbone), packaging plasmids (0.72 μg, psPAX2), and envelope plasmids (0.22 μg, pMD2.G) were co-transfected into Lenti-X 293T cells. For ecotropic retroviral production, transfer plasmid (0.95 μg, containing the CAR on the pMP71 vector backbone) was

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transfected into Platinum-E cells. After 72 h, the viral supernatant was concentrated by ultracentrifugation and stored at −80°C until transduction.

Concentrated lentiviruses were used to transduce E6-1 Jurkat cells and $58^{-/-}$ mouse hybridoma cells. E6-1 Jurkat cells and $58^{-/-}$ mouse hybridoma cells were routinely cultured in RPMI-1640 (Gibco, 11875093) or DMEM media respectively, supplemented with 10% fetal bovine serum (X&Y Cell Culture, FBS-500). During transduction, lentiviruses or ecotropic retroviruses were added to cells along with protamine sulfate (Millipore Sigma, P3369-10G) to a final concentration of 10 μg/mL. Cells were spinoculated at 800 × *g* for 60 min at room temperature. Then, cells were grown for 72 h before analysis of transduction efficiency by flow cytometry. Untransduced cells were used for gating.

Preparation of anti-CD19 CAR-transduced Jurkat clones

meGFP$^+$ Jurkat cell clones with stably low anti-CD19 CAR expression and mCherry$^+$ Jurkat cell clones with no CAR expression were used for (1) spike-in experiments to assay CAR-staining reagents for sensitivity and precision, and (2) magnetic enrichment experiments. To prepare these clones, a polyclonal lentivirally transduced Jurkat cell population was single-cell sorted into 96-well plates. For preparation of meGFP$^+$ Jurkat cell clones, cells with lower meGFP fluorescence were chosen for the sort. For preparation of the mCherry$^+$ Jurkat cell clone, cells with median mCherry fluorescence were chosen for the sort. After culturing for 1 month, ~10% of sorted single cells grew to high cell density. Cells were successively transferred to larger media volumes.

meGFP or mCherry expression in each clone was analyzed by flow cytometry and compared with that of the pre-sorted polyclonal population. To quantify the average number of CAR molecules per cell in the anti-CD19 CAR-transduced Jurkat clones, we used GFP flow cytometer calibration beads (Takara, 632,594), because meGFP is fused to the C terminus of the CAR. Two meGFP$^+$ clones (named low clone and mid clone) were found to have lower CAR expression than the pre-sorted polyclonal population. One mCherry$^+$ clone was chosen for having median-level mCherry expression as the pre-sorted polyclonal population. In both CAR-expressing clones, CAR expression was stable and remained similarly low after extensive culturing (>1 month). Before each experiment, CAR expression in these clones was checked to confirm the clones' integrity.

Purification of dodecamer base protein

The dodecamer base protein is a tetramer of an inactive streptavidin subunit mutagenized with an added C-terminal cysteine. The protein was expressed in BL21 *Escherichia coli* and refolded to form a tetramer with four cysteines. This tetramer was first purified by fast protein liquid chromatography using a Superdex 75x10/300 GL column (GE Healthcare, 17517401). Then, the four cysteines on the purified tetramer were reduced with tris(2-carboxyethyl)phosphine and biotinylated overnight at room temperature using maleimide chemistry with an extended linker by EZ-Link BMCC Biotin (Thermo Scientific, 21900) at ≥50:1 molar ratio. Subsequently, the reaction mixture containing biotinylated base protein was desalted twice into Hank's balanced salt solution (HBSS) buffer using 7K MWCO Zeba Spin Desalting Columns (Thermo Scientific, 89882). The biotinylation efficiency and concentration of the final base protein were assayed by the Pierce Fluorescence Biotin Quantitation Kit (Thermo Scientific, S20033) and the Qubit Protein Assay Kit (Invitrogen, Q33211) respectively.

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Generation of antigen multimers

Multimers were constructed from AviTag-biotinylated human His-tagged CD19 (Acro Biosystems, CD9-H82E9), AviTag-biotinylated human His-tagged HER2 (Acro Biosystems, HE2-H82E2), site-specifically biotinylated Tn-glycosylated podoplanin G(T*)KPPLEE peptide (glycosylation marked with an asterisk [*])[5], and biotinylated BSA (BioVision, 7097-5). Biotinylated dodecamer base protein and either Alexa Fluor 647-labeled streptavidin (BioLegend, 405237) or APC-labeled streptavidin (BioLegend, 405207) were used for the multimer scaffold.

To generate antigen tetramers, biotinylated antigen ligand was added to fluorescently labeled tetrameric streptavidin at a 4:1 molar ratio for 30 min at 4°C in the dark. This mixture was diluted with PBS to convenient concentrations for staining.

To generate antigen dodecamers, biotinylated tetrameric dodecamer base protein was incubated with fluorescently labeled tetrameric streptavidin at a 1:4 molar ratio for 30 min at 4°C in the dark. Subsequently, biotinylated antigen ligand was added at a 12:1 molar ratio for an additional 30 min at 4°C in the dark. This mixture was diluted with PBS to convenient concentrations for staining.

CAR-staining assays for cell lines

For staining assays with cell lines, 50,000 live cells were employed for all conditions. Cells were first washed with cold FACS buffer (PBS, 2% BSA, 0.05% sodium azide). Then, cells were incubated with CAR-staining reagents as described next.

For staining with antigen multimers, cells were incubated for 30 min at 4°C in the dark with a staining solution containing AF647-labeled antigen tetramers or antigen dodecamers at chosen concentrations.

For staining with polyclonal anti-IgG, cells were incubated for 30 min at 4°C in the dark with a staining solution containing AF647-labeled anti-mouse IgG (H + L) cross-adsorbed F(ab')2-Goat (Fisher Scientific, A21237) at 5 μg per 100 μL staining volume (optimal concentration determined by pre-titrating cell lines).

For staining with Protein L, cells were incubated for 30 min at 4°C in the dark with a staining solution containing biotinylated Protein L (Thermo Fisher Scientific, 29997) at 1 μg per 100 μL volume according to specifications from the literature.[19] Cells were subsequently incubated with 1 ng/μL AF647-streptavidin (BioLegend, 405237) in FACS buffer for 20 min.

For staining with anti-FMC63, cells were incubated for 30 min at 4°C in the dark with a staining solution containing biotinylated anti-FMC63 (clone Y45, Acro Biosystems, FM3-BY45) at chosen concentrations. Cells were subsequently incubated with 1 ng/μL AF647-streptavidin (BioLegend, 405237) in FACS buffer for 20 min.

For staining with monomeric target antigen, cells were incubated for 30 min at 4°C in the dark with a staining solution containing biotinylated target antigen at chosen concentrations. Cells were subsequently incubated with 1 ng/μL AF647-streptavidin (BioLegend, 405237) in FACS buffer for 20 min.

For all CAR-staining assays, after staining with CAR-staining reagents, cells were incubated with LIVE/DEAD Fixable Near-IR viability dye (Invitrogen, L34975) diluted

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1:1,000 in PBS for 5 min at room temperature. Finally, cells were washed three times in FACS buffer at 4°C before analysis by flow cytometry.

CAR-staining assays for patient biospecimens

Cryopreserved patient biospecimens were thawed in warm RPMI + 10% FBS. Cells were first washed with cold FACS buffer (PBS, 2% BSA, 0.05% sodium azide). Next, Fc receptors were blocked by incubation with Human TruStain FcX (BioLegend, 422301) at 1:50 dilution for 5 min at 4°C. Then, cells were incubated for 30 min at 4°C in the dark with a staining solution containing BV421-anti-CD3ε (clone SK7, BioLegend, 344833), PE-anti-CD4 (clone SK3, BioLegend, 344605), AF488-anti-CD8α (clone Hit8a, BioLegend, 300916), and either antigen multimers or biotinylated anti-FMC63 (clone Y45, Acro Biosystems, FM3-BY45). For staining with biotinylated anti-FMC63, cells were subsequently incubated with 1 ng/μL AF647-streptavidin (BioLegend, 405237) in FACS buffer for 20 min. Monoclonal antibodies were generally used according to manufacturer recommendations.

After staining, cells were incubated briefly with LIVE/DEAD Fixable Near-IR viability dye (Invitrogen, L34975) diluted 1:1,000 in PBS for 5 min at room temperature. Then, cells were washed three times in FACS buffer at 4°C before analysis by flow cytometry.

Antigen multimers for magnetic enrichment

CAR-meGFP-transduced Jurkat cell clones with stably low CAR expression (low clone and mid clone) were spiked into mCherry-transduced non-CAR Jurkat cell at ~0.2% prevalence. Subsequently, cells were stained with 3 nM APC-labeled CD19 tetramers or CD19 dodecamers for 1 h at 4°C in FACS buffer (PBS, 2% BSA, 0.05% sodium azide). Stained cells were washed three times and then incubated with anti-APC microbeads (Miltenyi Biotec, 130-090-855) at 1:5 dilution for 15 min at 4°C. Cells were then washed once with FACS buffer and applied onto magnetic columns (Miltenyi Biotec, 130-042-201). Columns were washed with three column volumes of FACS buffer. Then, the column was removed from the magnet, and cells were eluted under pressure from a plunger. Subsequently, cells were centrifuged and incubated briefly with LIVE/DEAD Fixable Near-IR viability dye (Invitrogen, L34975) diluted 1:1,000 in PBS for 5 min at room temperature. Finally, cells were washed once in FACS buffer at 4°C before analysis by flow cytometry.

CAR T cell stimulation assays

CAR-transduced Jurkat cells were incubated in T cell media (RPMI supplemented with 10% FBS, 1% Pen/Strep, 2 mM L-glutamine, 50 mM 2-mercaptoethanol) with antigen multimers or anti-idiotype antibodies. After 24 h at 37°C or 4°C, cells and supernatants were collected for the analyses of activation markers and cytokine production respectively.

To measure T cell activation markers, the cells were washed with FACS buffer (PBS, 2% BSA, 0.05% sodium azide) and incubated for 30 min at 4°C in the dark with a staining solution containing APC-anti-CD25 (clone M-A251, BioLegend, 356110) and BV510-anti-CD69 (clone FN90, BioLegend, 310936). Subsequently, cells were incubated with LIVE/DEAD Fixable Near-IR viability dye (Invitrogen, L34975) diluted 1:1,000 in PBS for 5 min at room temperature. Finally, cells were washed three times in cold FACS buffer before analysis by flow cytometry.

The supernatant was diluted 1:2 in PBS and analyzed for IL-2 secretion by an enzyme-linked immunosorbent assay (IL-2 Quantikine ELISA Kit, R&D Systems, D2050). For each ELISA, an eight-point standard curve was generated (R^2 > 99%).

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Single-cell omics assays

Patient peripheral blood mononuclear cells were collected from blood biospecimens by Ficoll-Paque PLUS (Cytiva, 95021-205), and cryopreserved (RPMI supplemented with 10% FBS and 10% DMSO) in liquid nitrogen until analyses.

Cryopreserved patient biospecimens were thawed in warm RPMI + 10% FBS. Cells were first washed with cold FACS buffer (PBS, 2% BSA, 0.05% sodium azide). Next, Fc receptors were blocked by incubation with Human TruStain FcX (BioLegend, 422301) at 1:50 dilution for 5 min at 4°C. Then, cells were incubated for 30 min at 4°C in the dark with a staining solution containing BV421-anti-CD3ε (clone SK7) and Alexa Fluor 647-labeled antigen tetramers at 3 nM final concentration for CAR detection. Subsequently, cells were incubated with LIVE/DEAD Fixable Near-IR viability dye (Invitrogen, L34975) diluted 1:1,000 in PBS for 5 min at room temperature. Finally, cells were washed three times in cold cell media before FACS. To draw sorting gates based on a biological control, cells from a healthy donor were similar stained and analyzed.

Twelve-thousand sorted endogenous CAR^- T cells and 7,853 sorted CAR^+ T cells were separately partitioned into droplets for single-cell omics assays via Chromium Next GEM Single-Cell 5′Kit v2 (10x Genomics, 1000263). From the resulting barcoded nucleic acids, TCR-seq libraries were prepared via the Chromium Single-Cell Human TCR Amplification Kit (10x Genomics, 1000252). Sequencing libraries were quantified via the Qubit dsDNA HS Assay Kit (Invitrogen, Q32851), quality checked for fragment sizes via a high-sensitivity D5000 ScreenTape (Agilent, 5067-5592), pooled, and sequenced (Illumina, NovaSeq-6000).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.matt.2021.09.027

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AUTHOR CONTRIBUTIONS

J.H. conceived the original concept for antigen multimers. Y. Hu, J.K., and J.H. designed all experiments. Y. Hu, X.C., and N. Asby conducted flow cytometry experiments. Y. Hu performed stimulation and magnetic selection experiments. X.H. performed ELISA. G.C. performed single-cell omics analysis. Y. Hu and A.R. analyzed staining titration data. A.T., A.R., N. Ankenbruck, Y. He, and Y. Hu cloned and transduced the CARs. J.K., P.R., and M.B. coordinated patient biospecimens for these

studies. J.K., P.R., M.B., and H.S. reviewed the manuscript. Y. Hu wrote and J.H. and J.K. edited the manuscript.

DECLARATION OF INTERESTS

The authors will consider filing a patent based on findings described in this manuscript.

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REFERENCES

1. June, C.H., O'Connor, R.S., Kawalekar, O.U., Ghassemi, S., and Milone, M.C. (2018). CAR T cell immunotherapy for human cancer. Science *359*, 1361–1365. https://doi.org/10.1126/science.aar6711.

2. Eshhar, Z., Waks, T., Gross, G., and Schindler, D.G. (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors. Proc. Natl. Acad. Sci. U S A *90*, 720–724. https://doi.org/10.1073/pnas.90.2.720.

3. Kochenderfer, J.N., Feldman, S.A., Zhao, Y., Xu, H., Black, M.A., Morgan, R.A., Wilson, W.H., and Rosenberg, S.A. (2009). Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J. Immunother. *32*, 689–702. https://doi.org/10.1097/CJI.0b013e3181ac6138.

4. Chmielewski, M., Hombach, A., Heuser, C., Adams, G.P., and Abken, H. (2004). T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. J. Immunol. *173*, 7647–7653. https://doi.org/10.4049/jimmunol.173.12.7647.

5. He, Y., Schreiber, K., Wolf, S.P., Wen, F., Steentoft, C., Zerweck, J., Steiner, M., Sharma, P., Michael Shepard, H., Posey, A., et al. (2019). Multiple cancer-specific antigens are targeted by a chimeric antigen receptor on a single cancer cell. JCI Insight *4*. https://doi.org/10.1172/jci.insight.130416.

6. O'Rourke, D.M., Nasrallah, M.P., Desai, A., Melenhorst, J.J., Mansfield, K., Morrissette, J.J.D., Martinez-Lage, M., Brem, S., Maloney, E., Shen, A., et al. (2017). A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci. Transl. Med. *9*, eaaa0984. https://doi.org/10.1126/scitranslmed.aaa0984.

7. O'Hara, M., Stashwick, C., Haas, A.R., and Tanyi, J.L. (2016). Mesothelin as a target for chimeric antigen receptor-modified T cells as anticancer therapy. Immunotherapy *8*, 449–460. https://doi.org/10.2217/imt.16.4.

8. Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., et al. (2017). Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. N. Engl. J. Med. *377*, 2531–2544. https://doi.org/10.1056/NEJMoa1707447.

9. Schuster, S.J., Bishop, M.R., Tam, C.S., Waller, E.K., Borchmann, P., McGuirk, J.P., Jäger, U., Jaglowski, S., Andreadis, C., Westin, J.R., et al. (2019). Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. N. Engl. J. Med. *380*, 45–56. https://doi.org/10.1056/NEJMoa1804980.

10. Abramson, J.S., Palomba, M.L., Gordon, L.I., Lunning, M.A., Wang, M.L., Arnason, J.E., Mehta, A., Purev, E., Maloney, D.G., Andreadis, C., et al. (2019). Pivotal safety and efficacy results from transcend NHL 001, a multicenter phase 1 study of lisocabtagene maraleucel (liso-cel) in relapsed/refractory (R/R) large B cell lymphomas. Blood *134*, 241. https://doi.org/10.1182/blood-2019-127508.

11. Abramson, J.S., Palomba, M.L., Gordon, L.I., Lunning, M.A., Wang, M., Arnason, J., Mehta, A., Purev, E., Maloney, D.G., Andreadis, C., et al. (2020). Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. Lancet *396*, 839–852. https://doi.org/10.1016/S0140-6736(20)31366-0.

12. Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., et al. (2018). Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N. Engl. J. Med. *378*, 439–448. https://doi.org/10.1056/nejmoa1709866.

13. Wang, M., Munoz, J., Goy, A., Locke, F.L., Jacobson, C.A., Hill, B.T., Timmerman, J.M., Holmes, H., Jaglowski, S., Flinn, I.W., et al. (2020). KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. N. Engl. J. Med. *382*, 1331–1342. https://doi.org/10.1056/nejmoa1914347.

14. Wagner, J., Wickman, E., DeRenzo, C., and Gottschalk, S. (2020). CAR T cell therapy for solid tumors: bright future or dark reality? Mol. Ther. *28*, 2320–2339. https://doi.org/10.1016/j.ymthe.2020.09.015.

15. Hu, Y., and Huang, J. (2020). The chimeric antigen receptor detection toolkit. Front. Immunol. *11*, 1–16. https://doi.org/10.3389/fimmu.2020.01770.

16. Demaret, J., Varlet, P., Trauet, J., Beauvais, D., Grossemy, A., Hégo, F., Yakoub-Agha, I., and Labalette, M. (2021). Monitoring CAR T-cells using flow cytometry. Cytom. Part B Clin. Cytom. *100*, 218–224. https://doi.org/10.1002/cyto.b.21941.

17. Seif, M., Einsele, H., and Löffler, J. (2019). CAR T cells beyond cancer: hope for immunomodulatory therapy of infectious diseases. Front. Immunol. *10*, 2711. https://doi.org/10.3389/fimmu.2019.02711.

18. Guedan, S., Calderon, H., Posey, A.D., and Maus, M.V.((2019). Engineering and design of chimeric antigen receptors. Mol. Ther. Methods Clin. Dev. *12*, 145–156. https://doi.org/10.1016/j.omtm.2018.12.009.

19. Zheng, Z., Chinnasamy, N., and Morgan, R.A. (2012). Protein L: a novel reagent for the detection of chimeric antigen receptor (CAR) expression by flow cytometry. J. Transl. Med. *10*. https://doi.org/10.1186/1479-5876-10-29.

20. Lobner, E., Wachernig, A., Gudipati, V., Mayrhofer, P., Salzer, B., Lehner, M., Huppa, J.B., and Kunert, R. (2020). Getting CD19 into shape: expression of natively folded "difficult-to-express" CD19 for staining and stimulation of CAR-T cells. Front. Bioeng. Biotechnol. *8*, 1–13. https://doi.org/10.3389/fbioe.2020.00049.

21. De Oliveira, S.N., Wang, J., Ryan, C., Morrison, S.L., Kohn, D.B., and Hollis, R.P. (2013). A CD19/Fc fusion protein for detection of anti-CD19 chimeric antigen receptors. J. Transl. Med. *11*. https://doi.org/10.1186/1479-5876-11-23.

22. Jena, B., Maiti, S., Huls, H., Singh, H., Lee, D.A., Champlin, R.E., and Cooper, L.J.N. (2013). Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. PLoS One *8*, e57838. https://doi.org/10.1371/journal.pone.0057838.

23. Laurent, E., Sieber, A., Salzer, B., Wachernig, A., Seigner, J., Lehner, M., Geyeregger, R., Kratzer, B., Jäger, U., Kunert, R., et al. (2021). Directed evolution of stabilized monomeric CD19 for monovalent CAR interaction studies and monitoring of CAR-T cell patients. ACS Synth. Biol. *10*, 712–717. https://doi.org/10.1021/acssynbio.1c00010.

24. Wooldridge, L., Lissina, A., Cole, D.K., Van Den Berg, H.A., Price, D.A., and Sewell, A.K. (2009). Tricks with tetramers: how to get the most from multimeric peptide-MHC. Immunology *126*,

147–164. https://doi.org/10.1111/j.1365-2567.2008.02848.x.

25. Davis, M.M., Altman, J.D., and Newell, E.W. (2011). Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. Nat. Rev. Immunol. *11*, 551–558. https://doi.org/10.1038/nri3020.

26. Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96. https://doi.org/10.1126/science.274.5284.94.

27. Huang, J., Zeng, X., Sigal, N., Lund, P.J., Su, L.F., Huang, H., Chien, Y., and Davis, M.M. (2016). Detection, phenotyping, and quantification of antigen-specific T cells using a peptide-MHC dodecamer. Proc. Natl. Acad. Sci. U S A *113*, E1890–E1897. https://doi.org/10.1073/pnas.1602488113.

28. Fairhead, M., and Howarth, M. (2015). Site-specific biotinylation of purified proteins using BirA. Methods Mol. Biol. *1266*, 171–184. https://doi.org/10.1007/978-1-4939-2272-7_12.

29. Ghorashian, S., Kramer, A.M., Onuoha, S., Wright, G., Bartram, J., Richardson, R., Albon, S.J., Casanovas-Company, J., Castro, F., Popova, B., et al. (2019). Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. Nat. Med. *25*, 1408–1414. https://doi.org/10.1038/s41591-019-0549-5.

30. Sharma, P., Marada, V.V.V.R., Cai, Q., Kizerwetter, M., He, Y., Wolf, S.P., Schreiber, K., Clausen, H., Schreiber, H., and Kranz, D.M. (2020). Structure-guided engineering of the affinity and specificity of CARs against Tn-glycopeptides. Proc. Natl. Acad. Sci. U S A *117*, 15148–15159. https://doi.org/10.1073/pnas.1920662117.

31. Brudno, J.N., Lam, N., Vanasse, D., Shen, Y.w., Rose, J.J., Rossi, J., Xue, A., Bot, A., Scholler, N., Mikkilineni, L., et al. (2020). Safety and feasibility of anti-CD19 CAR T cells with fully human binding domains in patients with B-cell lymphoma. Nat. Med. *26*, 270–280. https://doi.org/10.1038/s41591-019-0737-3.

32. Li, W., Qiu, S., Chen, J., Jiang, S., Chen, W., Jiang, J., Wang, F., Si, W., Shu, Y., Wei, P., et al. (2020). Chimeric antigen receptor designed to prevent ubiquitination and downregulation showed durable antitumor efficacy. Immunity *53*, 456–470. https://doi.org/10.1016/j.immuni.2020.07.011.

33. Walker, A.J., Majzner, R.G., Zhang, L., Wanhainen, K., Long, A.H., Nguyen, S.M., Lopomo, P., Vigny, M., Fry, T.J., Orentas, R.J., et al. (2017). Tumor antigen and receptor densities regulate efficacy of a chimeric antigen receptor targeting anaplastic lymphoma kinase. Mol. Ther. *25*, 2189–2201. https://doi.org/10.1016/j.ymthe.2017.06.008.

34. Yang, Y., Kohler, M.E., Chien, C.D., Sauter, C.T., Jacoby, E., Yan, C., Hu, Y., Wanhainen, K., Qin, H., and Fry, T.J. (2017). TCR engagement negatively affects CD8 but not CD4 CAR T cell expansion and leukemic clearance. Sci. Transl. Med. *9*. https://doi.org/10.1126/scitranslmed.aag1209.

35. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517. https://doi.org/10.1056/NEJMoa1407222.

36. Day, C.L., Seth, N.P., Lucas, M., Appel, H., Gauthier, L., Lauer, G.M., Robbins, G.K., Szczepiorkowski, Z.M., Casson, D.R., Chung, R.T., et al. (2003). Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. J. Clin. Invest. *112*, 831–842. https://doi.org/10.1172/JCI200318509.

37. Jose, E.S., Borroto, A., Niedergang, F., Alcover, A., and Alarcon, B. (2000). Triggering the TCR complexes causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism. Immunity *12*, 161–170. https://doi.org/10.1016/S1074-7613(00)80169-7.

38. Feucht, J., Sun, J., Eyquem, J., Ho, Y.J., Zhao, Z., Leibold, J., Dobrin, A., Cabriolu, A., Hamieh, M., and Sadelain, M. (2019). Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. Nat. Med. *25*, 82–88. https://doi.org/10.1038/s41591-018-0290-5.

39. Rossi, J., Paczkowski, P., Shen, Y.W., Morse, K., Flynn, B., Kaiser, A., Ng, C., Gallatin, K., Cain, T., Fan, R., et al. (2018). Preinfusion polyfunctional anti-CD19 chimeric antigen receptor T cells are associated with clinical outcomes in NHL. Blood *132*, 804–814. https://doi.org/10.1182/blood-2018-01-828343.

40. Landgraf, K.E., Williams, S.R., Steiger, D., Gebhart, D., Lok, S., Martin, D.W., Roybal, K.T., and Kim, K.C. (2020). convertibleCARs : a chimeric antigen receptor system for flexible control of activity and antigen targeting. Commun. Biol. *3*, 296. https://doi.org/10.1038/s42003-020-1021-2.

41. Cook, W.J., Choi, Y., Gacerez, A., Bailey-Kellogg, C., and Sentman, C.L. (2020). A chimeric antigen receptor that binds to a conserved site on MICA. ImmunoHorizons *4*, 597–607. https://doi.org/10.4049/immunohorizons.2000041.

42. Davis, S.J., and van der Merwe, P.A. (2006). The kinetic-segregation model: TCR triggering and beyond. Nat. Immunol. *78*, 803–809. https://doi.org/10.1038/NI1369.

43. Rafiq, S., Hackett, C.S., and Brentjens, R.J. (2020). Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat. Rev. Clin. Oncol. *17*, 147–167. https://doi.org/10.1038/s41571-019-0297-y.

44. Hamada, M., Nishio, N., Okuno, Y., Suzuki, S., Kawashima, N., Muramatsu, H., Tsubota, S., Wilson, M.H., Morita, D., Kataoka, S., et al. (2018). Integration mapping of piggyBac-mediated CD19 chimeric antigen receptor T cells analyzed by novel tagmentation-assisted PCR. EBioMedicine *34*, 18–26. https://doi.org/10.1016/j.ebiom.2018.07.008.

45. A Ajina, J.M. (2018). Strategies to address chimeric antigen receptor tonic signaling. Mol. Cancer Ther. *17*, 1795–1815.

46. Prommersberger, S., Hudecek, M., and Nerreter, T. (2020). Antibody-based CAR T cells produced by lentiviral transduction. Curr. Protoc. Immunol. *128*, e93. https://doi.org/10.1002/cpim.93.

47. Deng, Q., Han, G., Puebla-Osorio, N., Ma, M.C.J., Strati, P., Chasen, B., Dai, E., Dang, M., Jain, N., Yang, H., et al. (2020). Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nat. Med. *26*, 1878–1887. https://doi.org/10.1038/s41591-020-1061-7.

48. Sheih, A., Voillet, V., Hanafi, L.A., DeBerg, H.A., Yajima, M., Hawkins, R., Gersuk, V., Riddell, S.R., Maloney, D.G., Wohlfahrt, M.E., et al. (2020). Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. Nat. Commun. *11*, 1–13. https://doi.org/10.1038/s41467-019-13880-1.

49. Fraietta, J.A., Lacey, S.F., Orlando, E.J., Pruteanu-Malinici, I., Gohil, M., Lundh, S., Boesteanu, A.C., Wang, Y., O'Connor, R.S., Hwang, W.T., et al. (2018). Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat. Med. *24*, 563–571. https://doi.org/10.1038/s41591-018-0010-1.

50. Morrissey, M.A., Williamson, A.P., Steinbach, A.M., Roberts, E.W., Kern, N., Headley, M.B., and Vale, R.D. (2018). Chimeric antigen receptors that trigger phagocytosis. eLife *7*, e36688. https://doi.org/10.7554/elife.36688.