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Inclusion of *Strep*-tag II in design of antigen receptors for T-cell immunotherapy

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Adoptive immunotherapy with genetically engineered T cells has the potential to treat cancer and other diseases. The introduction of *Strep*-tag II sequences into specific sites in synthetic chimeric antigen receptors or natural T-cell receptors of diverse specificities provides engineered T cells with a marker for identification and rapid purification, a method for tailoring spacer length of chimeric receptors for optimal function, and a functional element for selective antibody-coated, microbead-driven, large-scale expansion. These receptor designs facilitate cGMP manufacturing of pure populations of engineered T cells for adoptive T-cell therapies and enable *in vivo* tracking and retrieval of transferred cells for downstream research applications.

Adoptive transfer of chimeric antigen receptor (CAR)- and T-cell receptor (TCR)-engineered T cells has shown striking efficacy for the treatment of particular human malignancies[1–5]. Current approaches administer cell products comprising a mixture of transduced and non-transduced T cells, and expression of introduced receptors on T cells is variable. Ideally, engineered receptors would be designed to facilitate purification or selective expansion of receptor-bearing T cells and enable their *in vivo* tracking and reisolation for functional analysis. Here we design such multifunctional receptors through incorporation of modified *Strep*-tag II sequences at various locations in the extracellular region of the CAR or TCR (*Strep*-tag CAR; *Strep*-tag TCR)[6]. We selected *Strep*-tag II to evaluate as a receptor-intrinsic marker because binding reagents for *Strep*-tag are used in clinical cell processing[7].

We introduced one or more *Strep*-tag II sequences with Gly/Ser linkers at the N terminus, between the VL and VH, or between the single-chain Fv (scFv) and the hinge of CD19 CARs with 4-1BB/CD3ζ or CD28/CD3ζ signaling domains (**Supplementary Fig. 1a**)[8]. The constructs were encoded in lentiviral vectors with truncated epidermal growth factor receptor (EGFRt) downstream of a T2A sequence to provide an independent transduction marker[9]. CD19-Hi, a conventional CD19-CAR without *Strep*-tag II, served as a control for functional assays (**Supplementary Fig. 1a**). We transduced human CD8$^+$ T cells, sorted for EGFRt expression and evaluated CAR surface expression by staining with an anti-*Strep*-tag II monoclonal antibody (mAb). All *Strep*-tag CAR-T cells were stained with anti-*Strep*-tag II mAb, independent of the position or number of *Strep*-tag II sequences, and staining intensity was highest for CAR-T cells that contained three *Strep*-tag II sequences (**Fig. 1a**). All the *Strep*-tag II CAR-T cells lysed K562/CD19 cells (K562 erythroleukemia cells transduced with a lentiviral vector encoding the human *CD19* gene) and Raji-ffluc (Raji lymphoma cells transduced with a lentiviral vector encoding the FFLuc-eGFP fusion gene) as efficiently as T cells expressing the CD19-Hi CAR, and did not recognize control K562/ROR1 cells (K562 cells transduced with a lentiviral vector encoding the human *ROR1* gene) (**Fig. 1b**). CD19-specific recognition by *Strep*-tag II CAR-T cells was confirmed by the production of interleukin 2 (IL-2) and interferon (IFN)-γ after co-culture with CD19$^+$ tumor cells (**Fig. 1c**). We then investigated whether *Strep*-tag II could be introduced into a TCR specific for the breast cancer antigen NY-BR-1 (ref. 10). *Strep*-tag II TCRs were expressed in primary CD8$^+$ T cells as determined by staining with anti-*Strep*-tag mAb or human leukocyte antigen (HLA) tetramer, and conferred equivalent function as the introduction of the wild-type NY-BR-1 TCR (**Supplementary Fig. 1b–f**). These data indicate that inclusion of *Strep*-tag II did not interfere with CAR or TCR expression or *in vitro* function.

The length and composition of the spacer between the scFv and the T-cell membrane can affect CAR-T cell recognition[8]. CD19 CAR-T cells with short (IgG4 hinge), intermediate (hinge-C_H3) and long (hinge-C_H2-C_H3) spacers lysed CD19$^+$ tumor cells *in vitro*; however, the short spacer CAR (CD19-Hi) resulted in higher cytokine production (**Supplementary Fig. 2a**). A hierarchy of cytokine production and T-cell proliferation was also observed for CAR-T cells containing one, two or three *Strep*-tag II sequences between the scFv and IgG4 hinge, and was independent of the co-stimulatory domain (4-1BB or CD28) in the CAR (**Supplementary Fig. 2b,c**). The CD19 CAR with one *Strep*-tag II contained 19 additional amino acids in the spacer compared with the CD19-Hi CAR, yet conferred significantly ($P < 0.05$) greater cytokine production and cell proliferation after tumor recognition, suggesting that the incorporation of *Strep*-tag II and Gly/Ser sequences into CAR design could optimize spacer length and/or add flexibility to improve signaling (**Supplementary Fig. 2b–d**).

We evaluated the *in vivo* antitumor activity of CD19 *Strep*-tag II CAR-T cells in cohorts of NSG (non-obese diabetic (NOD)/severe combined immune deficient (SCID)/IL-2rγ (null)) mice engrafted with Raji lymphoma. Mice treated with T cells transduced with the CD19-Hi CAR, or with CD19 CARs containing one or three *Strep*-tag II sequences in the spacer region had complete tumor elimination in <28 d, but tumors progressed in mice treated with control T cells (**Fig. 1d**). Staining with anti-EGFRt and anti-*Strep*-tag II was used to track CAR-T cells in blood samples obtained after the T-cell infusion (**Fig. 1e**),

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and a time-course analysis of CAR-T cells in blood demonstrated that the *Strep*-tag II and CD19-Hi CAR-T cells proliferated and contracted similarly during tumor elimination (**Fig. 1f**).

We speculated that anti-*Strep*-tag II mAbs could be used to isolate CAR-expressing T cells from blood after transfer to evaluate changes in their gene expression *in vivo*. We sorted CAR-T cells with the

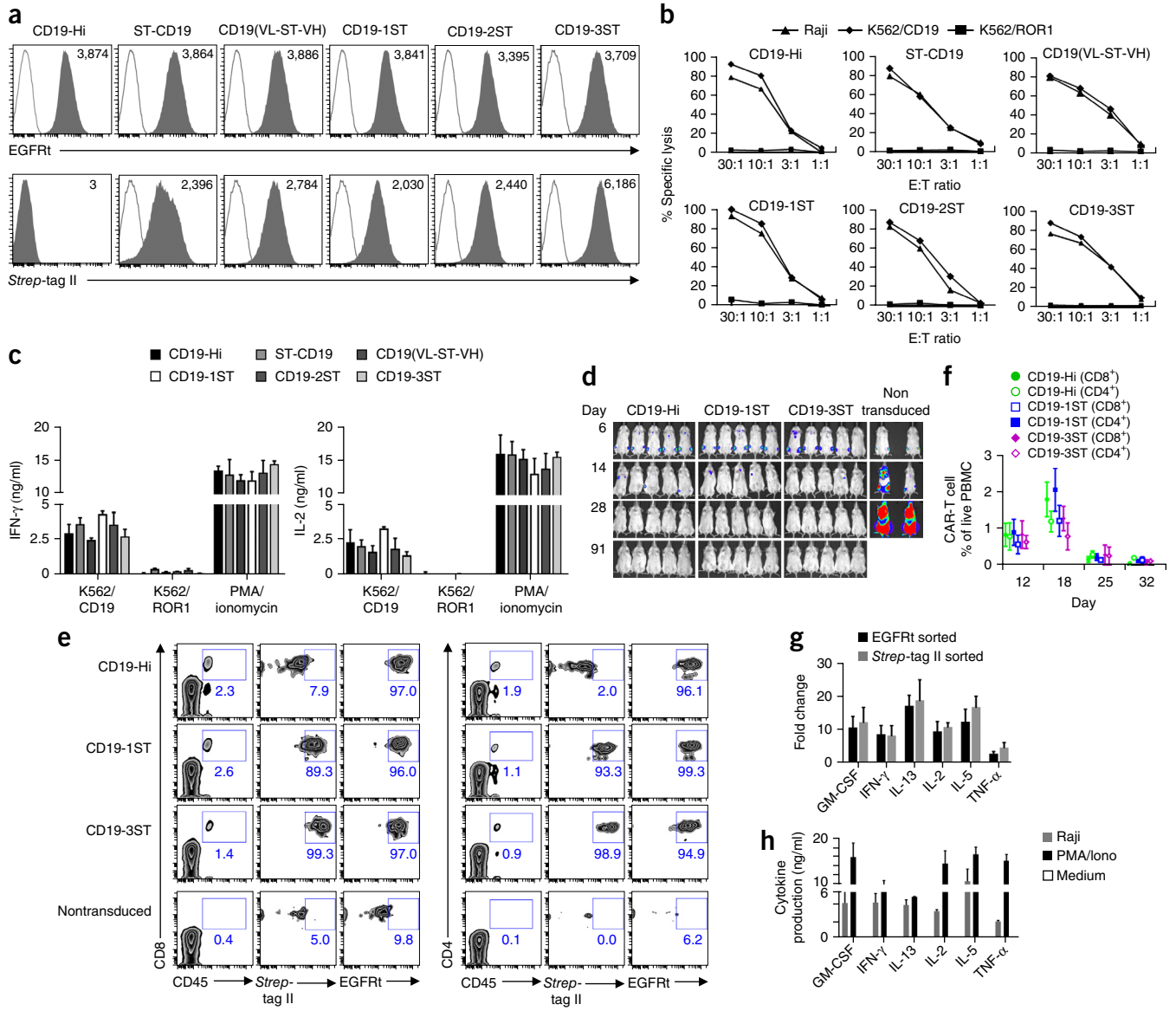

Figure 1 Expression and function of CD19 CARs that contain the *Strep*-tag II. (**a**) Analysis of CD19 CAR expression. Primary human CD8+ T cells were transduced with vectors encoding a CD19-Hi CAR or CD19 CARs with *Strep*-tag II in various extracellular locations and sorted for EGFRt+ cells by fluorescence-activated cell sorting; purity was confirmed by staining with anti-EGFR (gray, top panels). Cell surface expression of the *Strep*-tag II CARs was evaluated by staining with anti-*Strep*-tag II antibodies (gray, bottom panels). Nontransduced cells (white) served as controls for staining. ST, *Strep*-tag II. (**b**) Cytolytic activity of CD19-Hi and *Strep*-tag II 4-1BB/CD3ζ CAR-T cells. After sorting for EGFRt expression, CD8+ T cells transduced with each of the CARs were tested for lysis of K562/CD19 or K562/ROR1 at various effector/target (E:T) ratios. (**c**) IFN-γ and IL-2 production by CD19-Hi and *Strep*-tag 4-1BB/CD3ζ CAR-T cells 24 h after stimulation with K562/CD19 and K562/ROR1. PMA/ionomycin-treated T cells were used as a positive control. The data in **a**–**c** are representative of three experiments with CD8+ T cells from different donors. (**d**) Tumor progression and distribution evaluated by serial bioluminescence imaging in cohorts of NSG mice inoculated with Raji-ffluc and then CD19 4-1BB/CD3ζ CAR-T cells with Hi, 1ST or 3ST spacers. CAR-T cells were formulated in a CD8/CD4 ratio of 1:1. (**e**) Tracking CAR-expressing T cells *in vivo* by staining with anti-*Strep*-tag II mAb. Blood obtained from mice after the T-cell infusions was stained with anti-human CD45, CD8, CD4, anti-*Strep*-tag II and anti-EGFR mAbs, and analyzed by flow cytometry. Expression of *Strep*-tag II and EGFRt on CD45+CD8+ and CD45+CD4+ T cells is shown. (**f**) Kinetics of expansion and contraction of CD19 CAR-T cells in the blood after adoptive transfer to NSG mice bearing Raji tumors. The mean frequency of CD45+CD8+EGFRt+ and CD45+CD4+EGFRt+ human T cells in the blood of the mice ($n = 5$) of each group at various times after the T-cell infusion is shown. The data in **d**–**f** are representative of three experiments. (**g**) Fold-change in expression of selected cytokine genes in CD19-1ST/4-1BB/-CD3ζ CAR-T cells after infusion to Raji tumor-bearing and nontumor-bearing NSG mice and staining with anti-EGFR or anti-*Strep*-tag mAb. Gene expression was analyzed using a human common cytokine PCR array. The mean fold-change values of cytokine genes in the sorted CAR-T cells from NSG/Raji compared to sorted CAR-T cells from nontumor-bearing NSG mice were calculated. Samples were run in triplicate and data are presented as the mean fold increase ±s.d. (**h**) Cytokine production by CD8+ T cells expressing CD19-1ST/4-1BB/CD3ζ CARs after stimulation with Raji cells *in vitro*. PMA/ionomycin-treated and nontreated T cells were used as positive and negative controls, respectively. The results are representative of the mean values obtained in three experiments with transduced T cells from different donors. The error bars in **c**, **f** and **h** indicate the s.d. in replicate experiments.

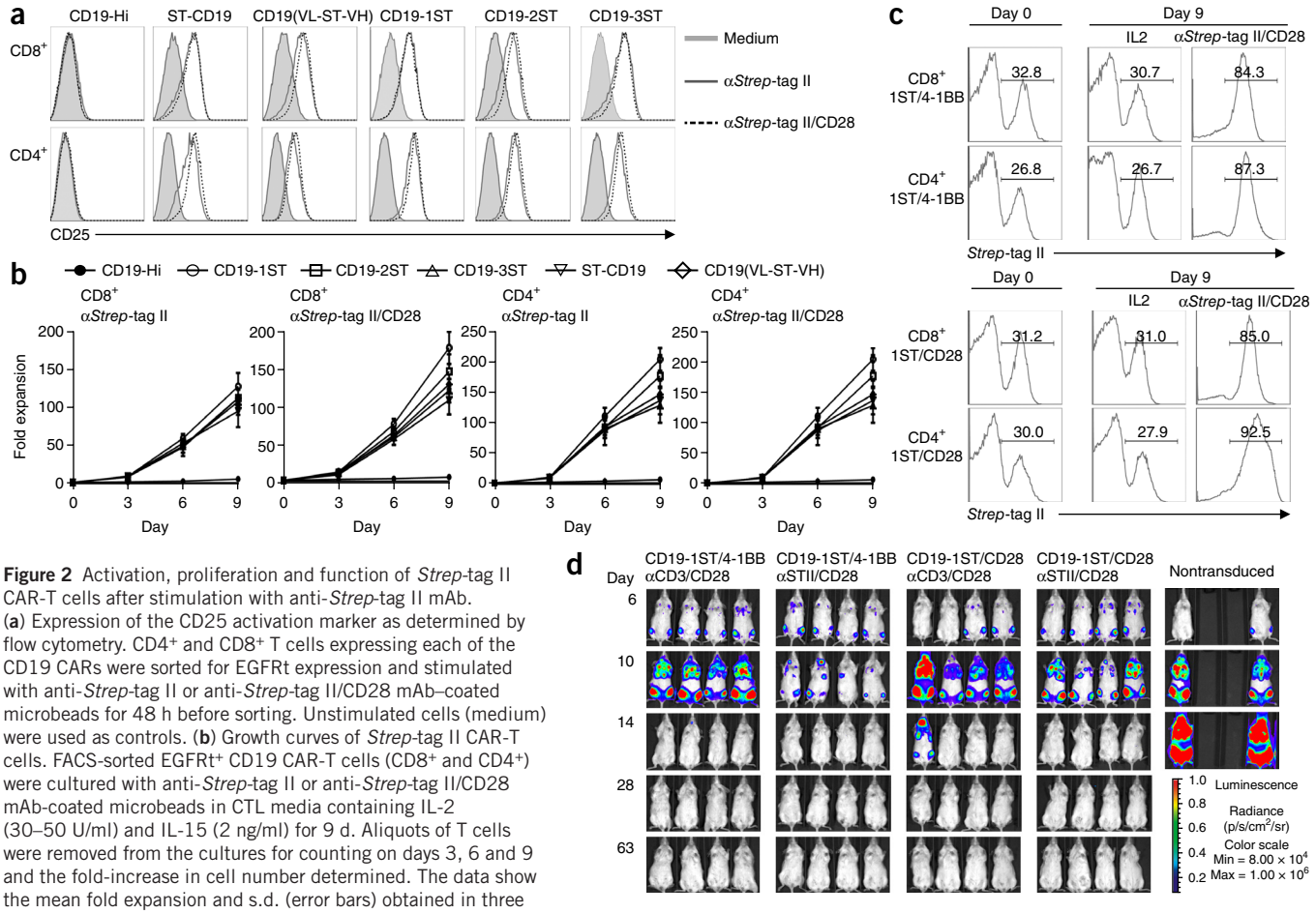

Figure 2 Activation, proliferation and function of *Strep*-tag II CAR-T cells after stimulation with anti-*Strep*-tag II mAb. (**a**) Expression of the CD25 activation marker as determined by flow cytometry. CD4+ and CD8+ T cells expressing each of the CD19 CARs were sorted for EGFRt expression and stimulated with anti-*Strep*-tag II or anti-*Strep*-tag II/CD28 mAb–coated microbeads for 48 h before sorting. Unstimulated cells (medium) were used as controls. (**b**) Growth curves of *Strep*-tag II CAR-T cells. FACS-sorted EGFRt+ CD19 CAR-T cells (CD8+ and CD4+) were cultured with anti-*Strep*-tag II or anti-*Strep*-tag II/CD28 mAb-coated microbeads in CTL media containing IL-2 (30–50 U/ml) and IL-15 (2 ng/ml) for 9 d. Aliquots of T cells were removed from the cultures for counting on days 3, 6 and 9 and the fold-increase in cell number determined. The data show the mean fold expansion and s.d. (error bars) obtained in three experiments with T cells from different donors. (**c**) Percentage of *Strep*-tag II–positive cells at day 0 (before stimulation) and day 9 (after stimulation) as measured by flow cytometry. CD8+ and CD4+ T cells were transduced with CD19 1ST/4-1BB/CD3ζ and 1ST/CD28ζ CARs and 10 d later stimulated with anti-*Strep*-tag II/CD28 microbeads plus IL-2 or cultured with IL-2 alone for 9 additional days. The results are representative of three experiments. (**d**) Anti-tumor activity of CD19 1ST/4-1BB/CD3ζ or 1ST/CD28ζ CAR-T cells in Raji-ffluc cell–engrafted NSG mice as evaluated by serial bioluminescence imaging.

anti-*Strep*-tag II mAb or anti-EGFRt mAb to >95% purity from Raji tumor-bearing NSG mice and from NSG mice that were not engrafted with Raji lymphoma but received *Strep*-tag II CAR-T cells, and cytokine gene expression was determined using the Qiagen (Hilden, Germany) Human Common Cytokines PCR Array. IFN-γ, IL-2 and tumor necrosis factor (TNF)-α were markedly upregulated in CAR-T cells from tumor-bearing compared to nontumor-bearing mice (**Fig. 1g**), consistent with elevated levels of these cytokines in the serum of patients with B-cell malignancies treated with CD19 CAR-T cells. We also observed upregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-13 and IL-5, which we confirmed in supernatants of CAR-T cells stimulated with Raji tumor cells *in vitro* (**Fig. 1g**,**h**). To ensure that anti-*Strep*-tag II mAb could be similarly used to detect CAR-T cells in human blood, we spiked peripheral blood mononuclear cells (PBMCs) and whole blood with CAR-T cells and found that the T cells were readily detected by anti-*Strep*-tag II mAb staining (**Supplementary Fig. 3a–c**). Thus, *Strep*-tag II labeling can be used to track CAR-T cells and analyze their gene expression *in vivo* during an antitumor immune response.

Anti-CD3/CD28 mAb-coated beads are used to nonselectively activate T cells and facilitate transduction with viral vectors, but at the same time induce proliferation of both transduced and nontransduced T cells[11,12]. We hypothesized that multivalent binding of *Strep*-tag II would selectively activate CAR signaling and induce proliferation only of transduced T cells. Co-culture of *Strep*-tag II CAR and control CAR-T cells with microbeads coated with anti-*Strep*-tag II mAb alone or combined with anti-CD28 mAb induced CD25 upregulation only in *Strep*-tag II CAR-T cells (**Fig. 2a**), and expanded CD4 and CD8 *Strep*-tag II CAR-T cells >100-fold in 9 d of culture (**Fig. 2b**). Activation and proliferation was independent of scFv specificity and costimulatory domains in the CAR (**Supplementary Fig. 4a–c**). The frequency of *Strep*-tag II CAR-T cells increased from ~26–33% to 84–92% in cultures stimulated with anti-*Strep*-tag II/CD28 beads, but did not increase in cultures with IL-2 alone (**Fig. 2c**). Analysis of TCR diversity of CAR-T cells before and after expansion with anti-*Strep*-tag II/CD28 showed that a diverse repertoire was retained, and the expanded T cells expressed CD62L, CD28 and CD27, maintained CAR-directed cytolytic function, and proliferated in response to tumor cells (**Supplementary Fig. 5a–d**). CAR-T cells expanded with anti-*Strep*-tag II/CD28 stimulation were as effective in eliminating Raji lymphoma in NSG mice as those expanded with anti-CD3/CD28 mAbs (**Fig. 2d**). Receptor tyrosine kinase-like orphan receptor-1 (ROR1)–specific CAR-T cells expanded with anti-*Strep*-tag II/CD28 also retained function *in vitro*, and *in vivo* against ROR1+MDA−MB-231 breast cancer xenografts in NSG mice, demonstrating that this approach can be applied broadly in CAR-T cell therapy (**Supplementary Fig. 6a–c**).

Strep-tag II CAR-T cells could be expanded 1×10^6-fold by repeated stimulation with anti-*Strep*-tag II/CD28 beads every 9–12 d for

applications where large numbers of T cells are necessary. Expanded T cells continued to express the CAR, CD62L and CD28 and retained specificity for CD19$^+$ targets after three stimulations, albeit with a decrease in cytokine production after the second stimulation (**Supplementary Fig. 7a–d**).

The ideal manufacturing process for T-cell therapy would rapidly generate pure cell products for patient administration. Current procedures for obtaining CAR-T cells require 10–20 d of culture, and provide cell products with a variable proportion of transduced cells[1,2,13]. The high binding affinity of *Strep*-tag II for StrepTactin suggested that StrepTactin-coated beads might be used as a simple strategy for rapid selection of *Strep*-tag II-expressing CAR-T cells. D-biotin, which has even higher affinity for StrepTactin could then be used to elute enriched *Strep*-tag CAR-T cells. We examined whether *Strep*-tag CAR-T cells could be selected using an automated device (T-CATCH/IBA), in which agarose beads were functionalized with

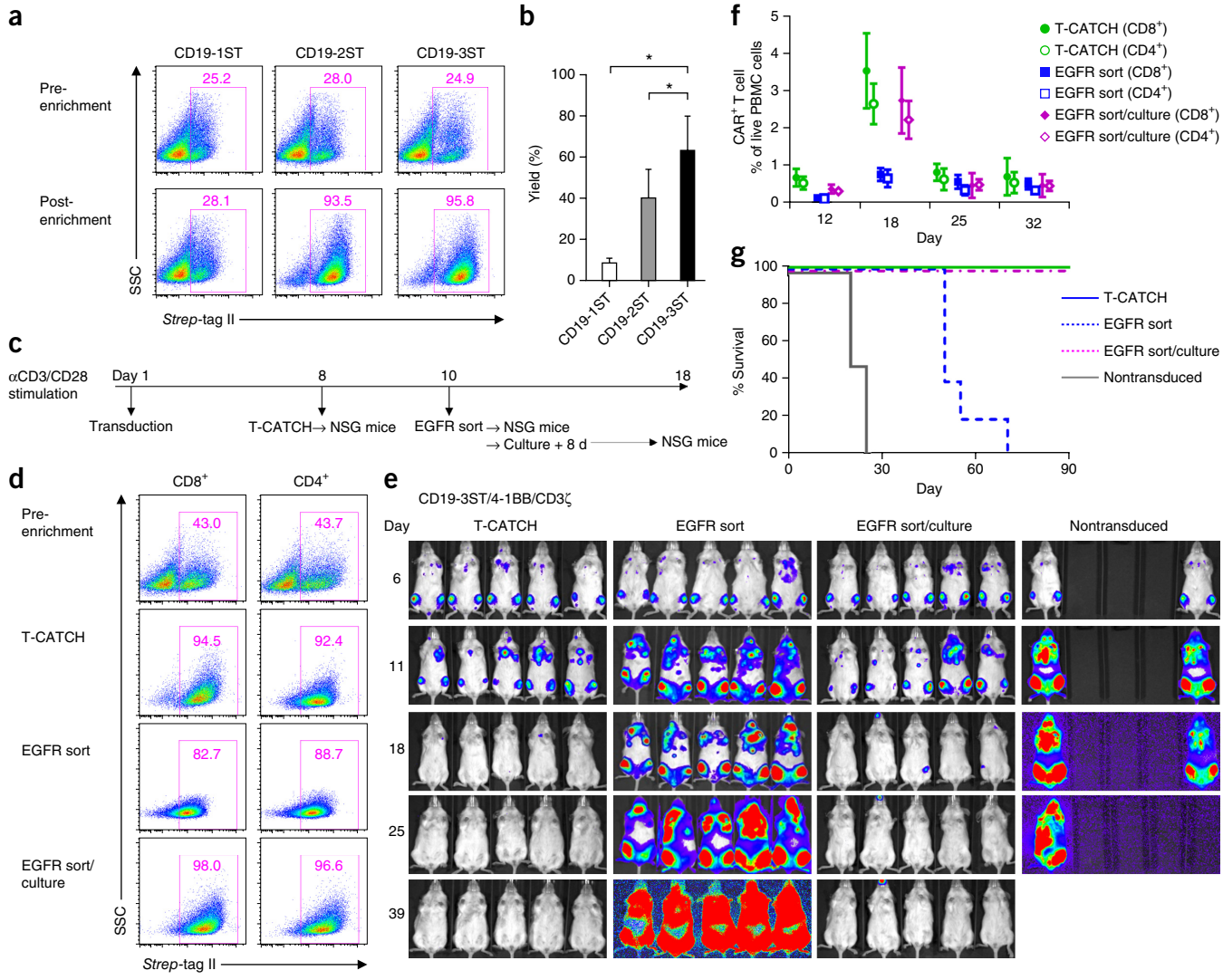

Figure 3 *Strep*-tag CAR-T cells can be enriched and exhibit potent anti-tumor activity *in vivo*. (**a**) Flow cytometric analysis of the frequency of *Strep*-tag CAR-T cells before and after enrichment on T-CATCH device using anti-*Strep*-tag II staining. Data are representative of six experiments using T cells from three donors. (**b**) Yield of *Strep*-tag CAR-T cells after T-CATCH enrichment. Yield was determined by the absolute numbers of *Strep*-tag CAR-T cells in the enriched fraction divided by the absolute numbers of *Strep*-tag CAR-T cells in the starting population. Data are derived from four experiments and expressed as means ± s.d. Statistical analysis was performed using the Student's *t*-test. $^*P < 0.05$. (**c**) Experimental scheme for adoptive transfer of *Strep*-tag II CAR-T cells enriched by StrepTactin selection or using EGFR mAb. CD8$^+$ and CD4$^+$ T cells were stimulated in independent cultures with anti-CD3/CD28 microbeads and transduced with the CD19-3ST/41BB/CD3ζ CAR. Cultures were established at different times so that T-cell administration into tumor-bearing mice occurred simultaneously. Anti-CD3/CD28 beads were removed at day 5 in all groups and CAR-T cells were prepared for inoculation into mice either by selection on the T-CATCH at day 8, FACS sorting for EGFRt$^+$ cells on day 10, or FACS sorting of EGFRt$^+$ cells followed by 8 d of culture on irradiated CD19$^+$ LCL cells with IL-2 to remove residual bound anti-EGFR mAb. (**d**) Flow cytometric analysis of CD19 CAR expression using *Strep*-tag II staining of CD8$^+$ and CD4$^+$ CAR-T cells before enrichment, after T-CATCH purification, after EGFR mAb sorting, and after EGFR mAb sorting followed by culture. (**e**) Tumor progression and distribution seen by serial bioluminescence imaging of NSG mice engrafted with Raji-ffluc cells and treated with CAR-T cells selected by T-CATCH, EGFR sorting or EGFR sorting, formulated in a CD4/CD8 ratio of 1:1. (**f**) Persistence of CD19 CAR-T cells in each cohort of NSG/Raji mice. Flow cytometric analysis of CD4$^+$ and CD8$^+$ CAR T cells in the peripheral blood of each group of mice after staining with CD45, CD8, CD4 and EGFR mAbs at different time points after T-cell infusion. The frequency of CAR-T cells is presented as a percentage of live peripheral blood cells. (**g**) Survival of mice treated with different CAR-T cell products or with nontransduced T cells depicted as Kaplan-Meier curves. The data in **d**–**g** are representative of two independent experiments.

immobilized StrepTactin and loaded into plastic tips. After washing to remove unbound T cells, *Strep*-tag CAR-T cells were released from the beads by the addition of D-biotin. *Strep*-tag CAR-T cells containing two or three *Strep*-tag II sequences were enriched from 26–28% to >90% purity, with yields of 40–60% (**Fig. 3a**,**b**). T cells transduced with the *Strep*-tag II NY-BR-1 TCR with two *Strep*-tag II sequences were also enriched to high purity using StrepTactin beads (data not shown). CAR-T cells containing one *Strep*-tag II were not enriched, perhaps because the affinity of a single *Strep*-tag II in the CAR was insufficient for stable binding to StrepTactin beads.

We then compared *in vivo* antitumor efficacy of CD19 CAR-T cells containing three *Strep*-tag II sequences that were enriched 8 d after transduction with the T-CATCH device with that of CAR-T cells selected using an anti-EGFR mAb, and either directly transferred to mice or cultured to remove the bound anti-EGFRmAb (**Fig. 3c**). T-CATCH-enriched T cells were of comparable purity to T cells sorted using anti-EGFRmAb (**Fig. 3d**), and eliminated Raji tumors in all mice. In contrast, CAR-T cells sorted with anti-EGFR mAb did not eliminate Raji tumors (**Fig. 3e**), their survival *in vivo* was compromised (**Fig. 3f**) and additional *in vitro* culture was required for optimal efficacy (**Fig. 3e**–**g**).

A theoretical disadvantage of including *Strep*-tag II in the CAR that could be evaluated in immunodeficient mice is that, in addition to the mouse scFv and fusion sites, the foreign *Strep*-tag II sequences could elicit T-cell and/or humoral immune responses. Humoral immunity can be examined only by *in vivo* studies in immunocompetent hosts; however, to address the issue of T-cell immunogenicity, we screened the *Strep*-tag II, linker and candidate flanking sequences in the CAR before construction of vectors using NetMHC3.4 to identify HLA-binding peptides. We did not identify sequences likely to bind to common HLA class I alleles with high affinity. Collectively, our studies show that the addition of *Strep*-tag II provides a receptor-intrinsic surface marker that endows engineered T cells with multiple functionalities, facilitating cost-effective and efficient current good manufacturing practice (cGMP), *in vivo* monitoring and analysis of therapeutic cell products in clinical applications.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.L. and S.R.R. conceived this study and designed the experiments. L.L., A.C., P.K., D.S. and T.H. performed experiments. L.L. and S.R.R. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Vector construction and preparation of viral vectors. CD19 and ROR1-specific CARs were constructed as previously described[14]. Spacer domains consisted of either a short (12 aa) IgG4 hinge, intermediate (119 aa) IgG4 hinge-C_H3 or a long (229 aa) IgG4 hinge-C_H2-C_H3 domain as described[8]. *Strep*-tag II CARs were designed by incorporating one or more copies of 9 aa *Strep*-tag II (NWSHPQFEK) fused with a Gly/Ser (G4S)2 linker N-terminal to the scFv, between VL and VH, or N-terminal to the hinge region. All *Strep*-tag II CARs used the short IgG4 hinge fused to human CD28 transmembrane domain and to a signaling module derived from either CD28 or 4-1BB and the cytoplasmic domain of CD3ζ as described[14]. The construct encoded a T2A sequence and a truncated epidermal growth factor receptor (EGFRt) sequence downstream of the CAR[9].

Codon-optimized nucleotide sequences encoding each transgene were synthesized (Life Technologies; IDT DNA Technologies) or generated by overlap extension PCR and cloned into the epHIV7 lentiviral vector[15]. CD19 and ROR1 CAR lentivirus supernatants were produced in 293T cells co-transfected with each of the lentiviral vector plasmids and the packaging vectors pCHGP-2, pCMV-Rev2 and pCMV-G using CalPhos transfection reagent (Clontech). Medium was changed 12 h after transfection, and lentivirus collected after 24, 48 and 72 h.

The NY-BR-1 TCR was derived from a T-cell clone isolated from a healthy donor as described[10]. The sequences of the variable regions of the TCR chains were determined by sequencing 3′-RACE-PCR products and then combined with constant regions containing modifications to increase TCR expression and stability[16,17]. The TCR-chains were linked by a P2A element, codon-optimized (Life Technologies), and cloned into the retroviral vector MP71 (ref. 18). To generate a *Strep*-tag II containing TCR, two *Strep*-tag sequences were included at the N terminus of the TCRα-chain[19]. Virus supernatant was generated as described[18].

Transduction of T cells with CAR lentiviral and TCR retroviral vectors. Blood samples were obtained from donors who provided written informed consent for research protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center and peripheral blood mononuclear cells were prepared by Ficoll-Hypaque gradient centrifugation. $CD8^+$ and $CD4^+$ T cells were isolated from PBMC using $CD8^+$ or $CD4^+$ T Cell Isolation Kits (Miltenyi Biotec), activated with anti-CD3/CD28 beads (Life Technologies) according to the manufacturer's instructions, transduced on day 1 or 3 after activation by exposure to lentiviral supernatant supplemented with 0.8 μg/ml polybrene (Millipore), and centrifuged at 800*g* for 45 min at 32 °C. T cells were transduced with retrovirus as described before[18]. T cells were expanded in RPMI, 10% human serum, 2 mM L-glutamine, 1% penicillin-streptomycin and 50 μM β-mercaptoethanol (CTL medium), supplemented with recombinant human IL-2 to a final concentration of 50 U/ml. Aliquots of transduced CAR-T cells were stained with biotin-conjugated anti-EGFR antibody and streptavidin-PE (Miltenyi), and EGFRt-positive T cells were sorted on a FACS-Aria II (Becton Dickinson). After sorting, CD19 CAR-T cells were expanded for some experiments by stimulation with irradiated (8,000 rad) $CD19^+$ B-lymphoblastoid cell line cells (LCL) at a T cell/LCL ratio of 1:7 in CTL supplemented with 30–50 U/ml IL-2 for another 8~10 d to remove the residual-bound antibody. R12 CAR-T cells after EGFRt sort and NY-BR-1 TCR–transduced T cells after Vβ22 sort, were expanded using a rapid expansion protocol (REP) as described[20].

Flow cytometric analysis. Conjugated antibodies specific for CD4, CD8, CD25, CD137, CD45, CD62L, CD27, CD28 (BD Biosciences), *Strep*-tag II (Genscript), EGFR (ImClone Systems) and Vβ22 (Beckman Coulter) were used for staining analysis by flow cytometry. Biotinylated antibodies were stained with streptavidin-PE (BD Biosciences, San Jose, CA). T cells transduced with the NY-BR-1 TCR were analyzed by flow cytometry after staining with antibodies specific for CD8, *Strep*-tag II, Vβ22 and major histocompatibility complex (MHC)-tetramers loaded with NY-BR-1 peptide (FHCRC Immune Monitoring Lab). Staining with propidium iodide (PI, BD Biosciences) was used for live/dead cell discrimination as directed by the manufacturer. Flow analyses were done on a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using FlowJo software (Treestar).

Chromium release, cytokine secretion and CFSE proliferation assays. Target cells were labeled with Cr^{51} (PerkinElmer) for 2 h, washed and incubated in triplicates at 1×10^3 cells/well with effector T cells at various effector/target (E:T) ratios for 4 h of culture. Specific lysis was determined using the standard formula. For analysis of cytokine secretion, target and effector cells were plated in triplicate wells at an E:T ratio of 2:1 (Raji, peptide-pulsed T2 cells) or 4:1 (K562/CD19 and K562/ROR1), and cytokine production was measured in supernatants removed after 24 h incubation by multiplex cytokine immunoassay (Luminex) or enzyme-linked immunosorbent assay (ELISA). For analysis of proliferation, the T cells were labeled with 0.2 μM carboxy fluorescein succinimidyl ester (CFSE, Invitrogen), washed and plated in triplicate wells with stimulator cells at a ratio of 2:1 (Raji, peptide-pulsed T2 cells and MDA-MB231) or 4:1 (K562/CD19 and K562/ROR1) in CTL medium without exogenous cytokines. After 5 d of incubation, the cells were labeled with anti-CD8 or CD4 mAb and PI to exclude dead cells from analysis. Samples were analyzed by flow cytometry for cell division of live T cells determined by dilution of CFSE.

Generation of anti-*Strep*-tag mAb and bead stimulation of *Strep*-tag CAR-T cells. An anti-*Strep*-tag II mAb was raised in the FHCRC Antibody Development Core by immunizing BALB/c mice with the NWSHPQFEK peptide conjugated to KLH using the Maleimide Activated BSA/KLH Conjugation Kit (Sigma, MBK-I). Spleen cells from immunized mice were fused with mouse myeloma cells, and supernatants from hybridomas screened by ELISA on a BSA conjugate of the above peptide, and by flow cytometric analysis on *Strep*-tag and control CAR-T cells. One clone termed 3E8, which secretes an lgG2b antibody was selected based on its affinity to *Strep*-tag II.

An optimized concentration of anti-*Strep*-tag II mAb (150 μg) alone or with anti-CD28 mAb (50 μg) was covalently coated on 50-mg customized functional magnetic beads (3.0~3.9 μm) according to product manual (Spherotech)[12]. For activation and expansion of *Strep*-tag CAR-T cells, anti-*Strep*-tag II mAb-coated microbeads were added to T cells in CTL medium supplemented with IL-2 (50 U/ml) alone or plus IL-15 (2 ng/ml). During T-cell expansion, culture medium was changed every 3 d.

Enrichment of *Strep*-tag II CAR-T cells using StrepTactin-coated resin. T cells containing various proportions of *Strep*-tag II CAR-T cells were washed with IS buffer (IBA) and aliquoted into multiple wells of the Tip-based Cell Affinity Chromatograph (T-CATCH) (IBA) device that selects cells based on binding to nonmagnetic resin with immobilized StrepTactin on the surface and is preloaded into sterile plastic tips. Sample loading, cell selection, washing and elution are performed automatically using computer-controlled preformatted software. T cells bound to the resin were released from the tips using IS buffer with 50 μM D-biotin and residual bound T cells were flushed out from the tips using IS buffer. The selected T cells were washed twice with PBS with 0.5% human serum before assaying for purity and *in vitro* and *in vivo* function.

Adoptive transfer of T cells in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice engrafted with Raji-ffluc or MDA-MB231-ffluc cells. The FHCRC Institutional Animal Care and Use Committee approved all mouse experiments. 6- to 8-week-old female NSG mice were obtained from the Jackson Laboratory or bred in-house. Mice were injected intravenously (i.v.) with 0.5×10^6 Raji-ffluc tumor cells through the tail vein, and received injections of CAR-modified or control T cells i.v. 7 d after tumor inoculation.

MDA-MB231 breast cancer xenografts were established in NSG mice by injecting 5×10^5 $ROR1^+$ MDA-MB231-ffluc cells subcutaneously (s.c.). Mice received i.v. injections of CAR-modified or control T cells 7 d after tumor inoculation. Tumor progression and distribution were evaluated by serial bioluminescence imaging as described[14]. All the animal experiments were repeated at least once. Five mice were used in each experimental group to provide 81% power to detect an effect size of 1.75, based on a *t*-test with a one-sided 0.05 level of significance.

RT^2 profiler human common cytokine PCR array. The cytokine profile of CD19-1ST/4-1BB/CD3ζ CAR-T cells *in vivo* was examined 2 d after adoptive transfer into NSG mice ($n = 3$) with and without Raji-ffluc tumor cell xenografts using the Human Common Cytokines PCR Array, which profiles

the expression of 84 human cytokine genes (SABiosciences, Frederick, MD). CD19 CAR-T cells were sorted from blood, bone marrow and spleen using anti-*Strep*-tag II or EGFR mAb staining, RNA was extracted using RNeasy Plus Mini Kit (QIAGEN) and cDNA was synthesized using RT^2 First Strand Kits (QIAGEN). The PCR array was performed according to the manufacturer's protocol for the ABI7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR data were analyzed using Qiagen PCR Array Data Analysis Software (Excel & Web based). For each PCR reaction, the samples were run in triplicates, data were normalized to five housekeeping genes average C_t values, and a mean fold-change ($2\text{-}\Delta\Delta^{CT}$) and s.d. were calculated to examine the gene upregulation in the CAR-T cells infused into NSG mice previously engrafted with Raji tumor cells compared to CAR-T cells infused into NSG mice that were not inoculated with Raji tumor cells. Only genes with C_t values <35 in T cells from the tumor-bearing mice were selected to focus the comparison on abundant or moderately abundant target mRNAs.

TCR sequencing. *Strep*-tag II CAR-T cells were sorted with anti-*Strep*-tag II mAb and an aliquot was submitted to TCRB repertoire analysis using the ImmunoSeq Assay[21,22] (Adaptive Biotechnologies). A second aliquot was expanded on anti-*Strep*-tag II/CD28-microbeads and then submitted for TCRB repertoire analysis. TCR clonotypes at a frequency of > 0.01% were used to conduct overlap analysis and Pearson product-moment correlation coefficient (Pearson's *r*) was calculated to measure linear correlation between two cell products.

Statistical analyses. Statistical analyses were conducted using Prism Software (GraphPad). Student's *t*-test was conducted as a two-sided paired test with a confidence interval of 95% and results with a *P* value less than 0.05 were considered significant.

Antibodies. CD45-FITC (BD Pharmagen; Cat: 555482); CD8-PE-Cy7 (BD Pharmagen; Cat: 557746); CD4-APC (BD Pharmagen; Cat: 555349); *Strep*-tag II Antibody (biotin) (Genscript: Cat: A10737); CD28-PE-Cy7 (BioLegend; Cat: 302925); CD27-PE (eBioscience; Cat: 12-0279); CD62L-APC-Cy7 (BioLegend; Cat: 304813); EGFR antibody, ERBITUX (cetuximab) (Bristol-Myers Squibb; NDC: 66733094823); CD25-APC (BD Pharmagen; Cat: 555434); StrepTavidin-PE(BD Pharmagen; Cat: 554061); CD28 (BioLegend; Cat: 302902); Propidium Iodine (BD Pharmagen; Cat: 556463).

Cell lines. Raji, K562 and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Raji-ffluc and MDA-MB-231-ffluc tumor cells were generated by transducing tumor cells with a lentiviral vector encoding the FFLuc-eGFP fusion gene, and then sorted for GFP^+ cells. K562/CD19 cells were generated by transducing the K562 erythroleukemia cell line with a lentiviral vector encoding the human *CD19* gene, and then sorted for $CD19^+$ cells. K562/ROR1 cells were generated by transducing K562 cells with a lentiviral vector encoding the human *ROR1* gene, and then sorted for $ROR1^+$ cells. All cell lines were tested regularly for mycoplasma and shown to be negative.

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