Traceless aptamer-mediated isolation of CD8+ T cells for chimeric antigen receptor T-cell therapy

Nataly Kacherovsky1,4, Ian I. Cardle1,2,4, Emmeline L. Cheng1, Jonathan L. Yu1, Michael L. Baldwin2, Stephen J. Salipante3, Michael C. Jensen1,2,4* and Suzie H. Pun1*

Chimeric antigen receptor T-cell therapies using defined product compositions require high-purity T-cell isolation systems that, unlike immunomagnetic positive enrichment, are inexpensive and leave no trace on the final cell product. Here, we show that DNA aptamers (generated with a modified cell—SELEX procedure to display low-nanomolar affinity for the T-cell marker CD8) enable the traceless isolation of pure CD8+ T cells at low cost and high yield. Captured CD8+ T cells are released label-free by complementary oligonucleotides that undergo toehold-mediated strand displacement with the aptamer. We also show that chimeric antigen receptor T cells manufactured from these cells are comparable to antibody-isolated chimeric antigen receptor T cells in proliferation, phenotype, effector function and antitumour activity in a mouse model of B-cell lymphoma. By employing multiple aptamers and the corresponding complementary oligonucleotides, aptamer-mediated cell selection could enable the fully synthetic, sequential and traceless isolation of desired lymphocyte subsets from a single system.

The clinical impact of T-cell therapies is being rapidly realized with two recent FDA approvals for chimeric antigen receptor (CAR) T-cell therapies treating acute lymphoblastic leukaemia and diffuse large B-cell lymphoma (Novartis’s Kymriah and Gilead–Kite’s Yescarta, respectively), as well as many promising results in clinical trials. CAR T cells have also been generated as potential anti-HIV therapies. The production and administration of autologous CAR T-cell therapy involves harvesting and genetically manipulating T cells before reintroducing the engineered cells back into the patients. The first step in this process, cell harvesting, requires high-purity isolation of the desired cell populations. For example, CAR T cells with defined 1:1 CD4+ to CD8+ cell populations have been reported to be more potent than CD4+ or CD8+ cells alone, or unselected populations in animal models of leukaemia, and are also very effective in human clinical trials for acute lymphoblastic leukaemia.

T cells for CAR T-cell manufacturing are typically isolated from peripheral blood mononuclear cells (PBMCs) collected by leukapheresis. One method reported for use in clinical-scale T-cell isolation is the sequential isolation of CD8+ and CD4+ T cells from the apheresis product by immunomagnetic positive enrichment (CliniMACS, for example). This approach can achieve high purity and yield but may suffer from: high costs associated with biologically produced antibodies; potential safety concerns stemming from a final cell population that may still be associated with antibody-coated magnetic beads; and low throughput due to the need for multiple selection apparatuses in sequence. Furthermore, the magnetic beads retained on the cells may prevent the downstream selection of cell subsets that could be beneficial for therapy. Although clinical selection strategies that immunodeplete undesired cell populations allow for the isolation of untouched cells and downstream positive selection of specific cell subsets, they also: introduce more costs by relying on a large panel of antibodies for depletion; reduce the yield by half as the apheresis product has to be split to obtain separate subsets of both CD4+ and CD8+ T cells; and may result in target cells with a low purity.

Streptamer-based cell-selection technology that avoids some of these undesirable outcomes through fragment antigen-binding constructs (Fabs) fused with peptide tags that bind reversibly to magnetic beads coated with engineered streptavidin has been reported. Fabs can be released from the beads as a result of competition with high-affinity d-biotin and therefore must be engineered with relatively low receptor-binding affinity so that they dissociate rapidly from the cell once released in the monovalent form. While the extent of Fab internalization into the cells after release from the solid support is unclear, Fabs engineered with relatively low receptor binding are not substantially retained on the cell surface. However, this method is still costly due to its reliance on biologically produced engineered streptavidin and modified Fabs. Additionally, all the aforementioned approaches have low throughput and high supply requirements for CD4+ and CD8+ T-cell isolation and rely on multiple selection apparatus in either sequence or in parallel. Thus, despite technological advances in cell selection, an overall approach that has low cost, traceless selection and high throughput while maintaining a reasonable yield and purity has remained elusive.

Nucleic acid aptamers (single-stranded oligonucleotides capable of binding target molecules) are an attractive alternative to antibodies and Fabs for cell selection. First developed in the 1990s, aptamers can possess binding affinities comparable to (or even higher than) antibodies. Importantly, aptamers are produced synthetically as well-defined, low-variability products with a long storage stability, making them inexpensive and easy to manufacture. Aptamers are commonly discovered through a library selection method known as systematic evolution of ligands by exponential enrichment (SELEX) and can be further optimized for chemical stability. With their favourable attributes, as summarized in Supplementary Table 1, the application field for aptamers has escalated in the last quarter.

1Department of Bioengineering, University of Washington, Seattle, WA, USA. 2Ben Towne Center for Childhood Cancer Research, Seattle Children’s Research Institute, Seattle, WA, USA. 3Department of Laboratory Medicine, University of Washington, Seattle, WA, USA. 4These authors contributed equally: Nataly Kacherovsky, Ian I. Cardle. *e-mail: michael.jensen@seattlechildrens.org; spun@uw.edu
of a century to encompass areas including sensing, purification, diagnostics, drug delivery and therapeutics\textsuperscript{24}.

Here, we report a reversible aptamer-selection technology for the isolation of label-free CD8\textsuperscript{+} T cells. Three main facets of the work are presented. First, we identified several high-affinity DNA aptamers specific for CD8 using a modified cell–SELEX procedure and validated the binding characteristics of selected aptamers. We compared magnetic-activated cell sorting (MACS) with one of the aptamers to the antibody-based CD8 Microbead system that is used clinically and found that the aptamer isolated CD8\textsuperscript{+} T cells from PBMCs with efficiencies comparable to the standard method. Second, we developed a method to reverse aptamer binding using a complementary oligonucleotide reversal agent to disrupt aptamer folding. We show that CD8\textsuperscript{+} cells can be released from aptamer-immobilized supports with high yield and purity using this approach. Finally, we generated and fully characterized CAR T cells from both our reversible aptamer selection approach and standard, antibody-based selection. CAR T cells isolated using traceless aptamer selection were phenotypically similar to those isolated using antibodies and exhibited nearly identical effector functions, both in vitro and in vivo. This aptamer-based selection approach therefore enables highly efficient, label-free and inexpensive selection of T cells for potential clinical-scale cell therapy applications. With the future discovery of other T-cell-specific aptamers, such as a CD4 aptamer, this technique could be readily expanded for high-throughput, serial selection of multiple T-cell populations from a single apparatus using a panel of aptamers and corresponding reversal agents.

Results
Identification of T-cell-binding aptamers by cell–SELEX incorporating competitive and counter selection. The generation of highly specific aptamers is critical for cell isolation applications. As our initial efforts to identify T-cell-specific aptamers using either traditional protein–SELEX with recombinant proteins or cell–SELEX\textsuperscript{25–26} with engineered cell lines were unsuccessful and yielded aptamers with poor specificity, we hypothesized that both the native display of receptors on cell surfaces afforded by cell–SELEX and the increased stringency of selection provided by competitive selection\textsuperscript{27} would be required to discover a T-cell-specific aptamer. We therefore modified the published cell–SELEX protocol\textsuperscript{28} to include both competitive selection (inclusion of the presence of relevant undesired targets) and counter selection (the depletion of aptamers binding undesired targets) (Fig. 1). After an initial round of positive selection against T cells using a single-stranded DNA library with a 52 base pair (bp) random region (10\textsuperscript{16} variants), the selected aptamer pool underwent multiple rounds of competitive/counter selection. Each round included the competitive selection of T-cell-binding aptamers, which was achieved by incubating aptamer pools with PBMCs and then isolating untouched T cells and extracting the bound aptamers. Collected aptamers were then subjected to counter selection by incubation with J.RT3-T3.5, a CD3-specific antibody, robustly outcompeting concentrations of an unlabelled CD8-specific antibody (clone RPA-T8), but not a CD3-specific antibody, robustly outcompeting all three aptamers for binding to CD8\textsuperscript{+} T cells during a coincubation (Supplementary Table 5). CD8a knockdown (75\%) by siRNA (Supplementary Table 6) in primary CD8\textsuperscript{+} T cells was confirmed by antibody staining and correlated with a 73–77\% reduction in the binding of all three aptamers (Fig. 2b). Transient expression of CD8a from a green fluorescent protein (GFP) reporter plasmid in CD8\textsuperscript{-} Jurkat immortalized human lymphocyte cells introduced aptamer binding specifically to GFP\textsuperscript{+} cells (Fig. 2c).

To validate aptamer binding to the CD8a protein itself, we measured association and dissociation kinetics by biolayer interferometry (BLI), in which a serial dilution of recombinant extracellular CD8a protein (Ser22-Asp182) was screened against aptamers immobilized on streptavidin–coated BLI sensors. Whereas the RN aptamer negative control exhibited no detectable association with the CD8a protein (data not shown), the A1, A3 and A8 aptamers bound the protein with binding affinities ($K_D$ values) of 20.1 ± 0.2, 14.7 ± 0.1 and 5.59 ± 0.11 nM, respectively (Fig. 2d and Supplementary Table 7). Interestingly, the A3 aptamer had both the highest association ($K_A$) and dissociation ($K_D$) rate constants of the three aptamers, whereas A8 had the lowest $K_D$. We also evaluated the apparent $K_D$ values of aptamer binding to CD8\textsuperscript{+} T cells by
flow cytometry, and the A1, A3 and A8 aptamers have apparent $K_D$ values of $18.3 \pm 4.6$, $1.9 \pm 0.8$ and $2.4 \pm 0.9$ nM, respectively (Fig. 2c). The observed differences in $K_D$ values determined by the two methods were expected. Mass transport limitations not present with BLI but present when staining cells in static wells favour re-binding and thereby limit dissociation, improving the apparent $K_D$ of the faster-dissociating A3 relative to the other aptamers. Furthermore, at saturating concentrations of aptamer, where binding advantages stemming from high $K_w$ values are diminished given enough time (that is, binding at or near steady-state), the A8 aptamer displays increased binding over A3, consistent with the large difference in $K_w$ between the two aptamers. Regardless, all three aptamers have a high binding affinity to CD8α protein and CD8⁺ T cells, with $K_D$ values comparable to monoclonal antibodies.

Reversing aptamer binding with a complementary oligonucleotide. To achieve traceless cell isolation using an aptamer-based affinity agent, a method to reverse aptamer binding to the cell is needed in the cell recovery step. Aptamer binding can be disrupted by nuclease-mediated degradation of the aptamer, applied force, competitive binding or denaturing the secondary structure, either through heat or complementary oligonucleotide binding. Of these methods, complementary oligonucleotide displacement is a preferred approach due to its advantages of being gentle (compared to heat or force), with a high yield (compared to competitive binding) and relatively low cost (compared to nuclease degradation). We therefore designed a CD8-binding aptamer that could be released from cells by binding with a complementary displacement strand (reversal agent).

Two major considerations in the design of the selection aptamer and reversal agent are: high-affinity binding to target cells by the selection aptamer; and rapid disruption of the aptamer secondary structure, which is critical for receptor binding by the reversal agent. We therefore chose the A3 aptamer for cell selection, not only due to its low apparent $K_D$ for CD8⁺ T cells, but also because of its high $K_w$ and $K_m$ for CD8α protein. As multivalent display of the aptamer on a selection medium would potentially mitigate passive cell dissociation, because there are aptamers in cis to retain cell binding even if a few aptamers dissociate, we rationalized that faster association kinetics would probably be more important for cell isolation. Furthermore, a high $K_w$ implies less stable binding, suggesting that strategies to reverse aptamer binding by complementary oligonucleotide displacement may be more successful. We also tested a reported CD8 aptamer from the literature; however, low binding was observed under the binding conditions in the published research and our study (Supplementary Fig. 7). A toehold region was then extended on the 3’ end of the original A3 sequence (A3t) to facilitate the initiation of cell release by the complementary reversal agent (Fig. 3a and Supplementary Table 5). Toeholds are single-strand sequences that allow for complementary sequence binding and the supplanting of prepared bases through a method known as strand displacement. In this case, the reversal agent would undergo strand displacement via the toehold to abrogate intrastrand base pairing in the aptamer necessary for its secondary structure. It has been previously described that the rate constant of strand displacement depends critically on the toehold length, varying over up to six orders of magnitude, with maximum rates reached with toeholds over six bases in length; an octanucleotide toehold was therefore used in our CD8-aptamer selection agent. The reversal agent was designed to be 36 bases in length based on the predicted change in secondary structure on binding (Fig. 3b and Supplementary Table 5).

We demonstrated effective and rapid aptamer release from cells by the reversal agent using a fluorescently labelled aptamer with flow cytometry analysis. To determine the appropriate conditions for release, various concentrations of reversal agent (ranging from...
Fig. 2 | A1, A3 and A8 bind to CD8a glycoprotein. a. Flow cytometry plots of 50 nM random (RN), A1, A3 and A8 aptamer binding to CD4+ and CD8+ T cells in a mixed T-cell population. The plots represent one independent experiment. b. Flow cytometry analysis of CD8a antibody (CD8a Ab) and 10 nM RN, A1, A3 and A8 aptamer binding to CD8+ T cells 24 h after nucleofection with non-specific siRNA or CD8 siRNA duplexes. Left, five graphs showing three independent experiments with technical triplicates. Right, a chart showing the percentage of GFP+ Jurkat cells that were also positive for antibody or aptamer binding. Red, dashed horizontal line represents binding to non-specific siRNA-treated controls to which the CD8 siRNA data points were normalized (that is, 100% binding). Horizontal lines and error bars represent the mean ± s.d.; n = 3 independent experiments. *P < 0.05 and **P < 0.01 (one-way analysis of variance (ANOVA) with Bonferroni correction). c. Flow cytometry analysis of CD8a Ab and 10 nM RN, A1, A3 and A8 aptamer binding to CD8+ Jurkat cells 24 h after nucleofection with a CD8a-hnRNP-M-EGFP plasmid. Left, flow cytometry plots representing three biological replicates with technical triplicates. Right, a chart indicating the percentage of GFP+ Jurkat cells relative to non-specific siRNA-treated controls. Red, dashed horizontal line represents the mean of RN binding. Horizontal lines and error bars represent the mean ± s.d.; n = 3 biologically independent samples. *P < 0.05, ***P < 0.001 and ****P < 0.0001 (paired one-way ANOVA with Dunnett’s test). d. BLI-measured association and dissociation kinetics of serially diluted CD8a protein binding to immobilized A1, A3 and A8 aptamers. The association phase is illustrated from 0–1,200 s, whereas dissociation is shown from 1,200–1,800 s (separated by the vertical red line). Kd values were calculated by performing a global fit of the kinetic data at the different concentrations of CD8a protein to a 1:1 binding model. Kd values are the mean ± s.d.; n = 3 individual concentrations for A1 and 4 individual concentrations for A3 and A8. e. The flow cytometry binding curves of A1, A3 and A8 aptamers to CD8+ T cells, normalized to 200 nM of A8 binding. The curves represent a non-linear regression assuming one-site total binding of three independent experiments with technical triplicates. Kd values were calculated by averaging the individual regression values of the independent experiments. Data points and error bars, and Kd values, are the mean ± s.d.; n = 3 independent experiments. APC, allophycocyanin; PerCP/Cy5.5, peridinin-chlorophyll-protein complex: cyanine-5.5 conjugate; FAM, carboxyfluorescein; NS, non-specific; NA-DL633, neutravidin DyLight 633.

Kd = 20.1 (± 0.2) nM
Kd = 14.7 (± 0.1) nM
Kd = 5.59 (± 0.11) nM
Fig. 3 | Complementary reversal agent designed to occlude binding of A3 aptamer with modified toehold. a, The minimum free energy secondary structure of a modified A3 aptamer with a 3′ 8 bp toehold (A3t), predicted using NUPACK (temperature = 4 °C; Na+ = 137 mM; Mg2+ = 5.5 mM). The orange line represents the 36 bp region that a complementary reversal agent was designed to anneal. b, The predicted minimum free energy secondary structure of the A3t aptamer after strand displacement with the reversal agent (temperature = 20 °C; Na+ = 137 mM; Mg2+ = 5.5 mM).

An aptamer-based strategy for traceless T-cell isolation. Before applying the A3t aptamer to a cell selection process, it was imperative to ensure that the aptamer selectively binds T cells when other types of PBMCs are present. At the concentration used for cell isolation (5 nM), minimal binding to CD3-CD56-CD14+ monocytes and CD3-CD56-CD19+ B cells was observed and binding to these cell populations was not above the RN aptamer control (Supplementary Fig. 9). Binding to B cells was especially low (close to 0%)—a desirable trait given that the transduction of a single, highly competent leukaemic B cell with a CAR was recently shown to induce resistance to therapy48. In addition to CD3+CD56- T cells, the A3t aptamer displayed substantial binding to CD3+CD56+ natural killer T (NKT) cells and CD3-CD56+ natural killer (NK) cells (as expected), which are known to have subsets that express CD8. As NKT cells have been found to improve CAR T-cell therapy49, binding to these cells was seen as beneficial. Importantly, the median fluorescent intensity (MFI) of aptamer binding on A3t-positive monocytes, B cells and NK cells is much lower compared to that of A3t-positive T and NKT cells, suggesting that binding events would minimally capture the contaminant cells at this aptamer concentration.

We predicted that the selection aptamer A3t and its cognate reversal agent could be used to achieve traceless T-cell isolation in a completely synthetic system in which immobilized aptamers are used to isolate T cells that are then released by the addition of the reversal agent, which disrupts the secondary structure of the aptamer that is critical for binding (Fig. 4a). We tested this strategy using the A3t aptamer immobilized on immunomagnetic Anti-Biotin Microbeads (Miltenyi Biotec). We compared the aptamer strategy to antibody-based CD8 Microbeads (Miltenyi Biotec) in terms of their abilities to isolate CD8+ T cells with high purity and yield from three healthy donor PBMC populations, as CD8 Microbeads are the only selection technology approved for clinical-scale CAR T-cell manufacture at present. PBMCs were incubated with functionalized beads and applied onto a column under a magnetic field, after which the flow-through fraction was collected. Antibody-isolated cells were then removed from the column using a column flush whereas aptamer-isolated cells were exposed to a 100-fold excess of reversal agent for 10 min on the column. The column was then unplugged and washed, which constituted the reversal agent elution (RAE) fraction. Any remaining cells on the column were removed using a column flush. The fractions of both isolation methods were analysed via flow cytometry (Supplementary Fig. 10).

We observed near-complete depletion of CD8+ cells from the flow-through fraction using aptamer-loaded microbeads, comparable to that obtained using antibody-based CD8 Microbeads. This corresponded to the enrichment of CD8+ cells in the RAE and column flush fractions (Fig. 4b). Further analysis of the CD8+ cells in the RAE fraction showed that these cells were predominantly CD3+CD16− T cells (>97%), with only a small fraction of the population being CD8+CD16+ monocytes and NK cells (Fig. 4c). Importantly, this purity analysis is conservative, as it does not account for CD3+CD16+ NKT cells. On average, the combined RAE and column flush of the aptamer-based isolation yielded 97.5% of the CD8+ T cells from the starting PBMC population (comparable to that obtained from antibody-based isolation) and the traceless isolation alone (RAE fraction) yielded 72.3% (Fig. 4d). Even if contaminating CD8- cells are included, the average purity of CD8+ T cells in the RAE fraction was 95.6%, illustrating that the A3t aptamer displays minimal non-specific binding to CD8- cells in PBMCs (Fig. 4e). The CD8 staining of aptamer-isolated CD8+ T cells in the RAE fraction with the RPA-T8 antibody clone was comparable to that of CD8+ T cells in the starting PBMC population, whereas that of antibody-isolated CD8+ T cells was lower (Fig. 4f). Given that the antibody-based method depletes nearly the whole CD8+ T-cell population (Fig. 4b,d), the lower CD8 staining is probably a result of the cell-bound CD8 Microbeads hindering the binding of the staining antibody clone used (Fig. 4f). This further emphasizes certain advantages of the traceless nature of reversal agent strand displacement, which can enable accurate, downstream phenotyping of capture antigens without needing to optimize the staining antibody clone. Interestingly, aptamer-isolated CD8+ T cells in the column flush fraction that were not stripped off in the RAE were just as pure as those in the RAE fraction (Fig. 4e). However, they also had higher CD8 expression than those in the RAE fraction (Fig. 4f), albeit not significantly so, indicating that cells with higher CD8 expression and thus aptamer-bead labelling may be more difficult to remove from the column by RAE. Although simply flushing the column would provide a similarly pure product with higher yield than using a reversal agent, RAE allows the isolation of cells free of magnetic beads in a manner that is specific to each selection aptamer used. In addition to providing traceless cell recovery, this approach affords the potential for both further downstream selection of different cell subsets and the serial selection of different cell types from a single selection process.

To confirm that aptamer-isolated cells are similar to antibody-isolated cells, we compared the CD8+ T cells in the RAE fraction of the aptamer-based isolation to those of the antibody-based isolation using flow cytometry phenotyping and NanoString nCounter transcript profiling. Whereas CD8+ T cells in the aptamer-isolated RAE fraction were phenotypically identical to those in PBMCs, antibody-isolated CD8+ T cells comprised a slightly larger percentage of cells in a transitional stage of dual CD45RA/RO expression that coincided with a small reduction in effector memory cells (Supplementary Fig. 11a). Transcriptionally, however, aptamer- and antibody-isolated cells were identical (Supplementary Fig. 11b). No gene transcripts were differentially expressed in significantly higher or lower amounts, suggesting that there are no immediate side effects to briefly exposing the cells to high reversal agent concentrations.
Fig. 4 | Isolation of label-free CD8^+ T cells from PBMCs using a reversible, aptamer-based selection strategy. a, A schematic representation of the traceless selection of CD8^+ T cells using the A3t aptamer. Biotinylated aptamer (5 nM) preloaded onto Miltenyi Anti-Biotin Microbeads was incubated with PBMCs to magnetically label CD8^+ T cells. The cell suspension was applied onto an LS column under a magnetic field, in which unlabelled cells were removed in the flow-through fraction. Microbead-labelled CD8^+ T cells that remained on the column were incubated with a 100-fold excess of the complementary reversal agent and released CD8^+ T cells were washed off the column in the RAE fraction. Any remaining cells on the column were removed using a plunger column flush in the absence of the magnetic field.

b, Flow cytometry graphs of CD8 expression in the different fractions of standard, antibody-based Miltenyi CD8 Microbead isolation (left) and traceless aptamer-based isolation (right). The graphs represent three independent experiments with technical triplicates.

c, Flow cytometry plots of CD3 and CD16 expression in CD8^+ antibody-isolated column flush (left) and aptamer-isolated RAE cell fractions (right) to distinguish between CD3^+CD16^- T cells and CD3^-CD16^+ monocytes and NK cells. The plots represent three independent experiments with technical triplicates.

d–f, Flow cytometry analysis of yield (d), purity (e) and CD8 MFI (f) of CD3^+CD8^+CD16^- T cells in different fractions of antibody- and aptamer-based isolations. The circles, squares and triangles represent different donors from separate isolation experiments, and all data were collected in technical triplicates. The red, dashed horizontal line represents the corresponding mean value of the antibody group (d–e) or PBMC group (f). Horizontal lines and error bars represent the mean ± s.d.; n = 3; P > 0.05 and *P < 0.05 (paired one-way ANOVA with Tukey’s test). APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.
Generation of CAR T cells from aptamer-based traceless cell isolates. Few differences were observed between aptamer- and antibody-isolated cells immediately after isolation and we sought to confirm whether this would stay true for a final CAR T-cell product generated using these different isolation methods. We thus generated CD8+ CAR T cells from both the antibody-isolated cells and the traceless aptamer-isolated cells (RAE fraction) shown in Fig. 4d–f and fully compared their outgrowth, phenotype, gene expression and effector function. Antibody-isolated CD4+ T cells were not included in these studies so as not to convolute any differences between the CD8+ T cells isolated from the different methods. We transduced cells with the PLAT-02 lentiviral vector, which encodes a second generation CD19scFv-41BB-CD3ζ CAR (and the truncated version of the epidermal growth factor receptor, EGFRt, as a surrogate transduction marker) that is used in ongoing clinical trials (Fig. 5a). CD19 CAR T cells were manufactured using a sequential two week stimulation bead outgrowth from days S1D0 to S1D14 and a two week rapid expansion protocol (REP) with irradiated CD19+ feeder cells from days S1R1D0 to S1R1D14, as summarized in Supplementary Fig. 12, where S1 denotes the bead stimulation, R1 indicates the REP and D# signifies the number of days since stimulation or REP onset. After transduction, high expression of the surrogate transduction marker EGFRt (>60%) was observed on S1D9 without additional selection and was further increased using immunomagnetic enrichment and over the REP period to at least 94% on S1R1D13 before functional assays were run (Fig. 5b). Importantly, although transduction copy number was probably variable, given the variation in surrogate transduction marker expression, there was no difference in the mean EGFRt MFI between antibody- and aptamer-isolated cells (Fig. 5b).

Over the two week stimulation period, we observed no differences in the outgrowth between untransduced antibody- and aptamer-isolated mock T cells (Fig. 5c). This was consistent with the similar Ki-67 expression between both mocks and CD19 CAR T cells from the different isolation methods at the end of the two week stimulation outgrowth (Fig. 5d) and unsurprisingly the cells grew identically during the REP (Supplementary Fig. 13). On S1D14, while staining for PD1, TIM3 and LAG3 coexpression (markers of both activation and exhaustion) we observed small differences in the accumulation of these markers between cells from the different isolation methods at the end of the two week stimulation outgrowth (Fig. 5e and Supplementary Fig. 14). Whereas aptamer-isolated CD19 CAR T cells that had a loss of TIM3+ PD1−TIM3− cells and a gain of PD1+TIM3+ cells compared to antibody-isolated cells, opposite trends were seen in the mock cells between the two isolation methods, suggesting that these differences are probably artefacts of transduction and the stimulation process and not the isolation strategy. Consistent with the exhaustion/activation data, aptamer-isolated CD19 CAR T cells from the same day exhibited more differentiation than the antibody-isolated cells, as indicated by the greater proportion of CD45RA−CD62L+, but the mock cells of the two isolation methods were equivalently differentiated (Fig. 5f (left) and Supplementary Fig. 15). However, after a two week REP process, the aptamer-isolated CD19 CAR T cells were less terminally differentiated than the antibody-isolated cells while the mocks remained the same, again suggesting that the isolation strategy is not the principal cause of these small differences (Fig. 5f (right) and Supplementary Fig. 15). NanoString nCounter transcript profiling of immune-associated genes further confirmed that there were minimal differences between the two isolation methods with zero genes being differentially expressed in significantly higher or lower amounts between aptamer- and antibody-isolated mock and CD19 CAR T cells at the end of the REP before functional testing (Supplementary Fig. 16). These results reaffirm that the aptamer selection strategy did not have any durable, long-term side effects on CAR T-cell fitness.

We evaluated the antitumour effector function of these cells against both myelogenous leukaemia K562 cells lines that were transduced to stably express OKT3 Fab and CD19 for CD3 and CAR engagement, respectively, as well as B lymphoma Raji cells that constitutively express moderate levels of CD19 (Supplementary Fig. 17). On tumour challenge in vitro, aptamer-isolated CD19 CAR T cells lysed all three cell types to similar extents to antibody-isolated cells and secreted identical amounts of the effector cytokines tumour necrosis factor (TNF)-α and interferon (IFN)-γ (Fig. 5g,h). Thus, CAR T cells derived from an aptamer-based traceless isolation strategy perform in vitro to the standard of cells derived from the widely used antibody-based isolation.

Aptamer-isolated CAR T-cell performance in systemic Raji tumour mouse model. In vitro cytotoxicity results with CAR T cells do not always corrobore with in vivo results50. Thus, despite observing little difference in the effector function of antibody- and aptamer-isolated CD8+ CAR T cells in vitro, it was important to further show that this would translate in vivo. To this end, we used a less stringent version of the previously described CAR T-cell stress test61, in which Raji-bearing NOD/SCID/IL-2R−y null (NSG) mice were treated with a non-curative dose of CD8+ CD19 CAR T cells from the different isolation methods at the end of the REP (S1R1D14). Mice were injected with 5×107 GFP-fluc CD19+ Raji cells and treated 7 days later with 10⁴ antibody- or aptamer-isolated S1R1D14 CD8+ CAR T cells, as previously described53. CD8+ mock T cells from both isolation methods were included as placebo controls.

Administration of 2×10⁷ CD8+ CAR T cells was previously shown to be only 50% curative long term with this model due to the absence of the CD4+ CAR T-cell subset critical for therapy persistence52. The CD8+ CAR T cells in this study were also expanded by both a two week bead stimulation and two week REP, and thus displayed markedly more exhaustion and differentiation than the one or two week expanded CAR T cells described in other publications50,53. Consequently, using only 10⁴ CD8+ CD19 CAR T cells in the same model was found to be non-curative with a maximal survival of approximately ten weeks (A. Johnson, unpublished observations). We therefore postulated that this T-cell stress study design would be able to rigorously identify any differences in antitumour effector function between antibody- and aptamer-isolated CAR T cells. Even so, in vivo, we continued to observe identical antitumour activity between both antibody- and aptamer-isolated CD8+ CAR T cells. Tumour regression and relapse kinetics, as measured using the photon flux of the tumour, overlapped between mice that received CAR T cells derived from the different isolation methods across multiple donors. (Fig. 6a). Although the therapy was non-curative for both CAR T-cell treatment groups, indicative of a successful stress test model, mice receiving aptamer-isolated CAR T cells exhibited similar prolonged survival compared to mice receiving antibody-isolated CAR T cells as determined by both biological significance (median survival time) and log-rank statistical significance (Fig. 6b). These results further illustrate that traceless aptamer-based cell isolation with strand displacement is a viable replacement for antibody-based isolation in the initial production step of CAR T-cell therapy, having negligible downstream impact on the quality of the final cell product.

Discussion

A challenge with CAR T-cell therapy is the time and cost associated with manufacturing a clinical product. As recent literature has shown that the selection of different T-cell subsets for therapy may provide improved and consistent clinical outcomes compared to undefined products starting from heterogenous PBMCs, it is increasingly imperative that efficient and cost-effective selection approaches are developed to meet the demands of these new therapeutic compositions54. At present, CD8+ and CD4+ T cells are
Fig. 5 | Characterization of CD19 CAR T cells generated from antibody- and aptamer-isolated cells. a. The second generation CD19 CAR T-cell construct with EGFRt reporter that was used to retrovirally transduce isolated T cells. b. Left, flow cytometry analysis of EGFRt expression in antibody- and aptamer-isolated T cells 9 d after initial bead stimulation (S1D9) and 13 d after the REP (S1R1D13) with irradiated CD19+ TM-LCL cells. The timeline for cell expansion is shown in Supplementary Fig. 12. The flow graphs represent one independent experiment. Right, a chart indicating EGFRt reporter MFI at S1R1D13, using the symbols from Fig. 4d–f. Horizontal lines and error bars represent the mean ± s.d.; n = 3; P > 0.05 (two-sided paired t-test). c. The growth of non-transduced mock T cells after bead stimulation. The symbols are as in Fig. 4d–f; n = 3; P > 0.05 (paired two-way ANOVA with Bonferroni correction). The curves represent a least-squares fit to the exponential growth equation. d. Flow cytometry analysis of Ki-67 expression in mock and CD19 CAR T cells on S1D14 immediately before the REP. The symbols are as in Fig. 4d–f; n = 3; P > 0.05 (paired two-way ANOVA with Bonferroni correction). e. Flow cytometry analysis of PDI/TIM3/LAG3 expression in mock and CD19 CAR T cells on S1D14 immediately before the REP. Individual donor values can be found in Supplementary Fig. 14. The pie charts show the mean phenotype of the cells; n = 3 biologically independent samples; P > 0.05 and **P < 0.01 (paired two-way ANOVA with Bonferroni correction). f. Flow cytometry analysis of CD62L/CD45RA expression in mock and CD19 CAR T cells on S1D14 immediately before the REP and on S1R1D14 immediately after the functional assays. Individual donor values can be found in Supplementary Fig. 15. The pie charts show the mean phenotype of the cells; n = 3 biologically independent samples; P > 0.05 and **P < 0.01 (paired two-way ANOVA with Bonferroni correction). g. In vitro antitumour cytotoxicity and cytokine release of mock and CD19 CAR T cells. For h, the symbols are as in Fig. 4d–f. Data points and error bars (g) and graph bars and error bars (h) represent the mean ± s.d.; n = 3 biologically independent samples; P > 0.05 (paired two-way ANOVA with Bonferroni correction (g); paired two-way ANOVA with Sidak correction (h)). SA–PE, streptavidin–phycoerythrin; PE-Cy7, phycoerythrin: cyanine-7 tandem conjugate.
which adds supply-chain risk to a cell therapy manufacturing process. Perishable and frequently have a sole supplier of consumables, and similar avidity-reliant Fab multimerization strategies have partially mitigated these issues by enabling label-free isolation; however, these approaches still rely on expensive, biologically produced selection agents and require two or more apparatus in sequence to isolate pure, separate cell subsets. These technologies are also perishable and frequently have a sole supplier of consumables, which adds supply-chain risk to a cell therapy manufacturing process using immunomagnetic positive selections. Both approaches are far from perfect: the former wastes a portion of the cells in the starting population by splitting the cells, but saves time with parallel isolations; the latter is time consuming as it uses the whole population in each step. Furthermore, labels remaining on the cells prevent further selection and present regulatory barriers for clinical translation into patients. Two recent reports have used DNA-labelled antibodies followed by strand displacement to isolate multiple cell populations; however, antibodies and DNA tags remain attached to selected cells. The advent of Streptamer technology and similar avidity-reliant Fab multimerization strategies have partially mitigated these issues by enabling label-free isolation; however, these approaches still rely on expensive, biologically produced selection agents and require two or more apparatus in sequence to isolate pure, separate cell subsets. These technologies are also perishable and frequently have a sole supplier of consumables, which adds supply-chain risk to a cell therapy manufacturing process that is often time-sensitive for patients with refractory disease.

DNA aptamers and complementary strand displacement with a reversal agent present a unique opportunity to improve the selection of T cells for use in adoptive cell therapy. Aptamers are synthetic and thereby inexpensive to produce at large scales. Furthermore, their manufacture can be outsourced to one of many available companies and they have a long-shelf life, so they bear minimal supply-chain risk. High-affinity DNA aptamers can be developed against multiple targets and their sequences can be further modified after SELEX to include unique toeholds and stems for aptamer-specific strand displacement. Thus, panels of aptamers against diverse T-cell antigens can be developed with corresponding unique reversal agents for sequential label-free isolation of different cell subsets off the same column. As an example, microbeads loaded with CD4 and CD8 aptamers that have unique toeholds and sequences could be added at the same time to one whole leukapheresis product, and CD4+ and CD8+ T cells could be serially eluted off one column by sequential incubations with the corresponding reversal agents. A similar outcome can be achieved using a CD3 aptamer in combination with either a CD4 or CD8 aptamer. Alternatively, one could deplete CD8+ T cells and other unwanted cell subsets using a CD8-specific aptamer and other aptamers that bind monocytes, B cells and NK cells, respectively. Untouched CD4+ T cells could then be enriched in the flow-through fraction and CD8+ T cells could be selectively eluted from the column using a reversal agent specific to the CD8 aptamer. The key to executing these strategies is the identification of highly cell-specific aptamers.

In this study, we report the discovery of high-affinity CD8a-specific aptamers and the successful application of one aptamer with a reversal agent in a traceless CD8+ T-cell isolation system. Using the aptamer alone, we report an equivalent CD8+ T-cell selection yield compared to a widely used antibody-based approach. With a reversal agent for label-free elution, we observed a >70% selection yield and >95% purity of CD8+ T cells. Given that a 350–450 ml apheresis product from a person with acute lymphoblastic leukaemia will have 3–9 × 10^9 T cells, and only 200–600 × 10^9 CD8+ T cells are needed to manufacture a 1:1 CD4+:CD8+ CAR T-cell therapy, the trade of lower yield for high purity, lower cost and label-free selection with this approach has little consequence but many benefits. Importantly, on the basis of anecdotal observations of the maximal capacity of this system at a small scale, we estimate that only US$5–10 of aptamer would be needed for reliable clinical-scale
isolated. CD19-directed CAR T cells manufactured from label-free, aptamer-isolated cells also exhibited identical performance to CAR T cells generated from antibody-selected cells in assays designed to measure antitumour effector function, showing that aptamer-based traceless cell isolation is a practical selection strategy for CAR T-cell therapy.

Although the competitive SELEX approach was designed to identify multiple T-cell-specific aptamers, only CD8-specific aptamers were discovered with this strategy. We speculate that this occurred due to the strong partitioning of the library towards the high-affinity CD8 aptamers. SELEX strategies using unmodified CD4+ primary T cells or the CD8+ Jurkat T-cell line may thus be required to identify aptamers that bind to alternative T-cell antigens like CD3 or CD4. Furthermore, due to the limited binding chemistry available to unmodified DNA aptamers, certain proteins may not be amenable to high-affinity aptamer discovery using unmodified DNA libraries. For these targets, increased chemical diversity including one or two modified base pairs in the library design may be needed for the successful partitioning of high-affinity binders.

In the future, aptamers could be readily functionalized for attachment to solid supports for affinity chromatography separation, thus resulting in a purely synthetic isolation system without the need for recombinant proteins, magnetic supports and pre-incubation with selection agents. Cell-release efficiency could be potentially improved, for example by refining the strand displacement kinetics between aptamer and reversal agent through further sequence optimization (higher toehold GC content, aptamer truncation) and chemical modification of the aptamer and reversal agent (locked nucleic acids). Further discovery of aptamers against the T-cell markers CD3 and CD4 will also be required to realize a serial selection engineering strategies to prepare engineered CD4+ and CD8+ T cells through continuous flow methods, thus increasing the accessibility of T-cell immunotherapy.

Methods

Oligonucleotides. All oligonucleotides studied were synthesized by Integrated DNA Technologies. The ssDNA library used in the T-cell–SELEX process was purified using high-performance liquid chromatography and consisted of a 52bp random sequence flanked by two 18bp constant regions. The primers used for library amplification between SELEX rounds, with IDT modification codes, are as follows: forward 5′-/56-FAM/ATCCAGAGTGACGCAGCA-3′ and reverse 5′-5BiosG/ACTAAGCCACCGTGTCCA-3′. The individually synthesized ssDNA aptamers are listed in Supplementary Table 5.

Antibodies and flow cytometry. The following dyes, antibodies and secondarys were used to stain cells: Zombie Violet (1:500 in 100μl 10× cells, BioLegend), Zombie Yellow (1:500 in 100μl 10× cells, BioLegend), APC antihuman CD4 (1:100, 300514, BioLegend), PerC/Cy5.5 antihuman CD8a (1:100, 301031, BioLegend), APC antihuman CD8a (rhesus cross-reactivity, 1:100, 301014, BioLegend), CD8β–biotin (1:100, 130-098-556, Millenyi), antimouse CD16/CD32 Fc block (1:100, 14-0161-86, eBioscience), FITC antimouse CD3ε (1:100, 100305, BioLegend), BV421 antimouse CD8a (1:50, 100737, BioLegend), FITC mouse antimouse CD3ε (rhesus cross-reactivity, 1:20, 55611, BD Biosciences), purified antihuman CD3Δ (Clone UCHT1, 300402, BioLegend), purified antihuman CD8a (Clone RPA-T8, 301002, BioLegend), Super Bright 600 antihuman CD19 (1:20, 63-0191-42, BioLegend), Super Bright 702 antihuman CD56 (1:100, 67-0566-42, eBioscience), PE antihuman CD3 (1:100, 300308, BioLegend), APC/ Cy7 antihuman CD14 (1:40, 325819, BioLegend), FITC antihuman CD16 (1:150, 302006, BioLegend), Alexa Fluor 700 antihuman CD3 (1:50, 300424, BioLegend), Brilliant Violet 785 antihuman CD4 (1:30, 317442, BioLegend), PE/Cy7 antihuman CD8a (1:200, 300914, BioLegend), BVU737 mouse antihuman CD45RA (1:25, 564412, BD Biosciences), BVU393 mouse antihuman CD45RO (1:25, 564291, BD Biosciences), PE antihuman CD26 (1:400, 304806, BioLegend), Brilliant Violet 421 antihuman CCR7 (1:25, 355208, BioLegend), Eritux–biotin (1:50, Jensen Lab), PE/Cy7 mouse anti-Ki-67 (1:60, 561283, BD Biosciences), BVU737 mouse antihuman PDI (1:20, 565299, BD Biosciences), Brilliant Violet 785 antihuman TIM3 (1:20, 345032, BioLegend), PE mouse antihuman LAG3 (1:20, 565616, BD Biosciences), Brilliant Violet 785 antihuman CD45RA (1:160, 304140, BioLegend), NeutrAvidin Protein DyLight 633 (1:500, 22844, Invitrogen), Alexa Fluor 647 streptavidin (1:500, 405237, BioLegend) and PE streptavidin (1:500, 405204, BioLegend). OneComp eBeads (Invitrogen) were used to prepare single-colour compensation fluorophores for compensation, if needed. Stained samples were analysed with a MACSQuant Analyzer 10 (Miltenyi), Attune NxT (Invitrogen) or BD LSRFortessa (BD Biosciences) flow cytometer.

Cell line culture and PBMC isolation. The J.RT3-T3.5 and Jurkat (clone E6-1) cell lines were used for counter selection and nucleofection, respectively, were purchased from ATCC. The Epstein–Barr virus-transformed lymphoblastoid cell line (TM-LCL) used in the REP of T cells was made from mononuclear cells as previously described. The CD19+ and OKT3+ K562 cells used for functional assays were generated by lentivirally transducing parental K562 parental cells (ATCC) with CD19+ or OKT3-expressing constructs. Raji parental cells were also purchased from ATCC. All the above cell lines were cultured in RPMI 1640 medium ( Gibco) with 10% heat-inactivated FBS (Life Tech and VWR). Human PBMCs were isolated from Leukocyte Reduction System cones (Bloodworks Northwest) using Ficoll-Paque density gradient centrifugation (GE). The mixed or CD8+ T cells used in non-isolation experiments were a gift from Juno Therapeutics. Rhesus PBMCs were a gift from A. Taraseviciute (Seattle Children’s Research Institute).

Competitive cell–SELEX with T-cell depletion. The SELEX protocol was adapted from a reported method. A schematic of the SELEX procedure is shown in Fig. 1 and the conditions used in the individual rounds are summarized in Supplementary Table 2. In brief, selection rounds were carried out in 96-well plates, in which 4 × 10^5 thawed mixed T cells, depleted of dead cells (Millenyi), were incubated with 40 nmol of ssDNA library (~10^6 individual sequences) for 1 h at 4°C in binding buffer. Bound aptamers were extracted and amplified by PCR using Phusion High Fidelity DNA Polymerase (NEB) with forward and reverse primers. Strand separation was performed with High Capacity Neutravidin Agaroose Resin (Thermo Scientific), as described previously, and the FAM-labeled ssDNA aptamer pool was used in the next round. For rounds 2–5, the ssDNA aptamer pools were incubated with thawed PBMCs depleted of dead cells, a process termed competitive selection. After three washes, T cells and bound ssDNA sequences were then enriched using a Pan T Cell Isolation Kit (Millenyi). The ssDNA pool was then extracted and incubated with 10^6 CD3+ CD8+ J.RT3-T3.5 T cells at 4°C as a form of negative selection in each round, and unbound ssDNA sequences were PCR amplified and used to generate ssDNA aptamer pools for use in the sequential round. The wash and binding buffer formulations, as well as the growth conditions, are as described previously.

Aptamer binding assays. Cells (2 × 10^6) were incubated with 100 μl of folded FAM-labelled ssDNA pools or FAM/biotin-labelled individual aptamers for 20–30 min at 4°C in binding buffer at the indicated concentrations. For antibody competition and multicolour flow cytometry staining with antibodies, antibodies were added to the primary incubation with aptamer. Cells were washed twice (or three times for large flow panels) in 200 μl of wash buffer supplemented with 1% BSA to remove excess aptamer. If the aptamers or antibodies used were biotinylated, the cells underwent a second incubation with 100 μl of fluorescently labelled streptavidin or neutravidin secondary for 15–20 min at 4°C in wash buffer with 1% BSA and were washed twice. Stained cells were fixed in 200 μl of wash buffer with 1% BSA and 0.1% PFA before analysis via flow cytometry.

NGS and data analysis. The starting RN and ssDNA pools from each SELEX round were PCR amplified with the barcoded primers listed in Supplementary Table 3 for sequencing using the MiSeq Reagent Kit v2 (300 cycles) and MiSeq System (Illumina) according to the manufacturer’s instructions. Exported FASTA files were analysed with FASTAptamer v1.0.3. Specifically, FASTAptamer-Count was first used to determine rank and reads per million for each sequence, whereas FASTAptamer-Compare was used to conduct pairwise comparison of reads per million for sequences between adjacent rounds and thus calculated fold enrichment (Supplementary Table 4). Neighbour joining trees were constructed for the top 100 sequences from rounds 2–4 and were further analysed by both FigTree v1.4.3 (tree.bio.ed.ac.uk/software/figtree/) for phylogenetic tree generation and MEME Suite v4.12.0 for motif prediction. The NUPACK web application was used to generate predicted secondary structures of aptamer sequences.

Murine spleenocyte isolation and staining. For mouse spleen harvesting, animal work was conducted under protocol no. 4053-01 approved by the Institutional Animal Care and Use Committee at the University of Washington, 20-week-old Tg(Aldehy11-1EGFP–DTAD8RtII) male mice (Jackson Laboratory) were euthanized with avertin and perfused with 20 ml PBS to limit coagulation. Spleens were harvested, minced with scissors and dissociated by sieving over a 40-μm cell strainer (Falcon). Red blood cells were removed by incubation with ACK Lysing Buffer (Gibco) and cells were stained with both anti-mCherry and anti-mCD8 antibodies and aptamers.

sRNA knockdown. Some 10^6 thawed CD8+ T cells were activated with Dynabeads Human T-activator CD3/CD28 (Life Tech) at 1.5 × 10^6 cells ml^-1 for 3 d in complete
Articles Nature Biomedical Engineering

ethanol for NanoString nCounter analysis. The remaining cells were banked for in vitro expression and anti-CD8 antibody and aptamer binding via flow cytometry.

Plasmid transfection. CD8α-hnRNPA-M-EGFP was a gift from L. Lu (Addgene plasmid no. 86054). 2 × 10^6 CD8α Jurkat cells were nucleofected (Lonza) with 2 μg of the plasmid using the Nucleofector KIT V (Lonza) with Program T-023 according to the manufacturer’s instructions. Aptamer and anti-CD8 antibody staining, as discussed in the previous section, were performed 24 h later and analysed via flow cytometry.

Comparison to previously reported aptamer. CD8A1p7s, as described by Wang et al.1, was synthesized with the sequence 5’-T-CATACGTCCTGCTGGCTCTTTTGCTGAA-3’. Binding to CD8+ T cells was compared to our aptamer A3t (Supplementary Table 5), and CD8A1p7s binding buffer (A BB) and folding conditions were used in addition to our binding buffer (T-BB) and folding conditions. For the CD8A1p7s conditions, 1 μM of each aptamer was folded by denaturation at 95° C for 5 min and cooled to 37°C in its wash buffer (40 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.5). Binding was similarly carried out and analysed as detailed above but in CD8A1p7s binding buffer, which comprised the wash buffer supplemented with 5% FBS.

Reversal agent optimization. A 36 bp reversal agent was designed complementary to the 3’ end of aptamer A3t (Supplementary Table 5). Binding to CD8+ T cells with 5 nM aptamer A3t was first carried out with secondary fluorescent streptavidin labelling, as discussed above. Labelled cells were then incubated with varying fold excesses (over the amount of aptamer used) of 200 μl of reversal agent in wash buffer with 1% BSA for different times and temperatures. Cells were washed twice with the wash buffer with 1% BSA to remove eluted aptamers, fixed and analysed via flow cytometry.

Traceless selection of CD8+ T cells from PBMCs. For each PBMC donor, 200 μl of Anti-Biotin Microbeads (Miltenyi) were diluted to 1,000 μl in binding buffer with 5 nM aptamer A3t and incubated for 15 min at 4°C under gentle rotation. The aptamer-labelled bead suspensions were then added to 2 × 10^6 FicolI-isolated PBMCs and allowed to incubate for another 15 min at 4°C under gentle rotation. Cells were subsequently washed with 10 ml of autoMACS Rinsing Solution (Miltenyi) supplemented with 0.5% BSA, resuspended in the same buffer and applied over two LS columns in parallel on a QuadroMACS separator (Miltenyi) according to the manufacturer’s instructions. A flow-through fraction, which includes the flow through from the initial application of cells and the three subsequent 3 ml column washes, was collected. Afterwards, 1 ml of 500 mM reversal agent (100-fold excess) in autoMACS solution with 0.5% BSA and 5 mM MgCl₂ was applied to the column on the magnet containing cells labelled with aptamer-functionalized microbeads. Approximately 600 μl of the reversal agent solution passed through the column before it was plugged with a M/F Luer Lock Plug (Smiths Medical) for a 10 min incubation at room temperature. On removal of the plug, the column was washed three times with 3 ml of autoMACS solution with 0.5% BSA and 5 mM EDTA, which constituted the RAe fraction. The RAe cells were immediately spun down and resuspended in fresh buffer to remove any reversal agent. Remaining cells on the column were removed with a column flush according to the manufacturer’s instructions. In parallel, CD8+ cells from the same donor were isolated from 200 × 10^6 PBMCs in the column flush fraction with antibody-based CD8 Microbeads (Miltenyi), according to the manufacturer’s instructions. All fractions were counted and analysed via flow cytometry with two antibody panels: a yield panel staining for CD3, CD8 and CD16 expression. Furthermore, 10^6 cell pellets from both the antibody-isolated column flush fraction and aptamer-isolated RAe fraction were flash frozen on dry ice and ethanol for NanoString nCounter analysis. The remaining cells were banked for downstream CAR T-cell production.

CD19 CAR T-cell manufacturing. CD8+ T cells from both isolation methods from each donor were thawed and 3.5 × 10^6 cells for both mock and CD19 CAR T-cell groups were stimulated 1:1 with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) in 4 ml complete RPMI with 20 μM 1H-2 (Miltenyi) and 0.5 mM 1H-15 (Miltenyi) in a 12-well plate. After 2 d (S1D2), cells designated for CAR T-cell production were transduced with clinical-grade PLAT-02 CD19 CAR lentivector (a gift from the City of Hope) at a multiplicity of infection of 0.3 with 40 μg ml⁻¹ proteamine sulphate via spinoculation for 30 min at 800 g at 37°C. Thereafter, mock changes were conducted every 2–3 d to replenish cytokines and cells were moved to larger culture vessels when cell concentrations reached 1.5–2 × 10^6 cells ml⁻¹. The activator beads were removed 9 d posttransduction, termed S1D9, and cells were stained for EGFR surrogate marker expression to assess transduction efficiency. CAR+ cells were magnetically enriched 12 d posttransduction (S1D12) using biotinylated Eritux antibody and Anti-Biotin Microbeads according to the manufacturer’s instructions. On day 14 posttransduction (S1D14), cells were analysed via flow cytometry by staining for: activation/proliferation with Ki-67; exhaustion/activation with PD1, TIM3 and LAG3; and differentiation with CD62L and CD45RA.

The two-week-stimulated T cells were further expanded using a two week REP, as previously described. Briefly, 1.5 × 10^6 CD9 CAR T cells were co-incubated with 10.5 × 10^6 irradiated CD19+ TM-LCL feeder cells in 25 ml of complete RPMI supplemented with the aforementioned cytokine concentrations in T-25 flasks (Corning). Similarly, 1.5 × 10^6 mock T cells were co-incubated with 10.5 × 10^6 irradiated CD19+ TM-LCL feeder cells and 5 × 10^6 irradiated donor-mismatched PBMCs. The cells were analysed 24 h later. Supernatants were collected and analyzed via 3-plex Bio-Plex custom kit with flat magnetic plates (Bio-Rad) according to the manufacturer’s instructions, which selects the housekeeping genes that minimize the pairwise variation statistic. Each group has six samples: three biological replicates for antibody-based isolation and three biological replicates for aptamer-based isolation. Using Excel (Microsoft), mRNA probes that gave normalized counts of less than 25 for more than 50% of the samples in a group (four or more samples) were removed from the analysis as they were mostly below the background. The unadjusted values of the log fold changes in the probe counts of aptamer-isolated cells over antibody-isolated cells were determined using a paired two-tailed t-test in Excel and the threshold for significance was calculated using the Benjamin–Yekutieli multiple-testing correction in R software v3.3.2 (http://www.R-project.org/).
T-cell stress test mouse model. All animal work described in this Article complied with local animal ethical and welfare standards. The T-cell stress test mouse model was conducted under protocol no. 13853 approved by the IACUC at the Seattle Children’s Research Institute. 9- to 11-week-old NSG female mice (Jackson Laboratory) were inoculated with 5 × 10⁵ GFP-fluor Raji cells in 200 µl of PBS by tail vein injection, followed by 10⁵ SIR1D14 antibody- or aptamer-isolated CD8+ mock or CD91 CAR T cells 7 d later. The same three donors from the in vitro studies were tested and for each of the four T-cell populations evaluated, three mice were used per donor per recombinant protein per condition. Protein Expr. 10, 975–982 (1997).

14. Knobel, M. et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nat. Med. 8, 631–637 (2002).

15. Schmitt, A. et al. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. Transfusion 51, 591–599 (2011).

16. Stemberger, C. et al. Novel serial positive enrichment technology enables clinical multiparameter cell sorting. PLoS ONE 7, e35798 (2012).

17. Sabatino, M. et al. Generation of clinical-grade CD19-specific CAR-modified CD8+ memory stem cells for the treatment of human B-cell malignancies. Blood 128, 519–528 (2016).

18. Ellington, A. D. & Szostak, J. W. In vitro selection of RNA molecules that bind specific ligands. Nature 346, 818–822 (1990).

19. Tuerk, C. & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510 (1990).

20. Robertson, D. L. & Joyce, G. F. Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA. Nature 344, 467–468 (1990).

21. Bunka, D. H. & Stockley, P. G. Aptamers come of age - at last. Nat. Rev. Microbiol. 4, 588–596 (2006).

22. Hernandez, L. I., Machado, I., Schafer, T. & Hernandez, F. J. Aptamers overview: selection, features and applications. Curr. Top. Med. Chem. 15, 1066–1081 (2015).

23. Zhou, J. & Rossi, J. Aptamers as targeted therapeutics: current potential and challenges. Nat. Rev. Drug Discov. 16, 181–202 (2017).

24. Dunn, M. R., Jimenez, R. M. & Chaput, J. C. Analysis of aptamer discovery and technology. Nat. Rev. Drug Discov. 1, 1076 (2017).

25. Daniels, D. A., Chen, H., Hicke, J. L., Swiderek, K. M. & Gold, L. A tenacious aptamer identified by tumor cell SELEX: systematic evolution of ligands by exponential enrichment. Proc. Natl Acad. Sci. USA 100, 15416–15421 (2003).

26. Shangguan, D. et al. Aptamers evolved from live cells as effective molecular probes for cancer study. Proc. Natl Acad. Sci. USA 103, 11838–11843 (2006).

27. Ogasawara, D., Hasegawa, H., Kaneko, K., Sode, K. & Ikebukuro, K. Screening of DNA aptamer against mouse prion protein by competitive selection. Proc. Natl Acad. Sci. USA 1, 248–254 (2007).

28. Sefah, K., Shangguan, D., Xiong, X., O’Dohogne, M. B. & Tan, W. Development of DNA aptamers using Cell-SELEX. Nat. Protoc. 5, 1169–1185 (2010).

29. Alam, K. K., Chang, J. L. & Burke, D. H. FASTAptamer: a bioinformatic toolkit for high-throughput sequence analysis of combinatorial selections. Mol. Ther. Nucleic Acids 4, e230 (2015).

30. Caroli, J., Taccioli, C., De La Fuente, A., Serafini, P. & Bicciato, S. APTANI: a computational tool to select aptamers through sequence-structure motif analysis of HT-SELEX data. Bioinformatics 32, 161–164 (2015).

31. Bailey, T. L. et al. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208 (2009).

32. Chen, L. et al. Aptamer-mediated efficient capture and release of T lymphocytes on nanostructured surfaces. Adv. Mater. 23, 4376–4380 (2011).

33. Li, S., Chen, N., Zhang, Z. & Wang, Y. Endonuclease-responsive aptamer-functionalized hydrogel coating for sequential catch and release of cancer cells. Biomaterials 34, 460–469 (2013).

34. Xu, Y. et al. Aptamer-based microfluidic device for enrichment, sorting, and detection of multiple cancer cells. Anal. Chem. 81, 7436–7442 (2009).

35. Yoon, J. W. et al. Isolation of foreign material-free endothelial progenitor cells using CD31 aptamer and therapeutic application for ischemic injury. PLoS ONE 10, e0131785 (2015).

36. Zhu, J., Nguyen, T., Pei, R., Stojanovic, M. & Lin, Q. Specific capture and temperature-mediated release of cells in an aptamer-based microfluidic device. Lab Chip 12, 3504–3513 (2012).

37. Lao, M. et al. Aptamer and antisense-mediated two-dimensional isolation of specific cancer cell subpopulations. J. Am. Chem. Soc. 138, 2476–2479 (2016).

38. Sun, N. et al. Chitosan nanofibers for specific capture and nondestructive release of CTGs assisted by pCRMA brushes. Small 12, 5909–5907 (2016).

39. Wan, Y. et al. Capture, isolation and release of cancer cells with aptamer-functionalized glass bead array. Lab Chip 12, 4693–4701 (2012).

40. Zhang, Z., Chen, N., Li, R. & Wang, Y. Functionalizable hydrogels for controlled cell catch and release using hybridized aptamers and complementary sequences. J. Am. Chem. Soc. 134, 15716–15719 (2012).
41. Nozari, A. & Berezovsky, M. V. Aptomers for CD antigens: from cell profiling to activity modulation. Mol. Ther. Nucleic Acids 6, 29–44 (2017).
42. Wang, C.-W. et al. A novel nucleic acid–based agent inhibits cytotoxic T lymphocyte–mediated immune disorders. J. Allergy Clin. Immunol. 132, 713–722 (2013).
43. Seelig, G., Soloveichik, D., Zhang, D. Y. & Winfree, E. Enzyme-free nucleic acid logic circuits. Science 314, 1585–1588 (2006).
44. Yurke, B. & Mills, A. P. Using DNA to power nanostructures. Genet. Program. Evol. Mach. 4, 111–122 (2003).
45. Yurke, B., Turberfield, A. J., Mills, A. P. Jr., Simmel, F. C. & Neumann, J. L. A DNA-fuelled molecular machine made of DNA. Nature 406, 605–608 (2000).
46. Zhang, D. Y. & Seelig, G. Dynamic DNA nanotechnology using strand-displacement reactions. Nat. Chem. 3, 103–113 (2011).
47. Zhang, D. Y. & Winfree, E. Control of DNA strand displacement kinetics using toehold exchange. J. Am. Chem. Soc. 131, 17303–17314 (2009).
48. Ruella, M. et al. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat. Med. 24, 1499–1503 (2018).
49. Hehezy, A. et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. Blood 124, 2824–2833 (2014).
50. Eyquem, J. et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 543, 113–117 (2017).
51. Zhao, Z. et al. Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. Cancer Cell 28, 415–428 (2015).
52. Brentjens, R. J. et al. Eradication of systemic B-cell tumors by genetically modified T cells expressing chimeric antigen receptors. Nat. Med. 9, 279–286 (2003).
53. Dahotre, S. N., Chang, Y. M., Wieland, A., Stammem, S. R. & Kwong, G. A. Individually addressable and dynamic DNA gates for multiplexed cell sorting. Proc. Natl Acad. Sci. USA 115, 4357–4362 (2018).
54. Probst, C. E., Zrazhevskiy, P. & Gao, X. Rapid multistage immunomagnetic separation through programmable DNA linker displacement. J. Am. Chem. Soc. 133, 17126–17129 (2011).
55. Gawande, B. N. et al. Selection of DNA aptamers with two modified bases. Proc. Natl Acad. Sci. USA 114, 2898–2903 (2017).
56. Ni, S. et al. Chemical modifications of nucleic acid aptamers for therapeutic purposes. Int. J. Mol. Sci. 18, 1683 (2017).
57. Pelloquin, F., Lamelin, J. & Lenoir, G. Human blymphocytes immortalization by Epstein-Barr virus in the presence of cyclosporin a. In Vitro Cell. Dev. Biol. 22, 689–694 (1986).
58. Zadeh, J. N. et al. NUPACK: analysis and design of nucleic acid systems. J. Comput. Chem. 32, 170–173 (2011).
59. Tsai, H. H. et al. Regional astrocyte allocation regulates CNS synaptogenesis and repair. Science 337, 358–362 (2012).
60. Madugula, V. & Lu, L. A ternary complex comprising transportin1, Rab8 and the ciliary targeting signal directs proteins to ciliary membranes. J. Cell Sci. 129, 3922–3934 (2016).
61. Wang, J. et al. Optimizing adoptive polyclonal T cell immunotherapy of lymphomas, using a chimeric T cell receptor possessing CD28 and CD137 costimulatory domains. Hum. Gene Ther. 18, 712–725 (2007).

Acknowledgements
This work was supported by a sponsored research agreement from Juno Therapeutics. We are grateful to C. Ramsberg (Juno Therapeutics), A. Bianchi (Juno Therapeutics), J. Shi (Juno Therapeutics), C. Chan (Juno Therapeutics), B. Olden (University of Washington) and J. Gustafon (Seattle Children's Research Institute) for their critical discussion and helpful advice and to A. Mills (Juno Therapeutics) for manuscript feedback. We are also grateful to all Pull and Jensen Lab members, especially J. Yokoyama (Seattle Children's Research Institute) and A. Johnson (Seattle Children's Research Institute), for experimental support and helpful advice. We also thank the Baker Lab, especially B. Langan, for assistance with Octet BLI studies. We thank C. Sazby (University of Washington) and R. Mukherjee (Seattle Children’s Research Institute) for their valuable input regarding the statistical analysis. We thank H. Y. Lin for preparing the SELEX and cell isolation figures. I. Cardile was supported partly by the National Cancer Institute of the National Institutes of Health under award no. ST2CA080416-19 for research reported in this publication.

Author contributions
S.H.P. and M.C.J. conceived the idea and provided experimental advice and funding support. N.K., I.I.C. and S.H.P. designed the project. N.K. and I.I.C. conceived, performed and interpreted the experiments. N.K. designed and performed the SELEX procedure. I.I.C. and E.L.C. evaluated the binding of aptamer libraries and select aptamers and I.I.C., S.H.P., S.J.S. and N.K. analysed the NGS data. I.I.C. performed murine spleenocyte and thymus binding experiments. N.K., I.I.C. and E.L.C. conducted receptor binding studies using siRNA knockdown and gene transfection. I.I.C. and N.K. conducted antibody competition and Octet studies. I.J.Y. and E.L.C. performed binding curve studies and E.L.C. and I.I.C. evaluated aptamer binding to human PBMCs. N.K., I.I.C. and I.J.Y. optimized tracer agent and traceless cell isolation conditions. I.I.C. performed CAR T-cell production and characterization studies. M.I.B. conducted in vivo tumour studies and bioluminescence imaging. I.I.C. prepared the figures and performed statistical analyses. I.I.C., N.K., E.L.C. and S.H.P. wrote the manuscript.

Competing interests
S.H.P., M.C.J., N.K. and I.I.C. are co-inventors on two US provisional patent applications (nos. 62/699,438 and 62/779,946) for the aptamers and complementary reverse agents for traceless isolation described in this manuscript.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41551-019-0411-6.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to M.C.J. or S.H.P.
Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2019
Corresponding author(s): Suzie Pun

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- Confirmed
- The **exact sample size** (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - *Give P values as exact values whenever suitable.*
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
  - *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on **statistics for biologists** may be useful.

Software and code

**Policy information about availability of computer code**

**Data collection**

MACSQuantify Software, BD FACSDiva V8.0.1, Attune NxT Software v2.6, MiSeq Control Software v2.6.2.1, FortéBio Octet Data Acquisition Software, Bio-Plex Manager 6.1, Packard TopCount NXT Software, Living Image 4.5.

**Data analysis**

FlowJo V10, GraphPad Prism 7, Strawberry Perl 5.28.0.1, FASTAptamer v1.0.3, Ninja v1.2.1, FigTree v1.4.3, MEME Suite web application, NUPACK web application, FortéBio Octet Data Analysis 9.0 Software, Microsoft Excel 2016, NanoString nSolver 4.0, NanoString Advanced Analysis 2.0, R 3.3.2.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research **guidelines for submitting code & software** for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the main findings of this study are available within the paper and its Supplementary Information. All data generated for this study and relevant information are available from the corresponding authors upon reasonable request. The NanoString nCounter data have been deposited in the NCBI Gene Expression Omnibus (GEO), with accession code GSE130185.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Cells were derived from three donors for all T-cell studies, which is standard for the field given the cost and time associated with manufacturing CAR-T cells. No sample-size calculation was performed for animal studies. However, prior ‘CAR stress tests’ from the literature have used 5–14 mice per group (Zhao & Condomines et al., Cancer Cell, 2015), so 9 mice per treatment group (3 mice per donor) seemed reasonable. Furthermore, it was imperative to use the same number of mice per T-cell donor to prevent donor bias. Thus, the number of mice used per treatment group was restricted by the amount of CAR-T cells available in the donor with the lowest cell number after cell expansion. |
| Data exclusions | PBMC donors with low CD8 percentages were excluded from cell-isolation data analysis due to yielding insufficient cells required for flow-cytometry analysis, NanoString analysis, CAR-T cell production, and subsequent in vitro and in vivo effector function assays. A pre-exclusion criteria of yielding at least 15M CD8+ T cells from 200M PBMCs (both for antibody and traceless aptamer selection) was pre-established prior to carrying out the cell-isolation studies. |
| Replication | Attempts at replication were highly successful. Three independent experiments, or three biologically independent samples when appropriate, were performed for nearly all data presented in the main figures. Furthermore, three independent researchers performed the siRNA knockdown and flow-cytometry binding-curve experiments months apart with highly reproducible and consistent results. |
| Randomization | On day 6 post-tumour inoculation, mice were arranged into groups of 3 mice each for each donor and treatment group such that the average photon flux of the pre-established systemic tumours was approximately equal across all groups. |
| Blinding | Investigators were not blinded to group allocation during the assessment of tumour-imaging and tumour-survival endpoints; however, the efficacy of treatments and survival outcomes were readily apparent in tumour-flux quantification and in the behavior of mice. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | n/a | Involved in the study |
|----------------------------------|-----|-----------------------|
| ☒ Unique biological materials   | ☒ Unique biological materials |
| ☒ Antibodies                    | ☒ Antibodies |
| ☒ Eukaryotic cell lines         | ☒ Eukaryotic cell lines |
| ☒ Palaeontology                 | ☒ Palaeontology |
| ☒ Animals and other organisms   | ☒ Animals and other organisms |
| ☒ Human research participants   | ☒ Human research participants |

| Methods                         | n/a | Involved in the study |
|---------------------------------|-----|-----------------------|
| ☒ ChIP-seq                      | ☒ ChIP-seq |
| ☒ Flow cytometry                | ☒ Flow cytometry |
| ☒ MRI-based neuroimaging        | ☒ MRI-based neuroimaging |
Antibodies

Antibodies used

The following dyes, antibodies, and secondaries were used to stain cells: Zombie Violet (1:500 in 100 μL/10^6 cells, BioLegend), Zombie Yellow (1:500 in 100 μL/10^6 cells, BioLegend), APC anti-human CD4 (1:100, 300514, BioLegend), PerCP/Cy5.5 anti-human CD8α (1:100, 301031, BioLegend), APC anti-human CD8α (rhesus cross-reactivity, 1:100, 301014, BioLegend), CDB-biotin (1:100, 130-098-556, Milteny), anti-mouse CD16/CD32 Fc block (1:100, 14-0161-86, eBioscience), FITC anti-mouse CD3ε (1:50, 100305, BioLegend), BV421 anti-mouse CD8α (1:50, 100737, BioLegend), FITC mouse anti-human CD3ε (rhesus cross-reactivity, 1:20, 556611, BD Biosciences), Purified anti-human CD3 (Clone UCHT1, 304002, BioLegend), Purified anti-human CD8α (Clone RPA-T8, 301002, BioLegend), Super Bright 600 anti-human CD19 (1:20, 63-0198-42, eBioscience), Super Bright 702 anti-human CD56 (1:100, 67-0566-42, eBioscience), PE anti-human CD3 (1:100, 300308, BioLegend), APC/Cy7 anti-human CD4 (1:40, 325619, BioLegend), FITC anti-human CD16 (1:50, 302006, BioLegend), Alexa Fluor 700 anti-human CD3 (1:50, 300424, BioLegend), Brilliant Violet 785 anti-human CD4 (1:50, 317442, BioLegend), PE/Cy7 anti-human CD8α (1:200, 300914, BioLegend), BUV737 mouse anti-human CD45RA (1:25, 564442, BD Biosciences), BUV395 mouse anti-human CD55RO (1:25, 564291, BD Biosciences), PE anti-human CD62L (1:400, 304806, BioLegend), Brilliant Violet 421 anti-human CCR7 (1:25, 353208, BioLegend), Erbitux-biotin (1:500, Jensen Lab), PE-Cy7 mouse anti-Ki-67 (1:20, 561283, BD Biosciences), BUV37 mouse anti-human PD-1 (1:20, 565299, BD Biosciences), Brilliant Violet 785 anti-human TIM-3 (1:20, 345032, BioLegend), PE mouse anti-human LAG-3 (1:20, 565616, BD Biosciences), Brilliant Violet 785 anti-human CD45RA (1:160, 304140, BioLegend), NeutrAvidin Protein DyLight 633 (1:500, 22844, Invitrogen), Alexa Fluor 647 Streptavidin (1:500, 405237, BioLegend), and PE Streptavidin (1:500, 405204, BioLegend). OneComp eBeads (Invitrogen) were used to prepare single-color controls for compensation, if needed. Stained samples were analyzed with either a MACSQuant Analyzer 10 (Milteny), Attune NxT (Invitrogen), or BD LSRFortessa (BD Biosciences) flow cytometer.

Validation

Antibodies were either titrated on an appropriate cell type prior to studies reported herein to determine the optimal dilution for staining or diluted according to the manufacturer's instructions.

Links to manufacturer's validation statements of species and application:
- Zombie Violet (BioLegend, Cat# 423114): https://www.biolegend.com/en-us/products/zombie-violet-fixable-viability-kit-9341
- Zombie Yellow (BioLegend, Cat# 423104): https://www.biolegend.com/en-us/products/zombie-yellow-fixable-viability-kit-8514
- APC anti-human CD4 (BioLegend, Cat# 300514): https://www.biolegend.com/en-us/products/apc-anti-human-cd4-antibody-823
- PerCP/Cy5.5 anti-human CD8α (BioLegend, Cat# 301031): https://www.biolegend.com/en-us/products/percpcyanine55-anti-human-cd8α-antibody-4222
- APC anti-human CD8α (BioLegend, Cat# 301014): https://www.biolegend.com/en-us/products/apc-anti-human-cd8a-antibody-831
- CD8-biotin (Milteny, Cat# 130-098-556): https://www.miltenyibiotech.com/_Resources/Persistent/b0ddf194c59e08838b52d9e12d1e1510e2017.b0.pdf
- anti-mouse CD16/CD32 Fc block (eBioscience, Cat# 14-0161-86): https://www.thermofisher.com/antibody/product/CD16-CD32-antibody-clonal-93-MonoClonal-14-0161-86
- FITC anti-mouse CD3ε (BioLegend, Cat# 100305): https://www.biolegend.com/en-us/products/fictr-anti-mouse-cd3epsilon-antibody-23
- BV421 anti-mouse CD8α (BioLegend, Cat# 100737): https://www.biolegend.com/en-us/products/brilliant-violet-421-anti-mouse-cd8a-antibody-7138
- FITC mouse anti-human CD3ε (BD Biosciences, Cat# 55611): http://wwwbdbiosciences.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-non-human-primate-antibodies/cell-surface-antigens/fictr-mouse-anti-human-cd3-sp34/p/556611
- Purified anti-human CD3 (BioLegend, Cat# 300402): https://www.biolegend.com/en-us/products/purified-anti-human-cd3-antibody-867
- Purified anti-human CD8α (BioLegend, Cat# 301002): https://www.biolegend.com/en-us/products/purified-anti-human-cd8a-antibody-839
- Super Bright 600 anti-human CD19 (eBioscience, Cat# 63-0198-42): https://www.thermofisher.com/antibody/product/CD19-Antibody-clonal-SJ25Cl-MonoClonal-63-0198-42
- Super Bright 702 anti-human CD56 (eBioscience, Cat# 67-0566-42): https://www.thermofisher.com/antibody/product/CD56-NCAM-Antibody-clonal-TULY56-MonoClonal-67-0566-42
- PE anti-human CD3 (BioLegend, Cat# 300308): https://www.biolegend.com/en-us/products/pe-anti-human-cd3-antibody-753
- APC/Cy7 anti-human CD4 (BioLegend, Cat# 325619): https://www.biolegend.com/en-us/products/apc-cy7-anti-human-cd4-antibody-3959
- FITC anti-human CD16 (BioLegend, Cat# 302006): https://www.biolegend.com/en-us/products/fictr-anti-human-cd16-antibody-567
- Alexa Fluor 700 anti-human CD3 (BioLegend, Cat# 300424): https://www.biolegend.com/en-us/products/alexfluor-700-anti-human-cd3-antibody-3394
- Brilliant Violet 785 anti-human CD4 (BioLegend, Cat# 317442): https://www.biolegend.com/en-us/products/brilliant-violet-785-anti-human-cd4-antibody-7978
- PE/Cy7 anti-human CD8α (BioLegend, Cat# 300914): https://www.biolegend.com/en-us/products/pe-cy7-anti-human-cd8a-antibody-1916
- BUV737 mouse anti-human CD45RA (BD Biosciences, Cat# S64442): https://wwwbdbiosciences.com/eu/reagents/research/
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | J.RT3-T3.5, Jurkat Clone E6-1, K562, K562 + OKT3, K562 + CD19, and Raji cell lines were purchased from ATCC or derived from cell lines purchased from ATCC. TM-LCLs were made from PBMCs as previously described (Pelloquin et al. 1986). PBMCs were isolated from TRIMA LRS Chambers that were purchased from Bloodworks Northwest. Mixed and pure CD8+ T cells used in non-isolation experiments were a generous gift from Juno Therapeutics. Rhesus PBMCs were a generous gift from Dr. Agne Taraseviciute (Seattle Children’s Research Institute). |
| Authentication | Cell-line authentication information was provided by the vendor, and authentication was conducted using short-tandem repeat genotyping. |
| Mycoplasma contamination | The cell lines were tested by the vendor and also tested monthly. The cell lines were found to be negative for mycoplasma contamination. |
| Commonly misidentified lines | No commonly misidentified cell lines were used. |

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Tg(Aldh111-EGFP,-DTA)DBrth/J stock breeders were purchased from The Jackson Laboratory (JAX Stock No. 026033), and the mice used for spleen harvesting were male mice 20-weeks old. All animal experiment protocols, handling and reporting for these mice were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Washington (UW) under protocol number 4053-01. NSG mice stock breeders were purchased from The Jackson Laboratory (JAX Stock No. 005557), and the mice used in the in vivo study were F1 produced female mice 9-to-11-weeks old at the start of the experiment. All animal experiment protocols, handling, and reporting for these mice were conducted in compliance with the IACUC at Seattle Children’s Research Institute (SCRI) under protocol number 13853. Details are described in Methods section. |
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation       | Sample preparation is described in detail in Methods.       |
|--------------------------|-------------------------------------------------------------|
| Instrument               | MACSQuant Analyzer 10 (Miltenyi), Attune NxT (Invitrogen), or BD LSRFortessa (BD Biosciences) |
| Software                 | Flow-cytometry data were collected with either MACSQuantify Software, BD FACSDiva V8.0.1, or Attune NxT Software v2.6. Flow-cytometry data were analyzed with FlowJo V10. |
| Cell population abundance| Flow cytometry was used for quantification purposes only; no post-sorting fractions were collected. |
| Gating strategy          | Briefly, the desired cell population was selected by using forward and side scatter. Single cells were selected by using forward scatter area and height linearity. Live cells were selected as defined by Zombie Violet or Zombie Yellow negativity. Supplementary Fig. 10 illustrates further downstream gating strategies for select samples. |

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.