Scaffolds that mimic antigen-presenting cells enable ex vivo expansion of primary T cells

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Therapeutic ex vivo T-cell expansion is limited by low rates and T-cell products of limited functionality. Here we describe a system that mimics natural antigen-presenting cells (APCs) and consists of a fluid lipid bilayer supported by mesoporous silica micro-rods. The lipid bilayer presents membrane-bound cues for T-cell receptor stimulation and costimulation, while the micro-rods enable sustained release of soluble paracrine cues. Using anti-CD3, anti-CD28, and interleukin-2, we show that the APC-mimetic scaffolds (APC-ms) promote two- to tenfold greater polyclonal expansion of primary mouse and human T cells compared with commercial expansion beads (Dynabeads). The efficiency of expansion depends on the density of stimulatory cues and the amount of material in the starting culture. Following a single stimulation, APC-ms enables antigen-specific expansion of rare cytotoxic T-cell subpopulations at a greater magnitude than autologous monocyte-derived dendritic cells after 2 weeks. APC-ms support over fivefold greater expansion of restimulated CD19 CAR-T cells than Dynabeads, with similar efficacy in a xenograft lymphoma model.

T-cell-based therapies are a promising approach to treat various diseases1–3, and have shown unprecedented clinical success for the treatment of B-cell acute lymphoblastic leukemia4–7 and non-Hodgkin’s lymphoma8. However, the rapid ex vivo expansion of functional T cells, a key step in the production of T cells for adoptive cell transfer (ACT), remains a challenge. T-cell activation requires three signals: (1) T-cell receptor (TCR) stimulation, (2) costimulation, and (3) prosurvival cytokines9. In the body, these signals are provided by antigen-presenting cells (APCs), which present these cues to T cells in specific spatiotemporal patterns9–12.

Various approaches are used to expand T cells ex vivo for ACT1,13,14. Among these, synthetic artificial APCs (aAPCs) are particularly convenient for polyclonal T-cell expansion15–24. Currently, commercial microbeads (Dynabeads) functionalyzed with activating antibodies for CD3 (αCD3; TCR stimulus) and CD28 (αCD28; costimulatory cue) represent one of the most commonly used and clinically relevant synthetic systems14,25. These beads promote polyclonal T-cell activation with exogenous interleukin-2 (IL-2) supplementation. Although these cultures provide T cells with the three critical signals, the context in which these signals are presented is not representative of how they are naturally presented by APCs. This can lead to suboptimal T-cell expansion rates16,20 and T-cell products with limited or dysregulated functions26,27. In addition, these beads are non-degradable and must be separated from the cell product before infusion, which can increase cost and manufacturing challenges25, and they are not amenable to the presentation of larger sets of co-stimulatory cues (e.g., OX40L, 4-1BBL), which may be important for the generation of highly functional therapeutic T cells13,28. Autologous monocyte-derived dendritic cells (moDCs) represent another common system for the antigen-specific expansion of naive and memory T cells. While their expanded T-cell products have shown clinical success in cancer29–31, the use of moDCs are limited by lengthy cell manufacturing procedures, high variability among donor moDCs, and a need for routine restimulation32,33.

Here, we describe the development of a composite material comprised of supported lipid bilayers (SLBs) formed on high-aspect-ratio mesoporous silica micro-rods (MSRs). The SLBs enabled the presentation of combinations of T-cell activation cues at predefined densities on a fluid lipid bilayer. Functionalized SLBs have been used to study T-cell activation and signaling34, and MSRs have been used for drug delivery and vaccination35,36. MSR-SLBs facilitated the sustained paracrine release of soluble cues to nearby T cells, and following functionalization, enabled the presentation of both surface and soluble cues to T cells in a context analogous to that of natural APCs. In cell culture, the rods formed a three-dimensional (3D) scaffold, and these scaffolds formed from MSR-SLBs functionalized with T-cell activation cues are termed APC-mimetic scaffolds (APC-ms). APC-ms promoted greater polyclonal and antigen-specific expansion of primary mouse and human T cells, and CD19 CAR-T cells, than conventional expansion systems, and represent a flexible and tunable platform technology that could enable the rapid expansion of highly functional T cells for ACT.

RESULTS

Assembly and characterization of APC-ms
APC-ms were prepared (Fig. 1a) for (1) polyclonal T-cell expansion using activating antibodies against CD3 (αCD3) and CD28 (αCD28), and for (2) antigen-specific T-cell expansion using peptide-loaded major histocompatibility complex (pMHC) and αCD28 (Fig. 1b). High-aspect-ratio MSRs (70 μm length, 4.5 μm diameter, and 10.9 nm pores) were synthesized35,36 and adsorbed with IL-2 (Supplementary Fig. 1a).

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Figure 1  APC-ms. (a) Process for preparing APC-ms from MSRs. (b) For polyclonal T-cell expansion, activating antibodies against CD3 (αCD3) and CD28 (αCD28) are attached (left). For antigen-specific T-cell expansion, peptide-loaded MHC (pMHC) and αCD28 are attached (right). In both, IL-2 was released over time, resulting in paracrine delivery to local T cells. (c) Representative microscopy of MSRs (left), fluorophore-tagged phospholipid (middle), and co-localization of MSRs and lipids (right). Scale bar, 200 µm. (d) Retention of lipid on MSRs over time in either PBS or RPMI-1640 containing 10% serum (cRPMI) to mimic cell culture. (e) Representative images of lipid-coated MSRs maintained in cRPMI over time. Scale bar, 100 µm. (f) Recovery of fluorescence over time in a photobleached region of MSRs coated with a lipid. Quantification of eight MSR-SLB samples (left). Confocal fluorescence microscopy images at various time points from representative experiment (right). Scale bar, 100 µm. (g) IL-2 release in vitro (data points) with one-phase exponential fit (dashed line; $R^2 = 0.98$). (h) Attachment of various inputs of biotinylated IgG onto 500 µg lipid-coated MSRs containing 0.01 mol%, 0.1 mol%, or 1 mol% biotinylated lipid. Values above bars indicate µg of IgG. (i) β-galactosidase activity as a measure of B3Z reporter T-cell activation in response to culture with complete APC-ms, or material missing one or more components. (j) SEM image of primary human T cells with APC-ms. Scale bar, 10 µm. Data in d, f–i represent mean ± s.d. of at least three experimental replicates and are representative of at least two independent experiments. (g) Analyzed using a repeated measures one-way ANOVA, followed by Tukey’s HSD post hoc test. (i) Analyzed using a one-way ANOVA, followed by Tukey’s HSD post hoc test.
Liposomes (140 nm; Supplementary Fig. 1b) containing predefined amounts of a biotinylated lipid were prepared, and used to coated IL-2-laden MSRs, forming MSR-SLBs. Biotinylated cues for TCR stimulation and costimulation were attached to the MSR-SLB surfaces via a streptavidin intermediate. In cell culture, 3D scaffolds spontaneously formed through the settling and random stacking of the rods, making APC-ms (Supplementary Video 1). T cells infiltrated the interparticle space of the scaffolds (Supplementary Video 2). APC-ms presented cues for TCR stimulation and costimulation on the surface of the lipid bilayer, and sustained release of soluble IL-2 to infiltrating T cells, similar to how these cues are presented to T cells by natural APCs.

The liposomes used to coat MSRs were formed from phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), which is commonly used as a model for mammalian cell membranes. At low lipid-to-MSR ratios (e.g., 1:20 w/w), the lipid-MSR suspensions underwent substantial aggregation (Supplementary Fig. 1c), while at higher lipid-to-MSR ratios (e.g., 1:4 w/w), lipid-MSR structures maintained a well-dispersed, single-particle state (Fig. 1c). At this higher ratio, 34.1 ± 0.9% of the input POPC was initially associated with the MSRs. The POPC coating was slowly lost over time (Fig. 1d,e), corresponding to degradation of the MSRs. MSRs were also successfully coated with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). The amount of lipid associated with MSRs was directly related to the saturation of the lipid (Supplementary Fig. 1d), likely due to tighter packing of more highly saturated lipids. No substantial differences were observed in the stability of various lipid coatings (Supplementary Fig. 1e–g). Fluorescence recovery after photobleaching (FRAP) studies revealed rapid recovery, demonstrating that the MSR lipid coatings were continuous, fluid SLBs (Fig. 1f).

We next characterized the loading and release of soluble cues, and the loading of surface cues, on MSR-SLBs. When 500 µg of MSRs were loaded with 2 µg of IL-2 and coated with lipid, 50 ± 1% of the input IL-2 was retained. The loaded IL-2 was subsequently released in a controlled manner over 7 d. The trend could be well-approximated using a one-phase exponential function ($R^2 = 0.98$), indicating that the release followed first-order kinetics (Fig. 1g).

The cytokines IL-21 and TGF-β could also be loaded into MSR-SLBs with high efficiency and subsequently released in a sustained manner (Supplementary Fig. 1b).

The attachment of surface cues was next quantified as we varied the amount of the biotinylated lipid species in the formulation. Streptavidin was added at 30% of the molar amount of biotinylated lipid groups on the respective MSR-SLBs, and biotinylated IgG was added as a surface cue proxy. At saturation, the maximal amount of biotinylated IgG that could be loaded onto the various MSR-SLB formulations differed by a factor of ~10 (Fig. 1h), consistent with the relative differences in the amounts of biotinylated lipid in the various MSR-SLB formulations. This indicated that IgG surface density can be precisely controlled by changing the amount of biotinylated lipid. In all subsequent experiments, MSR-SLBs were saturated with surface cues as described (forming APC-ms), using various formulations (A1–A6; Table 1).

To confirm that surface cues attached in this way remained functional, we cultured either complete APC-ms (presenting αCD3 and αCD28) or MSR-SLBs missing one or more components with B3Z reporter T cells, which provide a readout for T-cell activation. Maximal activation of the B3Z cells required input of all the components (Fig. 1i). Lastly, to confirm that presentation of activation cues promoted T-cell interactions, we cultured T cells with either APC-ms or MSR-SLBs without cues. Whereas T cells largely ignored MSR-SLBs without surface T-cell cues, they interacted strongly with APC-ms, forming extensive, high-density cell-material clusters (Fig. 1j and Supplementary Fig. 2).

**Polycional expansion of primary mouse T cells**

APC-ms formulations were benchmarked against commercial CD3/CD28 T-cell expansion beads (Dynabeads) for the polycional expansion of primary mouse T cells isolated from C57BL/6J mice. Four permutations of APC-ms were generated by varying the density of surface cues and the amount of material input into the starting cell culture (Table 1). To evaluate whether higher Dynabead doses would promote increased T-cell expansion, we conducted a dose-response study, in which APC-ms formulations (A1–A4) were compared to Dynabeads added at either the manufacturer’s recommended dose of a 1:1 bead-to-cell-ratio (D1), or at a 5:1 (D2) or 25:1 (D3) bead-to-cell ratio. By day 4, progressively larger bead-cell clusters were noted in Dynabead conditions as a function of increasing Dynabead dose, but these clusters did not persist past day 7 (Fig. 2a). Culture with APC-ms similarly led to the formation of large cell-material clusters. The size, frequency, and persistence of these clusters was greater in APC-ms cultures than in Dynabead cultures, although these attributes varied depending on surface cue density and material input (Fig. 2b). Within the dose range tested, no substantial difference in expansion efficiency was observed among the various Dynabead conditions after 13 d of culture (Fig. 2c). In contrast, culture with the various APC-ms formulations yielded three- to fivefold greater T-cell expansion than all Dynabead conditions tested, with expansion efficiency dependent on the particular APC-ms formulation (Fig. 2c). T cells expanded in all conditions were highly viable (>80%; Supplementary Fig. 3a, and APC-ms lacking the activation cues αCD3/αCD28 did not promote the activation and expansion of T cells (Supplementary Fig. 3b). Notably, despite promoting nearly fivefold greater expansion than all Dynabead conditions tested after 13 d of culture, APC-ms formulation A4 yielded T cells that co-expressed the exhaustion markers PD-1 and LAG-3 at a frequency similar to that of the Dynabead product (Fig. 2d and 2f). The CD4-to-CD8 T-cell ratio was dependent on the Dynabead dose and the APC-ms formulation (Fig. 2e and Supplementary Fig. 3c). All tested APC-ms formulations promoted rapid and substantial CD8-biased skewing, with APC-ms formulations A3 and A4 promoting a culture that had >95% CD8+ T cells by day 13. In contrast, Dynabeads generally promoted primarily CD4-biased skewing except at very high Dynabead doses. We obtained similar results with T cells isolated from BALB/c mice (Supplementary Fig. 2d,e). CD8+ T cells that were expanded using APC-ms and Dynabeads upregulated Granzyme B to a similar extent (Supplementary Fig. 3f). No expansion of CD4+ FoxP3+ regulatory T cells was observed with either Dynabead or APC-ms (Supplementary Fig. 3g).

To compare more rigorously Dynabeads and APC-ms, we quantified the amount of αCD3/αCD28 presented by Dynabeads to match the dose of T-cell stimuli in APC-ms. We found that Dynabead dose D2 and APC-ms formulation A4 represented conditions in which the same total amount of αCD3/αCD28 was presented per cell (Supplementary Fig. 4). Under these αCD3/αCD28-matched conditions, APC-ms promoted substantially greater expansion of T cells than Dynabeads after 7 or 13 d of culture (Fig. 2c). To better understand the effect of IL-2 dose and loading IL-2 into APC-ms, we compared Dynabeads at dose D2 to a variant of APC-ms formulation A4 that was not pre-loaded with IL-2. These cultures were supplemented with the same amount of IL-2 as is typically loaded into A4 as a soluble bolus at the start of culture (D2/bIL2 and A4/bIL2, respectively). Under these matched conditions, APC-ms still promoted greater expansion of T cells than Dynabeads (Fig. 2f),...
while promoting similar or less upregulation of exhaustion marker co-expression (Fig. 2g). APC-ms also supported greater T-cell expansion when IL-2 was pre-loaded into the material (A4) than added as a soluble bolus (A4/bIL2), demonstrating the value of loading and release of IL-2 from the material (Fig. 2f).

**Polyclonal expansion of primary human T cells**

Next, we evaluated APC-ms for the polyclonal expansion of primary human T cells. Because we did not observe a dose-dependent effect with Dynabeads within the dose range tested for mouse T-cell expansion, we used Dynabeads at the manufacturer’s recommended dose of a 1:1 bead-to-cell ratio in all subsequent studies. Culture of primary human T cells led to the formation of consistently larger clusters in APC-ms than in Dynabead cultures, and the stability and persistence of clusters was dependent on surface cue density and material input (Fig. 3a). Culture with all the tested APC-ms formulations led to a two- to tenfold greater expansion than with Dynabeads (Fig. 3b), with >95% of cells viable at 14 d (Supplementary Fig. 5a). APC-ms formulations A1–A3, which presented relatively higher amounts of activating stimuli, promoted substantial CD4β-biased skewing after 14 d. In contrast, formulation A4, which presented a relatively lower amount of activating stimuli, promoted more balanced CD4α and CD8β T-cell expansion, similar to Dynabeads (Fig. 3c). Despite promoting nearly tenfold greater expansion than Dynabeads, APC-ms formulation A4 yielded a low frequency of PD-1 and LAG-3 co-expressing cells (<5%; Fig. 3d), similar to Dynabeads. No differences were observed between Dynabead- or APC-ms-expanded T-cell clusters in the co-expression of lymphoid homing molecules CCR7 and CD62L (Supplementary Fig. 5b), which has been shown to be important for function in *in vivo* transfer 19.

**Antigen-specific expansion of primary mouse T cells**

We next investigated whether APC-ms would enable antigen-specific expansion of primary mouse CD8+ T cells. Anti-CD3 was replaced with a biotinylated H-2K(b) MHC class I monomer presenting SIINFEKL to enable antigen-specific expansion of OT-1 cells. When OT-1 cells were cultured with these APC-ms formulations, extensive cell-material clusters formed (Fig. 4a). APC-ms formulations presenting SIINFEKL promoted robust expansion of OT-1 CD8+ T cells, even with surface cues as low as 0.01 mol% of the lipids (formulations A5 and A6; Fig. 4b). When cultured with B16-F10 melanoma cells pulsed with SIINFEKL peptide, the expanded T cells recognized their cognate antigen and secreted interferon (IFNγ) (Fig. 4c), upregulated the co-expression of IFNγ and TNFα (Fig. 4d), and killed target cells *in vitro* (Fig. 4e).

**Antigen-specific expansion of primary human T cells**

We next assessed whether APC-ms could be used for the antigen-specific enrichment and expansion of rare human T-cell subpopulations, which could be useful for the selective expansion of rare cancer antigen-specific T cells from tumors 40 or blood 40,41, when relevant epitopes of interest are known. APC-ms (formulation A3) presenting one of two peptides (CLGGLTMV or GLCTIVAML, abbreviated as CLG and GLC, respectively), from different Epstein–Barr virus (EBV)-associated proteins in the context of HLA-A*0201, were compared to moDCs. moDCs are used clinically for antigen-specific expansion of therapeutic T cells 29 and are considered the gold standard for such applications. CD8α T cells were isolated from leukapheresis samples from EBV-experienced, HLA-A2* donors and cultured with autologous moDCs or APC-ms that presented CLG or GLC, resulting in similar expansion of the relevant CD8α T-cell subpopulations after 7 d of culture. Whereas frequencies of the relevant antigen-specific subpopulations decreased from days 7 to 14 in moDC-stimulated cultures, they continued to increase in APC-ms-stimulated cultures (Fig. 5a–d and Supplementary Fig. 6a,b). When cultured with CLG-presenting APC-ms, the frequency of CLG-specific CD8α T cells increased from 0.12% at day 0 to 1.3% by day 7, and to 8.41% by day 14, corresponding to a ~31- and ~1,500-fold expansion, respectively (Fig. 5a,b and Supplementary Fig. 6a,b). When cultured with GLC-presenting APC-ms, the frequency of GLC-specific CD8α T cells increased from 1.0% at day 0 to 1.3% by day 7, and to 15% by day 14, corresponding to a ~3.40- and ~355-fold expansion, respectively (Fig. 5c,d and Supplementary Fig. 6a,b). Within each donor, the expansion of the relevant subpopulation was consistently greater than the expansion of the irrelevant subpopulation, indicating antigen-specific T-cell activation. Total cell numbers did not vary substantially (including non-CLG- or GLC-specific CD8α T cells) between APC-ms and moDC cultures (Supplementary Fig. 6c and Supplementary Table 1). CD8α T cells expanded with either CLG- or GLC-presenting APC-ms responded strongly to T2 cells presenting their cognate antigen, as indicated by IFNγ secretion (Supplementary Fig. 7a), IFNγ and TNFα co-expression (Fig. 5e and Supplementary Fig. 7b), and the *in vitro* killing of peptide-loaded target cells (Fig. 5f).

Next, we investigated whether APC-ms could be used to expand specific T-cell subpopulations directly from heterogeneous cell mixtures, such as peripheral blood mononuclear cells (PBMCs), obviating the need for T-cell isolation. PBMCs from EBV+ HLA-A2* donors were cultured with APC-ms that presented GLC. The frequency of GLC-specific CD8α T cells in APC-ms-treated PBMC cultures increased rapidly from 0.66% to 15% at day 7, with minimal changes in mock-treated samples (Fig. 5g), corresponding to a 60-fold expansion of GLC-specific CD8α T cells (Fig. 5h). These APC-ms-expanded cells responded robustly to T2 cells presenting their cognate antigen. Quantification of the frequency of cells co-expressing IFNγ and TNFα (Fig. 5i), and IFNγ secretion (Fig. 5j), demonstrated that CD8α T-cell populations expanded from PBMCs with GLC-presenting APC-ms responded robustly only to T2 cells that presented GLC.

To confirm that antigen-specific T-cell expansion in APC-ms-treated PBMC cultures was not due to peptide presentation by PBMC-derived APCs, we depleted CD14+ cells from PBMCs and cultured these monocyte-depleted populations with APC-ms presenting CLG or GLC. Rapid, antigen-specific T-cell expansion was again observed in these monocyte-depleted cultures, demonstrating that antigen-specific T-cell expansion was not due to antigen presentation.
by endogenous APCs. Similar to the experiments with CD8+ T-cell isolates, robust expansion of the relevant subpopulations was observed to continue from days 7 to 14 of culture (Supplementary Fig. 8a,b). Taken together, these data demonstrate the ability of APC-ms to robustly expand functional human T cells in an antigen-specific manner from either purified CD8+ T cells or heterogeneous cell populations such as PBMCs.

In vivo efficacy of APC-ms-expanded CAR-T cells
Finally, to evaluate whether T cells expanded with APC-ms are functional in vivo, human T cells expressing a chimeric antigen receptor (CAR) specific for CD19 (19BBz T cells) were polyclonally restimulated with APC-ms (formulation A4) or Dynabeads, which are used clinically for the expansion of CAR-T cells25. Following restimulation, APC-ms promoted over fivefold greater expansion of 19BBz T
cells than Dynabeads after 7 or 14 d of culture (Fig. 6a). After 7 d, the proportion of CAR⁺ T cells was higher with Dynabead expansion (59% versus 39%, Dynabeads vs. APC-ms), but there was no difference after 14 d (15% versus 12%, Dynabeads vs. APC-ms), presumably due to more efficient expansion of non-CAR⁺ T cells in the Dynabead condition (Fig. 6b). Whereas Dynabead-expanded 19BBz T cells underwent CD4-biased skewing, APC-ms-expanded 19BBz T cells preserved their initial CD4-to-CD8 ratio before restimulation (Fig. 6c and Supplementary Fig. 9a).

We tested the in vivo functionality of APC-ms-expanded 19BBz T cells in a disseminated xenograft model of Burkitt’s lymphoma using luciferized Raji cells (Fig. 6d). Prior to infusion of Dynabead-expanded 19BBz T cells, Dynabeads in the cell product had to be magnetically removed, whereas evaluation of silicon content in cell culture pellets over time confirmed the degradation of APC-ms before the time of cell infusion (Supplementary Fig. 9b). No adverse effects were observed in any of the mice treated with either 7-d or 14-d APC-ms-expanded 19BBz T cells. The anti-tumor efficacy of the cells was similar (Fig. 6c,f and Supplementary Fig. 9c,d).

**DISCUSSION**

Here, we demonstrate a multifunctional material that presents both surface and soluble cues to T cells in a manner that mimics how these cues are naturally presented in vivo. High-aspect-ratio particles were used to form APC-ms, which contrasts with most previously described synthetic aAPC materials. These particles spontaneously formed high-surface-area 3D scaffolds, which infiltrating T cells remodeled to form dense cell-material clusters, creating a microenvironment where T cells lie in close proximity to the material. The relatively large size and high aspect ratio of the rods likely contributed to the formation of the larger clusters observed in APC-ms versus Dynabead cultures, since many more T cells could interact with each individual rod (~70 µm length, ~4.5 µm diameter) than with the smaller spherical Dynabeads (4.5 µm diameter). The persistence of these clusters in APC-ms cultures was dependent on surface cue density and the amount of material in the culture, which likely contributed to the different phenotypes observed in the various APC-ms conditions.

In the present study we show that loading IL-2 into APC-ms is superior to adding the same amount of IL-2 into the media as a soluble bolus. This is consistent with prior work, which demonstrated that delivering cytokines such as IL-2 to T cells in a paracrine manner potentiates the effects of the cytokine. Current synthetic aAPC systems emphasize TCR clustering and subsequent T-cell activation via the static, high-density presentation of stimuli. Here we show that by presenting T-cell stimuli across the surface of a fluid lipid bilayer, emulating how these cues are presented on an APC plasma membrane, relatively lower surface cue densities can promote more rapid expansion rates and generate T cells with a more functional and less exhausted phenotype. The clustering of TCRs is only one step in a dynamic process and serves not only to enhance T-cell activation, but also to limit the duration of TCR signaling to protect against T-cell overstimulation.
In polyclonal mouse T-cell expansion studies, APC-ms promoted substantial CD8-biased skewing, with the degree dependent on the particular APC-ms formulation. This is consistent with previous observations that paracrine delivery of IL-2 enhanced the proliferation of mouse CD8+ T cells, but promoted activation-induced cell death in mouse CD4+ T cells24. In contrast, formulations that presented higher amounts of T-cell stimuli promoted substantial CD4-biased skewing in polyclonal human T-cell expansion, while a lower amount of T-cell stimuli promoted a more balanced CD4-to-CD8 ratio. Consistent with this observation, human CD19 CAR-T cells (19BBz T cells) expanded with APC-ms retained a more balanced CD4-to-CD8 ratio, similar to that of the starting population, whereas 19BBz T cells expanded with Dynabeads underwent substantial CD4-biased skewing. The discrepancy in CD8 versus CD4 bias observed between mouse and human T cells could indicate fundamental differences in mouse and human biology. A better understanding of this behavior could enable material formulations that bias mixed T-cell populations toward specific CD4-to-CD8 ratios, a property that has recently been shown to be important for the function of adoptively transferred T cells35.

The expansion of rare antigen-specific populations of T cells is a critical but challenging step in various disease treatments1-3. Although autologous moDCs represent the gold standard for expanding antigen-specific T cells, the need to isolate and culture moDCs on a donor-specific basis increases the resources and cost required to manufacture T-cell products. High donor-to-donor variability also makes it challenging to standardize the production process, and the initial frequency and quality of the moDCs can vary widely. Following a single stimulation, APC-ms promoted antigen-specific expansion of rare T-cell subpopulations similar to moDCs, but the degree of skewing differed between mouse and human T cells. A better understanding of these differences could enable material formulations that bias mixed T-cell populations toward specific CD4-to-CD8 ratios, a property that has recently been shown to be important for the function of adoptively transferred T cells35.

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We demonstrated that upon restimulation, APC-ms promoted more rapid expansion of 19BBz T cells than Dynabeads. We further demonstrate that 19BBz T cells expanded with these different materials showed no substantial functional differences in vivo in a disseminated lymphoma xenograft model. Notably, processes for CAR-T cell production that involve the use of Dynabeads require that beads be removed in a disseminated lymphoma xenograft model. Notably, processes for CAR-T cell production that involve the use of Dynabeads require that beads be removed in vivo.

to differences in the persistence of antigen presentation by APC-ms, for which the duration of antigen presentation is controlled by its degradation kinetics, compared to live moDCs which only transiently present exogenously loaded short peptides. Protocols employing moDCs frequently involve multiple restimulations to promote substantial enrichment of rare T-cell subpopulations.

Figure 5 Antigen-specific expansion of primary human T cells from donor leukapheresis samples (a–f) and from PBMCs (g–j). (a, c) Enrichment of live CD8+ cells specific for peptides CLGGLLTMV (CLG; a) and GLCTLVAML (GLC; c), respectively. Representative plots (left), and quantification (right), from HLA-A2+ donor T cells cultured with APC-ms presenting either CLG or GLC, followed by co-culture with T2 target cells either unpulsed (−peptide; left), pulsed with CLG (+CLG; middle), or pulsed with GLC (+GLC; right). (b, d) Fold expansion of CD8+ T cells specific for CLG (b) or GLC (d), cultured with APC-ms presenting CLG or GLC, or with autologous moDCs. (e) Frequencies of IFNγ+TNFα+CD8+ T cells cultured with APC-ms presenting CLG or GLC, following co-culture with T2 target cells either unpulsed (−peptide; left), pulsed with CLG (+CLG; middle), or pulsed with GLC (+GLC; right). (f) In vitro killing of T2 target cells unpulsed (−peptide), or pulsed with CLG (+CLG) or GLC (+GLC), by CD8+ T cells expanded with APC-ms presenting CLG or GLC. (g, h) Frequency (g) and number (h) of GLC-specific cells among CD8+ T cells from PBMCs cultured in 30 U/ml IL-2 (mock), or with APC-ms presenting CLG. Values above bars in g denote fold expansion (mean ± s.d.). (i, j) Frequency of IFNγ+TNFα+CD8+ T cells (i), and IFNγ secretion (j), from PBMCs cultured in 30 U/ml IL-2 (mock), or with APC-ms presenting CLG, following co-culture with T2 cells that were unpulsed (−peptide), pulsed with CLG (+CLG), or pulsed with GLC (+GLC). Data in a–d are from different donor samples and are representative of at least three independent experiments. APC-ms/CLG, n = 4; APC-ms/GLC, n = 6; moDC/CLG, n = 2; moDC/GLC, n = 4. (a–d) analyzed using a two-way ANOVA, followed by Tukey’s HSD post hoc test. Data in (e–j) represent mean ± s.d. of three experimental replicates and are representative of two independent experiments with n = 2 donor samples.
before administration. Although simple in theory, this can be challenging and inconsistent in practice, with at least one reported instance of an out-of-specification CAR-T cell manufacturing failure due to incomplete Dynabead removal25.

The need to rapidly generate therapeutically relevant numbers of functional T cells ex vivo is a challenge in personalized T-cell therapies43–44, and this study demonstrates that APC-ms provide an advancement toward meeting this need. APC-ms represents a multifunctional material platform that promotes more efficient polyclonal and antigen-specific cell expansion than widely used T-cell expansion systems (e.g., Dynabeads and moDCs). APC-ms is a modular platform technology that can be adapted to present diverse repertoires of cues and change the spatial and temporal context in which cues are presented. For example, MSR properties could be varied to tune the scaffold microenvironment or degradation kinetics. The lipid formulation could be altered to tune SLB stability45, fluidity46, or surface cue partitioning47–49, or enable the attachment of cues via different chemistries50–52. We envision that future iterations of APC-ms could present larger sets of both surface and soluble cues, enabling the generation of further optimized T cells for ACT18,28.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Figure 6 In vivo efficacy of restimulated 19BBz CAR-T cells in a disseminated lymphoma xenograft model. (a) Expansion of 19BBz T cells cultured with Dynabeads (dose D1) or with APC-ms (formulation A4; Table 1). (b) FACs quantification of CAR expression among live cells in the final infusion product cultured with Dynabeads or APC-ms. (c) Frequencies of CD4 and CD8 single-positive cells among live CD3+ cells in the final infusion product cultured with Dynabeads or APC-ms. (d) Experimental overview of disseminated Raji xenograft model. (e,f) Bioluminescent imaging of NSG mice inoculated with luciferized Raji cells, and then either mock treated (M), or treated with 19BBz T cells expanded for either 7 or 14 d with Dynabeads (D) or APC-ms (A). The dotted line in e denotes the average luminescence of mice not administered luciferin at the first measured time point (d7) and represents baseline. Representative bioluminescent images (e) and quantification of bioluminescent signal (f). Data in a represent mean ± s.d. of three experimental replicates. Data in f represent mean ± s.e.m. For 7 day-expanded 19BBz T cells: mock, n = 6; Dynabeads, n = 6; APC-ms, n = 6. For 14 day-expanded 19BBz T cells: mock, n = 6; Dynabeads, n = 7; APC-ms, n = 7.

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

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AUTHOR CONTRIBUTIONS

A.S.C. and D.J.M. conceived and designed the experiments. A.S.C., D.K.Y.Z., and S.T.K. performed the experiments. A.S.C., D.K.Y.Z., and D.J.M. analyzed the data. A.S.C., D.K.Y.Z., and D.J.M. wrote the manuscript. All authors discussed the results and commented on the manuscript. The principal investigator is D.J.M.
COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. Science 348, 62–68 (2015).
2. June, C.H., Riddell, S.R. & Schumacher, T.N. Adoptive cellular therapy: a race to the finish line. Sci. Transl. Med. 7, 280ps7 (2015).
3. Fesnak, A.D., June, C.H. & Levine, B.L. Engineered T cells: the promise and challenges of cancer immunotherapy. Nat. Rev. Cancer 16, 566–581 (2016).
4. Maude, S.L. et al. Chimeric antigen receptor T cells for sustained remissions in hematologic malignancies. N. Engl. J. Med. 371, 1419–1431 (2014).
5. Lee, D.W. et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385, 517–528 (2015).
6. Sunshine, R.J. et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 118, 4817–4828 (2011).
7. Turtle, C.J. et al. CD19 CAR T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J. Clin. Invest. 126, 2123–2138 (2016).
8. Neelapai, S.S. et al. Biodegradable nanoellipsoidal artificial antigen presenting cells complete remissions in patients with refractory diffuse large B-cell lymphoma (DLBCL): results from the pivotal phase 2 Zuma-1. Blood 128, LBA-6 (2016).
9. Hoppa, J.B. & Davis, M.M. T-cell-antigen recognition and the immunological synapse. Nat. Rev. Immunol. 3, 973–983 (2003).
10. Lee, K.-H. et al. The immunological synapse balances T cell receptor signaling and degradation. Science 302, 1218–1222 (2003).
11. Alarcón, B., Mestre, D. & Martínez-Martín, N. The immunological synapse: a cause or consequence of T-cell receptor triggering? Immunology 133, 420–425 (2011).
12. Minguet, S., Wölfli, M. & Greenberg, P.D. Antigen-specific activation and cytokine-facilitated expansion of naive, human CD8+ T cells. Nat. Protoc. 9, 950–966 (2014).
13. Alarcón, B. & Martinez-Martín, N. Endothelial cell-tumor interactions: from angiogenesis to immune cell recruitment. J. Immunother. 36, 566–581 (2016).
14. Eggermont, L.J., Tel, J. & Figdor, C.G. Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells. Immunology 149–161 (1997).
15. Perica, K. et al. Enrichment and expansion with nanoscale artificial antigen presenting cells for adoptive immunotherapy. ACS Nano 9, 6861–6871 (2015).
16. Mansal, S. et al. Polymer-based synthetic dendritic cells for tailoring robust and multifunctional T cell responses. ACS Chem. Biol. 10, 485–492 (2015).
17. Steenblock, E.R. & Fahmy, T.M. A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. Mol. Ther. 16, 765–772 (2008).
18. Fadel, T.R. et al. A carbon nanotube-polymer composite for T-cell therapy. Nat. Nanotechnol. 9, 639–647 (2014).
19. Sunyer, J.C., Perica, K., Schneck, J.P. & Green, J.J. Particle shape dependence of CD8+ T cell activation by artificial antigen presenting cells. Biomaterials 35, 269–274 (2014).
20. Fadel, T.R. et al. Enhanced cellular activation with single walled carbon nanotube bundles presenting antibody stimuli. Nano Lett. 8, 2070–2076 (2008).
21. Meyer, R.A. et al. Biodegradable nanoellipsoidal artificial antigen presenting cells for antigen specific T-cell activation. Small 11, 1519–1525 (2015).
22. Steenblock, E.R., Fadel, T., Labowsky, M., Pober, J.S. & Fahmy, T.M. An artificial antigen-presenting cell with paracrine delivery of IL-2 impacts the magnitude and direction of the T cell response. J. Biol. Chem. 286, 34893–34892 (2011).
23. 25. Novartis CTL019 Oncologic Drugs Advisory Committee Briefing Document (2017).
ONLINE METHODS

Cells and reagents. The B16-F10 murine melanoma cell line was obtained from American Type Culture Collection (ATCC), and confirmed to be negative for mycoplasma. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (HI-FBS) and 1% peni- cillin-streptomycin. The T2 (174 × CEM.T2) human lymphoblast cell line, was cultured in T-cell media (RPMI 1640 supplemented with 10% HI-FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 µM beta-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 1% penicillin-streptomycin). The B23 murine T-cell hybridoma cell line was cultured in T-cell media without HEPES and non-essential amino acids. Primary mouse and human T cells, and CD19 CAR-T cells were cultured in T-cell media supplemented with 20 U/ml recombinant mouse- or human-IL-2, respectively, unless otherwise stated. Luciferized Raji cells, were cultured in RPMI 1640 supplemented with 10% HI-FBS, 2 mM l-glutamine, 1% penicillin-streptomycin and 1% purocyn. 

Cell culture reagents were obtained from Gibco (e.g., DMEM, HI-FBS, penicillin-streptomycin), Lonza (e.g., RPMI 1640, t-glutamine, sodium pyruvate, non-essential amino acids), or Sigma (e.g., beta-mercaptoethanol, HEPES). Murine and human recombinant IL-2, IL-21, and TGF-β were purchased from Biolegend. All chemical reagents for MSR synthesis were purchased from Sigma-Aldrich. All lipids were purchased from Avanti Polar Lipids, including: DOPC (850375C), POPC (850457C), DSPC (850365C), PE-cap-biotin (870279C), PE-carboxyfluorescin (810332C). FoxP3 antibodies were from eBioscience. All other antibodies were purchased from Biolegend. Biotinylated peptide-loaded MHC monomers and fluorophore-labeled tetramers were obtained from the National Institutes of Health Tetramer Core Facility and MBL International. Mouse and human CD3/CD28 T-cell expansion Dynabeads were purchased from Thermofisher Scientific. The ovalbumin-derived peptide SIINFEKL was purchased from Anaspec. The EBDV-derived peptides CILGGLITMV and GLCTLVMVL were purchased from Proimmune.

Synthesis of MSRs. MSRs were synthesized as previously reported. Briefly, Pluronic P123 surfactant (average Mn ~5,800) was dissolved in 1.6 M HCl solution and stirred with tetraethyl orthosilicate (TEOS, 98%) at 40 °C, followed by aging at 100 °C. Subsequently, surfactant was removed by extraction in 1% HCl/ethanol (v/v). Particles were recovered by running the suspension through a 0.22-µm filter, washed with ethanol, and dried.

Primary T-cell isolation. C57BL/6 and BALB/c mice were used as primary mouse T-cell donors for polyclonal T-cell expansion studies and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were used for antigen-specific T-cell expansion studies, respectively. Primary human T cells were obtained from de-identified leukoreduction collars (Brigham and Women's Hospital Specimen Bank) and used within 24 h of initial collection (stored at room temperature (RT)). PBMCs were enriched from leukoreductions in a Ficoll gradient, then isolated using pan T cell or CD8a + T-cell isolation MACS kits (Miltenyi Biotec) to obtain CD3+ T cells or CD8+ T cells for polyclonal or antigen-specific T-cell expansion studies, respectively.

Preparation of APC-ms. MSRs and liposomes were prepared before APC-ms assembly. To prepare monodisperse liposomes, we first prepared lipid films composed of predefined lipid formulations by mixing lipid-chloroform suspensions, evaporating the bulk chloroform under nitrogen, and removing residual chloroform overnight in a vacuum chamber. For all functional studies, POPC was used as the primary lipid, and lipid formulations were doped with 0.01–1 mol% of either carboxyfluorescein-tagged or biotin-capped PE. For some characterization studies, DOPC and DSPC were alternatively used as the primary lipid. Lipid films were rehydrated in PBS and subsequently extruded through 100-nm polycarbonate filters using a Mini-Extruder (Avanti Polar Lipids). Liposome suspensions were stored at 4 °C and used within a week.

To prepare APC-ms, MSRs (10 mg/ml) were incubated with recombinant IL-2 (0.04 mg/ml) in PBS for 1 h at RT. Liposomes were added at lipid-to-MSR of 1:4 (w/w), and incubated for 1 h at RT with pipetting every 10 min to form MSR-SLBs. The material was washed and blocked with 0.25% bovine serum albumin (BSA) in PBS (w/v). Streptavidin was subsequently added at 30% theoretical molar saturation of the biotinylated lipid in the formulation (assuming 34% lipid retention for POPC) and the suspension was mixed. Biotinylated T-cell activating cues (1:1 molar ratio of TCR-activating cue-to-CD28) were added at a 80% molar saturation of the added streptavidin, and the suspension was mixed at RT. The material was washed and resuspended in cell culture media for in vitro assays. APC-ms was immediately used for T-cell expansion experiments, or stored at 4 °C and used within a week for characterization studies.

Characterization of MSR-SLB structure and stability. Brightfield and fluorescence microscopy, used to evaluate MSR-SLB lipid coating, dispensability, and degradation, were performed with an EVOS FL Cell Imaging System. Confocal microscopy was performed using a Zeiss LSM 710 confocal system. Lipid retention was evaluated using MSRs coated with lipid formulations containing 1 mol% fluorophore-tagged lipid, and quantified using a plate reader. Cultured material was recovered by centrifuging at 700 rcf for 5 min, and fluorescence intensity was normalized to the fluorescence intensity before culture. Fluorescence recovery after photobleaching (FRAP) experiments were carried out on MSRs coated with lipid formulations containing 10 mol% fluorophore-tagged lipid. Photobleaching was performed on the 488 nm laser line and images were taken every 1 s for at least 20 s. Fluorescence recovery was analyzed using Imager by normalizing the fluorescence intensity within the photobleached region to the fluorescence intensity in an unbleached region on a different rod.

Cytokine (IL-2, IL-21, and TGF-β) loading and release was quantified by loading 500 µg of MSRs with 2 µg of the specified cytokine, followed by coating with lipid as described. Cytokine-loaded MSR-SLBs were resuspended in release buffer (1% BSA in PBS (w/v)). At various time points, samples were spun down and supernatants collected and quantified via enzyme-linked immunosorbent assay (ELISA) (IL-2 ELISA from Biologend, IL-21 and TGF-β ELISA from R&D Systems). Subsequently, MSRs were resuspended in fresh release buffer and returned to culture. The total amount of cytokine cumulatively released upon reaching the asymptote of the release curve was assumed to be the loaded amount of cytokine. Cytokine loading efficiency was calculated as total mass of cytokine release divided by mass of cytokine input. To quantify loading, MSR-SLB samples were prepared using lipid formulations containing 0.01, 0.1, or 1 mol% biotinylated lipid. Streptavidin, at a 30% theoretical molar saturation of the retained biotinylated lipid (assuming 34% lipid retention for POPC), was added, followed by the addition of biotinylated IgG at 40% or 80% molar saturation of the added streptavidin. As controls, samples containing the same amount of biotinylated IgG but no material were also prepared. All samples were pelleted, and the amount of IgG in the supernatant fractions were quantified via ELISA (eBioscience). The biotinylated IgG stock was also used to prepare standard curves. The amount of IgG loaded onto the material was calculated by subtracting the amount of IgG detected in control sample supernatants from the amount of IgG detected in respective material sample supernatants.

Characterization of APC-ms. For scanning electron microscopy, cells were cultured with APC-ms overnight, fixed in 4% paraformaldehyde, and then spun down. Fixed samples were serially transitioned through a gradient of 0, 30, 50, 75, 90, 100% ethanol in water. Samples were submerged in hexamethyldisilazane (Electron Microscopy Sciences) and placed in a benchtop desiccator overnight. Dried coverslips were mounted on stubs, sputter-coated with 5 nm of platinum-palladium, and imaged using secondary electron detection (Carl Zeiss Supra 55 VP field emission scanning electron microscope).

For degradation studies, APC-ms was synthesized as described, and cultured with primary mouse T cells. At indicated time points, the mixture was harvested, and pelleted. Supernatant was removed and pellets washed.
lyophilized, and prepared for inductive coupled plasma atomic emission spectrometry (ICP-AES) to determine silicon (Si) content. ICP-AES was performed by Galbraith Laboratories. Percent detectable Si was calculated by normalizing the mass of Si at each time point to the mass of Si at 0 h. The reported limit of detection was 6.8 µg/sample.

**In vitro polyclonal T-cell expansion studies.** Isolated primary mouse and human T cells were mixed with activation stimuli (i.e., Dynabeads or APC-ms), and cultured for up to 2 weeks. Media was added to maintain the cells below a density of 2.5 × 10^6 cells/ml throughout the culture period. All mock-treated samples in polyclonal T-cell expansion experiments were cultured in basal T-cell media without IL-2 supplementation.

Commercial Dynabeads (ThermoFisher Scientific) were used according to the manufacturer-optimized protocol included with the kit. In some experiments, T cells were stimulated with Dynabeads at a bead-to-cell ratio of 5:1 or 25:1, or the manufacturer's recommended bead-to-cell ratio of 1:1. For Dynabead cultures, 1 × 10^5 cells were seeded in the starting culture with pre-washed Dynabeads at the indicated bead-to-cell ratio in T-cell media supplemented with 30 U/ml recombinant IL-2. Cells were counted every third day and fresh IL-2-supplemented media was added to bring the cell suspension to a density of 0.5–1 × 10^6 cells/ml. In some studies, Dynabead cultures were supplemented with IL-2 only at the start of culture as a soluble bolus.

For mouse and human polyclonal T-cell expansion studies, APC-ms were prepared that presented surface cues (αCD3/αCD28) on between 0.1–1 mol% of the lipids at a 1:1 molar ratio, and added into the starting culture at 33 µg/ml or 333 µg/ml. For APC-ms cultures, 2.5 × 10^4 cells were seeded in the starting culture in basal T-cell media without IL-2 supplementation. Starting on day 7, fresh media supplemented with 30 U/ml recombinant IL-2 was added as necessary to maintain cells below a density of 2.5 × 10^6 cells/ml. In some studies, APC-ms was prepared without pre-loaded IL-2, and IL-2 was instead added into the media at the start of culture as a soluble bolus.

Images of the T-cell cultures were taken and live cells manually enumerated with a hemocytometer using Trypan blue exclusion. Fold expansion was calculated by dividing the number of cells at the respective time point by the number of cells seeded at the start of culture. After enumeration, cell phenotype was evaluated using flow cytometry. Gates were set for each time point and sample independently based on fluorescence minus one (FMO) controls.

**In vitro antigen-specific T-cell expansion studies.** Antigen-specific mouse T-cell expansion experiments were carried out using primary CD8^+ T cells isolated from OT-I mice. Antigen-specific human T-cell expansion experiments were carried out using either CD8^+ T cells, PBMCs, or CD14^+ monocyte-depleted PBMCs derived from de-identified donor leukapheresis samples. Mouse T cells were mixed with APC-ms while human T cells, PBMCs, or CD14-depleted PBMCs were mixed with either APC-ms or moDCs. Prior to culture, donor samples were assayed for HLA-A*0201 expression via FACS, and prior EBV exposure via anti-EBV viral capsid antigen (VCA) ELISA (IBL International) of serum. Only HLA-A^2^, EBV-experienced donors were used for expansion studies. Baseline frequencies of CLG- and GLC-specific CD8^+ T cells in these samples were also assessed. Donors in which the baseline frequency was below 0.01% in 10,000 live single CD8^+ events were excluded from further analyses for the respective peptide (two incidences occurred).

For mouse antigen-specific studies, APC-ms were prepared that presented surface cues (SVYDFFFWVL/H-2K(b) or SIINFEKL/H-2K(b) and αCD28) on either 0.01 mol% or 0.1 mol% of the lipids at a 1:1 molar ratio, and added into the starting culture at 33 µg/ml or 333 µg/ml. For human antigen-specific studies, APC-ms were prepared that presented surface cues (CLGGLLTMV/HLA-A2 or GLCTLVAML/HLA-A2 and αCD28) on 0.1 mol% of the lipids at a 1:1 molar ratio, and added into the starting culture at 333 µg/ml. For mouse studies, 2.5 × 10^5 T cells were seeded with the specified amount of material in non-IL-2-supplemented media. For human studies, 5 × 10^5 T cells, PBMCs, or CD14-depleted PBMCs were cultured with the specified amount of material in media without IL-2. Starting on day 7, additional media that was added to APC-ms cultures was supplemented with 30 U/ml IL-2. Additional IL-2-supplemented media was added throughout the remainder of the culture period to maintain cells below a density of 2.5 × 10^6 cells/ml. Mock-treated samples in mouse expansion experiments were cultured in T-cell media without IL-2. Mock-treated samples in human antigen-specific expansion experiments were cultured in media with IL-2 supplementation.

For human antigen-specific moDC cultures, moDCs were generated via the Mo-DC generation toolbox I, human (Millenyi Biotec). Mature moDCs were either unpulsed or pulsed with 10 µg/ml peptide (mouse, SIINFEKL; human, CLGGLLTMV or GLCTLVAML) for 6 h at 37 °C. moDCs were washed and cultured with donor-matched, freshly isolated CD8^+ T cells in IL-2-supplemented media. 5 × 10^5 CD8^+ T cells were seeded with 3.3 × 10^5 autologous, peptide-pulsed or unpulsed moDCs in 96-well U-bottom plates. Media with IL-2 was added throughout the culture period to maintain cells below a density of 2.5 × 10^6 cells/ml.

Images of the T-cell cultures were taken and live cells manually enumerated with a hemocytometer using Trypan blue exclusion. After enumeration, frequencies of antigen-specific T cells were evaluated using tetramer staining and flow cytometry. Antigen-specific fold expansion was calculated as follows: (live cell number × percent CD8^+ × percent tetramer)^3/2/2/2/(number of cells seeded × percent CD8^+ × percent tetramer)^3/2/2/2.

**In vitro T-cell functional studies.** Stimulator cells (mouse, B16-F10; human, T2) were either unpulsed or pulsed with 1 µg/ml peptide (mouse, SIINFEKL; human, CLGGLLTMV or GLCTLVAML) for 30 min at 37 °C. Subsequently, 1 × 10^5 expanded cells were cultured with 2 × 10^6 stimulator cells for 1 h before adding Brefeldin A (BD Biosciences) to inhibit cytokine secretion, and then cultured for another 4 h. Cells were pelleted and fluorescence intensity of supernatant samples quantified using a plate reader. IFNy concentrations in supernatant samples were quantified via ELISA (Biolegend).

19B8z CAR T-cell restimulation studies. 19B8z CAR-T cells were obtained from Unum Therapeutics. 19B8z T cells were generated using the commercial vector pLent6.4/RRI/R2/V5-DEST (Invitrogen) with an EF1α promoter and the PGK protector and blasticidin S deaminase genes removed. The 19D9 CAR was cloned downstream of the EF1α promoter. The 19B8z CAR was previously described and is composed of a CD8α signal peptide, a CD19-targeting scFv, a CD8α hinge and transmembrane domain, a 4-1BB costimulatory domain, and a CD3ζ signaling domain. The CD19 CAR protein sequence is as follows: MALPTVALLPLALLHALARPDIGMTQMTTSSSLASLGDRTYTSRCTSRQASDQSKYNWFQVPDKTVKLLHLYTSLRHSQVPSFSGSGSGTDSLYSTLISNLEQEDIAFYQCGQNTLPTYTGGGTKLEITGGGGGSGGGGSGGGSGGSGKLEQSGCPGLAPSLSVTSCTSVGSPYDPSWIRPKQKLELGWLVIGWGETTYYNNALKSRILITIKDNSKQSVLFKMNQSLTDATTAYYCAHHYYYSYAMGDYWWQQTGTSVTQSTTTPRPPTPATIAQPSLRLPPEACRPAAGAVHTRGRLFADCIYIYIALPGMTCVGLLIVSITLYRCRGGKRLYIKFKQFMRPPVFTQTPQEDGCSRFREEEVEEGGCCELKVFSRRADAPAYQGGGQYNNELNLNGREEYDVLDRKRGRDPMEGKGPKRNPKQPLEYNLKQDKMAEAYSEIGMKERRGKHDGQYLQGLSSTKDTYDALMQLPPRRB. 19B8z T cells were polyclonally restimulated with APC-ms or Dynabeads as described above. CAR expression was evaluated by primary staining with biotin-Protein L (Genscript) and secondary staining with APC-streptavidin (Biolegend).

**Xenograft B-cell lymphoma model.** Female, NOD.Cg-PkdcreID122rgm1(Wj)/SzJ mice (NSG) mice (6 weeks, ~15 g; Jackson Laboratories) were used for all in vivo studies involving restimulated 19B8z T cells. NSG mice were inoculated with 1 × 10^6 luciferized Raji cells intravenously (day 0). On day 4, mice were randomized into treatment groups within each cage and intravenously administered vehicle (RPMI-1640) or 10^6 19B8z T cells restimulated with either APC-ms or Dynabeads for 7 or 14 d. The investigator performing the 19B8z T-cell infusions was blinded to the treatment group. Prior to cell infusion, cells were collected, washed twice with T-cell media and with RPMI-1640, and then resuspended at the appropriate concentration in RPMI-1640. Dynabeads were magnetically removed (Dynal) and cell products were visually inspected for viable cell viability.
Dynabead contamination before injection. 19BBz T cells restimulated with APC-ms were used without additional purification steps. Tumor burden was evaluated over time by intraperitoneal Luciferin (Gold Biotechnology) injection and luminescence measurements 10 min post-injection via IVIS (Perkin Elmer). Total flux (p/s) per mouse was quantified in regions-of-interest (ROI) defined around individual mice. A single ROI with the same dimensions was placed below the mice as a measure of background luminescence in the image field, due to substantial bleed-over from mice with high tumor burden within the chamber, and used to normalize the signal of all mice imaged within the chamber. Luminescence signal of each individual mouse was reported as normalized total flux, calculated as (total flux)_{mouse ROI}/(total flux)_{background ROI}. One animal was excluded from analysis as it was accidently administered a different dose. Mice were monitored daily for signs of discomfort and euthanized upon losing more than 10% of initial body weight, the development of hind-limb paralysis, or when graft-versus-host disease was evident (hair loss, behavioral changes, decline in overall health).

Statistical analysis. All values were expressed as mean ± s.d., unless otherwise indicated. The required sample size varied depending on the experiment, and for in vivo experiments was determined in G*Power using an a priori analysis, set at significance level 0.05 and power 0.9. The F-test was used to determine the equality of variances. For samples of equal variance, the significance between groups was measured using appropriate two-tailed, parametric tests (e.g., Student’s t-test, two-way ANOVA). For samples of unequal variance, the significance between groups was measured using appropriate two-tailed non-parametric tests (e.g., Mann–Whitney U test). Statistical testing was performed using GraphPad Prism and detailed statistical methods are stated in the text. In all cases, P < 0.05 was considered significant.

Data availability. The authors declare that the data supporting the findings of this study are available within the main manuscript, the supplementary figures, and the tables. Life Sciences and Flow Cytometry Reporting summaries for this article are available.

53. Imai, C. et al. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. Leukemia 18, 676–684 (2004).
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1. Sample size
   Describe how sample size was determined.
   The required samples size varied depending on the experiment and for in vivo experiments was determined in G*Power using an a priori analysis, set at significance level 0.05 and power 0.9.

2. Data exclusions
   Describe any data exclusions.
   All exclusion criteria were pre-established. In the human antigen-specific T cell expansion studies, data obtained from donors in which the baseline frequency of CLG- or GLC-specific CD8+ T cells was below 0.01% in 10000 live single CD8+ events were excluded from analysis for the respective peptide (two incidences occurred). In the in vivo CD19 CAR T cell study, one animal was excluded from analysis as it were given a different dose of therapeutic CAR T cells.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experiments with the exception of the in vivo study were conducted at least two times and could be reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Animals were randomized and allocated to different experimental groups within each cage.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   During the experiments, the investigator administrating the treatment to the animals was blinded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | A statement indicating how many times each experiment was replicated |
| ☑   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☑   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study. Sample size was calculated using G*Power v3 (for in vivo experiments) and statistical testing was performed using GraphPad Prism v6.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company. No unique materials were used in this study.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species). Antibodies were purchased Biolegend, with the exception of Foxp3, which was purchased from eBioscience. Antibodies used in this study included:

- anti-mouse CD3 (100243)
- anti-mouse CD28 (102103)
- anti-human CD3 (317319)
- anti-human CD28 (302903)
- anti-mouse CD3 (100227)
- anti-mouse CD4 (100405)
- anti-mouse CD8 (100711)
- anti-human CD3 (344803)
- anti-human CD4 (344611)
- anti-human CD8 (344705)
- anti-human CD62L (304827)
- anti-human CCR7 (353213)
- anti-mouse granzymeB (515405)
- anti-mouse FoxP3 (12-5773)
- anti-mouse PD-1 (135205)
- anti-human LAG-3 (369303)
- anti-human PD-1 (329919)
- anti-human HLA-A2 (343307)
- anti-human IFNg (506510)
- anti-human TNFa (502906)

Nature Biotechnology: doi:10.1038/nbt.4047
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      T2 cell line: ATCC
      B3Z T cell reporter cell line: ATCC
      B16-OVA: ATCC

   b. Describe the method of cell line authentication used.
      Cells lines were not authenticated.

   c. Report whether the cell lines were tested for mycoplasma contamination.
      The B16-OVA cell line tested negative for mycoplasma. The B3Z and T2 cell lines were not tested for mycoplasma contamination.

   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by iCLAC, provide a scientific rationale for their use.
      No such cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines.

11. Description of research animals
    Provide details on animals and/or animal-derived materials used in the study.
    All animals were female and between 6 and 9 weeks old at the start of the experiment. Specific strains included: C57BL/6J, C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I), BALB/c, and NOD.Cg-Prkdscid Il2rgtm1Wjl/SzJ mice (NSG).

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    No human research participants were involved in this study.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

**Data presentation**
For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. 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).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

**Methodological details**

5. Describe the sample preparation.

Mouse T cells were obtained from the spleen following mechanical disruption and T cell isolation (Miltenyi). Human PBMCs were obtained following a Ficoll-gradient separation. Human T cells or CD14-depleted PBMCs were obtained following an additional T cell isolation or CD14-depletion step (Miltenyi). Cells were washed and then stained directly with fluorophore-conjugated tetramers, antibodies, or live/dead exclusion stain.

6. Identify the instrument used for data collection.

BD LSR fortessa X-20 (5 laser)

7. Describe the software used to collect and analyze the flow cytometry data.

Treestar FlowJo v10

8. Describe the abundance of the relevant cell populations within post-sort fractions.

At least 10,000 relevant events were acquired for all FACS analysis. For the human antigen-specific T cell expansion experiments, donors in which the baseline frequency of CLG- or GLC-specific CD8+ T cells was below 0.01% in 10000 live single CD8+ events were excluded from further analyses for the respective peptide (two incidences occurred).

9. Describe the gating strategy used.

In general, cells were first gated by FSC-A/SSC-A to exclude debris, then FSC-A/FSC-H to exclude doublets, followed by live/dead staining (Thermo Fisher), and then followed by the specific antibody or tetramer stain (e.g., CD3, CD8, GLC tetramer, etc.). Boundaries between positively- and negatively-stained cells were defined based on fluorescence-minus-one (FMO) controls.

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