Expression of inducible factors reprograms CAR-T cells for enhanced function and safety

Highlights

- Uni-Vect combines constitutive and inducible expression in a single lentivirus
- IL-12 delivered by Uni-Vect safely enhances the efficacy of CAR-T cells in vivo
- Autonomous release of an IL-6R-blocking antibody in CAR-T cells ameliorates CRS
- Transient transcription factor expression improves CAR-T cell expansion in vivo

Authors

Anze Smole, Alexander Benton, Mathilde A. Poussin, ..., Beatriz M. Carreno, Monica Casucci, Daniel J. Powell, Jr.

Correspondence

anje.smole@nib.si (A.S.), poda@pennmedicine.upenn.edu (D.J.P.)

In brief

Smole et al. develop a single-vector lentiviral system that combines constitutive and antigen-inducible transgene expression, called Uni-Vect. The therapeutic impact of Uni-Vect is demonstrated in CAR-T cells using an immunostimulatory cytokine that safely enhances efficacy, an antibody that ameliorates cytokine-release syndrome (CRS), and a transcription factor that enhances expansion.
Expression of inducible factors reprograms CAR-T cells for enhanced function and safety

Anže Smole,1,2,3,10,13,* Alexander Benton,1,7,13 Mathilde A. Poussin,1 Monika A. Eiva,1 Claudia Mezzanotte,4 Barbara Camisa,1 Beatrice Greco,1 Prannda Sharma,1,11 Nicholas G. Minutolo,1,17 Falon Gray,1 Adham S. Bear,1,9 Miren L. Baroja,1,12 Casey Cummins,1,2 Chong Xu,1,11 Francesca Sanvito,5 Andrea Lang Goldgewicht,1 Tatiana Blanchard,1,14 Alba Rodriguez-Garcia,1,16 Michael Klichinsky,1 Chiara Bonini,6 Carl H. June,1,2,3 Avery D. Posey, Jr.,1,2,7,8 Gerald P. Linette,1,7,16 Beatriz M. Carreno,1,2,17 Monica Casucci,1 and Daniel J. Powell, Jr.1,2,3,14,*

1Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
2Parker Institute for Cancer Immunotherapy, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
3Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
4Innovative Immunotherapies Unit, Division of Immunology, Transplantation, and Infectious Diseases, IRCCS Ospedale San Raffaele Scientific Institute, 20132 Milan, Italy
5Pathology Unit, Division of Experimental Oncology, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy
6Experimental Hematology Unit, Division of Immunology, Transplantation, and Infectious Diseases, IRCCS Ospedale San Raffaele Scientific Institute and University Vita-Salute San Raffaele, 20132 Milan, Italy
7Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
8Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA, USA
9Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
10Present address: National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Immunology and Cellular Immunotherapy (ICI) Group, 1000 Ljubljana, Slovenia
11Present address: Department of Otorhinolaryngology – Head and Neck Surgery, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
12Present address: Department of Hematology and Oncology, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clinic, Barcelona, Spain
13These authors contributed equally
14Lead contact
*Correspondence: anze.smole@nib.si (A.S.), poda@pennmedicine.upenn.edu (D.J.P.)
https://doi.org/10.1016/j.ccell.2022.11.006

SUMMARY

Despite the success of CAR-T cell cancer immunotherapy, challenges in efficacy and safety remain. Investigators have begun to enhance CAR-T cells with the expression of accessory molecules to address these challenges. Current systems rely on constitutive transgene expression or multiple viral vectors, resulting in unregulated response and product heterogeneity. Here, we develop a genetic platform that combines autonomous antigen-induced production of an accessory molecule with constitutive CAR expression in a single lentiviral vector called Uni-Vect. The broad therapeutic application of Uni-Vect is demonstrated in vivo by activation-dependent expression of (1) an immunostimulatory cytokine that improves efficacy, (2) an antibody that ameliorates cytokine-release syndrome, and (3) transcription factors that modulate T cell biology. Uni-Vect is also implemented as a platform to characterize immune receptors. Overall, we demonstrate that Uni-Vect provides a foundation for a more clinically actionable next-generation cellular immunotherapy.

INTRODUCTION

Engineered T cell immunotherapies mediate unprecedented clinical responses in hematological malignancies.1–6 However, their therapeutic efficacy is limited by immunosuppression or T cell dysfunction.7 Furthermore, adoptive cellular immunotherapies can cause life-threatening complications such as cytokine-release syndrome (CRS) and neurological toxicity.8

Attempts to enhance the efficacy of redirected T cells often involve arming with soluble factors or engineered surface receptors.9 In particular, IL-12 is being developed for delivery in a constitutive10,11 or inducible, nuclear factor of activated T cells (NFAT)-driven12–15 manner. Tumor-infiltrating lymphocytes (TILs) transduced with NFAT-driven IL-12 were previously tested in human clinical trials.16 However, the trial was closed due to toxicity likely related to ectopic IL-12 expression by TILs, leading to the conclusion that further refinement would be necessary before this approach could be safely used in a clinical setting.

A key challenge is genetically integrating both an immune receptor and an accessory molecule in a clinically feasible and
safe manner. Transient expression of the introduced effector molecule is preferred over constitutive expression to avoid systemic toxicity or malignant transformation of the cellular product. Furthermore, non-human-derived genetic components should be avoided to reduce immunogenicity. The most common approach to achieve transient expression in Chimeric Antigen Receptor (CAR)-T cell is a dual vector system in which separate viruses enable constitutive expression of a CAR and inducible expression of an accessory molecule. These systems result in non-homogeneous genetic modifications of the cellular product, which require costly cell sorting and can limit translational potential.

Viral systems that combine NFAT-driven expression of one transgene with constitutive expression of a second transgene in a single vector are an improvement on two-vector systems. As a route toward clinical translation, we developed and characterized unique genetically integrated systems that combine autonomous antigen-induced production of an accessory molecule with constitutive immune receptor expression in a single vector, referred to as Uni-Vect. We first demonstrate the applicability of Uni-Vect as a research tool for developing immune receptors. Next, we focus on in vivo therapeutic applications and feature three approaches to upgrade CAR-T cell functions by (1) enhancing anti-tumor activity with human IL-12, (2) ameliorating CRS with secretion of an IL-6 receptor α (IL-6Rα) blocking antibody, and (3) programming T cell intrinsic features and expansion via transient transcription factor expression.

RESULTS

Design, development, and validation of Uni-Vect in primary human T cells

To develop Uni-Vect, a versatile platform for cellular immunotherapy, we combined a constitutive immune receptor (CAR or T cell receptor (TCR)) with inducible effector molecule expression in a single lentiviral vector system (Figure 1A). To achieve a transient response, we rewired endogenous T cell activation-mediated NFAT signaling, triggered by an immune receptor’s recognition of its cognate target, to the expression of an accessory molecule. Using fluorescent reporter proteins, we tested several designs with varied orientation of the inducible and constitutive modules (forward or reverse), activation-inducible promoters (P_CD69, P_CD137, and P_NFAT), and presence of the SV40 polyadenylation signal (SV40 pA) (Figures S1A–S1E).

Two configurations with NFAT-driven inducible modules emerged as the most relevant candidates (Figure 1B).

The first architecture, pASP4.2 (ieGFP + cmCherry, Table S1), places inducible EGFP upstream of a constitutive EF1α-driven mCherry module in a forward orientation. In both Jurkat (Figure S1C) and primary human T cells (Figure 1B), we observed coordinate expression of constitutive mCherry and inducible NFAT-driven expression of EGFP only upon activation, demonstrating that Uni-Vect functioned as designed. This architecture demonstrated tight regulation of the inducible module with no detectable expression of EGFP in an unstimulated state (Figure 1B), but resulted in relatively low virus titer (Figure S1C). To improve virus titers and the output of the inducible module, we tested a second architecture, pASPS (ieGFP + cmCherry,
Figure 1. Design and validation of Uni-Vect in primary human T cells
(A) Schematic representation of Uni-Vect recognizing a target antigen.
(B) Primary human T cells were transduced with pASP4.2 or pASP5 at MOI 5 and stimulated. Expression of reporter genes was monitored and quantified as MFI after 24 h (n = 3) (Welch’s ANOVA).
(C) Schematic representation of compared constructs/experimental groups.
(D and E) Primary human T cells were transduced with the vectors shown in (C). Cells were sorted into CAR+ and CAR−/C0 fractions and then stimulated. (D) EGFP expression of cells activated with phorbol 12-myristate 13-acetate (PMA) + ionomycin overnight. Histograms are shown for CAR+ and CAR−/C0 fractions. (E) Cells were left unstimulated or stimulated through TCRs or CARs for 24 h. MFI of EGFP is shown (n = 3) (Student’s t test). MFI, mean fluorescence intensity. All data with error bars are presented as mean ± SD. All data are representative of two or more experiments. See also Figures S1 and S2 and Table S1.
Figure 2. T cell receptor knockout in Uni-Vect CAR-T cells

(A) Uni-Vect CAR-T cells may be stimulated by either the introduced CAR or the endogenous TCR. Endogenous TCR knockout prevents expression of the accessory molecule induced by endogenous TCR signaling.
T cells, the CAR+ population homogeneously expressed EGFP, to integrate into the same genomic locus at equimolar ratios, since system, both the accessory and the CAR modules are expected 

neous modification of the cellular product whereby both CAR+ two-component system resulted in separate integration events (CAR, Table S1) (Figure 1C). With Uni-Vect and the constitutive single-vector system (constitutive eGFP-2A-HER2-41BB CAR, Table S1) (Figure 1D). This heterogeneity also resulted in reduced output of the inducible module when CAR+ cells were activated through either CARs or TCRs (Figure 1E). Together, these results demonstrate how Uni-Vect delivers more accessory molecule expression in CAR+ cells and less dysregulated expression in CAR− cells than two-vector systems. Apart from a simplified modular assembly of lentiviral transfer plasmid and virus production where only one viral product is manufactured, Uni-Vect also removes any need for sorting of the cellular product.

Like Uni-Vect, the constitutive system is expected to integrate accessory and CAR modules into the same genomic locus at equimolar ratios. While the constitutive system generated a homogenous product, its basal expression of EGFP in the unstimulated state exceeded that of Uni-Vect T cells that were stimulated through the CAR via HER2 beads (Figure 1E). In contrast, the ability of Uni-Vect to transiently achieve the levels of expression that are basal in constitutive systems demonstrates its potential to deliver potent immune modulators with an enhanced safety profile.

Endogenous TCR knockout renders CAR signaling an exclusive activator of the system

CAR-T cells express endogenous TCRs that can recognize epitopes presented in pMHCs (Peptide-major histocompatibility complex) to trigger T cell activation, thus upregulating NFAT. Because the inducible Uni-Vect module senses NFAT signaling, it may be activated by target antigen stimulation via the CAR or through the T cell’s endogenous TCR upon recognition of specific pMHC (Figure 2A). Stimulation through the TCR causes undesired accessory molecule expression independent of the presence of a target tumor antigen.

To reduce potential toxicity related to unintentional triggering of NFAT-inducible accessory molecule expression, the endogenous TCR was knocked out using a CRISPR-Cas9 method compatible with lentiviral transduction of Uni-Vect (Figure S3A). Using sgRNAs (single guide RNA) targeting genes encoding TCRα and TCRβ, the endogenous TCR was knocked out in primary human T cells with 80%–90% efficiency (Figure S3B). These primary T cells were then transduced with pASP85 (ieGFP+chER2-41BB CAR, Table S1), (ieGFP+cHER2-41BB CAR, Table S1), and the remaining TCR+ cells were removed by negative selection (Figure S3B). The expansion of edited cells was only partially impaired compared with mock, due to the negative selection step following TCR knockout during expansion (Figure S3C).

Following expansion, pASP85-transduced TCR+ and TCR− cells were stimulated through TCRs and analyzed by flow cytometry (Figure 2B). While unedited TCR+ cells produced a high EGFP mean fluorescence intensity (MFI), the response of TCR− cells remained comparable to that of unstimulated controls, indicating functional knockout. Importantly, TCR+ and TCR+ CAR-T cells responded similarly to a HER2+ cell line (Figure 2C). Cytotoxic T cell function of the edited CAR-T cells was not affected (Figure 2D), indicating that TCR-knockout Uni-Vect cells retain CAR-mediated effector functions. These data provide a proof of concept for the knockout of an endogenous TCR when introducing Uni-Vect into primary human T cells for the creation of CAR-T cells with precise transgene expression. This may improve the safety of armored CAR-T therapies, especially when potent accessory molecules are utilized.

Uni-Vect expression cassettes for characterization of T cell receptors

We predicted that a built-in NFAT-inducible reporter of T cell activation in the Uni-Vect system could provide a platform to functionally assess TCRs. Therefore, we developed a Jurkat E6.1 cell line deficient in endogenous TCR expression and with introduced CD8αβ (to enable major histocompatibility complex MHC class I recognition by the introduced TCR) and a Uni-Vect system featuring NFAT-inducible EGFP in the forward (pASP89, Table S1) or reverse (pASP90, Table S1) architecture (Figure 3A). Cells were sorted for the TCR+/CD8αβ/mCherry (Uni-Vect) population to generate JpASP89 and JpASP90 cell lines. Melanoma neoantigen (PORCN H364Y)-specific TCRs and β chains were introduced into these cell lines and βTCR expression was confirmed. Expression of the TCR conferred reactivity to a peptide (LLHGFSFYL/HLA-A*02:01; p/HLA) multimer, demonstrating assembly and antigen specificity (Figures 3B and S4A).

Next, we studied PORCN H364Y-specific JpASP90 or JpASP89 using titrated peptide concentrations in the presence of antigen-presenting cells (monoallelic HLA-A*02:01-expressing K562). Increasing peptide concentrations led to increased EGFP expression, demonstrating the Uni-Vect system’s utility as a platform to monitor TCR activation (Figures 3B, S4A, and S4B). Antigen stimulation of JpASP90 cells resulted in EGFP expression in up to 80% of cells, accompanied by a 5-fold increase in MFI. CD69 upregulation and production of GM-CSF, IL-2, TNF-α, and IL-8 correlated with EGFP expression as a readout of T cell activation (Figure S4C).

To further evaluate our platform, six distinct TCRs directed at HLA-A*02:01-restricted neoantigen peptide complexes (Table S2) were introduced into JpASP90 cells. All, except for
SEC24A P469L-specific TCRs, had similar expression (Figure 3C). However, a range of MFI intensities was observed when p/HLA multimers were used as staining reagents, suggesting these TCRs may display different avidities for their corresponding p/HLA complexes. This was confirmed by titration of neoantigenic peptides that demonstrated a range of avidities among TCR-engineered JpASP90 cells, with those cells expressing SEC24A P469L TCR requiring the highest peptide concentration to achieve 50% cell activation (Figure 3D and Table S2). In addition, two TCRs (PORCN H346Y and TMEM48 F169L) recognized wild-type peptide at high peptide concentrations. This finding is consistent with our previous reports demonstrating cross-reactivity in primary PORCN H346Y- and TMEM48 F169L-specific CD8+ T cells with these receptors.26,27

Finally, for discovery of tumor-reactive TCRs, the ability of this platform to be activated by endogenously processed and presented antigens is of critical importance. To this end, DM6 melanoma cells were engineered to express tandem minigene constructs (TMCs) encoding a mutated (MUT) or wild-type (WT) peptide inserted into a 27-amino-acid framework, allowing for minimal epitope processing and presentation. As shown in Figure 3E, JpASP90 cells expressing the various TCRs upregulated EGFP expression when co-cultured with DM6 cells expressing the MUT but not WT TMC. Only cells expressing PORCN H346Y-specific TCRs were activated by the WT TMC, a finding consistent with prior results obtained when loaded peptides were used as antigens (Figure 3D). Overall, these results demonstrate that the Uni-Vect NFAT sensor represents a reliable platform to characterize expression, specificity, and functional avidity of TCRs.

**IL-12-enabled Uni-Vect improves anti-tumor efficacy of CAR-T cells in vivo**

IL-12 is a pleiotropic pro-inflammatory cytokine, but the severe side effects associated with systemic administration have limited its use in cancer patients.12,28 However, CAR-T cells expressing constitutive IL-12 are currently under clinical investigation (NCT02498912) and are considered a current state-of-the-art treatment. We hypothesized that localized CAR-inducible secretion of IL-12 would increase the efficacy of CAR-T cells without causing significant toxicity (Figure 4A). To test our hypothesis we first placed human IL-12 into the low-leakage forward architecture of Uni-Vect to generate construct pASP18 (iIL-12+cHER2, Table S1). To test for reversible expression of IL-12, pASP18 T cells were stimulated for 2 days, followed by 2 days of rest, and restimulated for 2 days, each time replacing the medium. Secreted hIL-12 was detected only when cells were stimulated, demonstrating a tightly regulated response that was also fully reversible and capable of responding to a second activation event (Figure 4B). Control CAR-T cells secreted no IL-12 after stimulation (Figure S5A).

We generated iIL-12-CAR-T cells by combining inducible hIL-12 with a constitutive HER2 targeting CAR in construct pASP38 (iIL-12+cHER2-41BB CAR, Table S1). As controls, we generated HER2-targeting CAR-T cells with ieGFP (pASP26, ieGFP+cHER2-41BB CAR, Table S1), constitutive hIL-12 expression (chIL-12, chIL-12+cHER2-41BB CAR, Table S1), and a two-vector system (Figure 4C). Following in vitro expansion, we found that the phenotypic effector memory compartment was consistently enriched in cellular products that comprised engineered IL-12 either in inducible or in constitutive mode of expression (Figure S5B), presumably due to previously described mechanisms.30 Similar lysis was observed between all cellular products in co-culture with a target cell line (Figure 4D). Inducible expression of hIL-12 in the presence of HER2+ SKOV3 cells was higher in Uni-Vect compared with the two-component system, demonstrating greater on-target delivery. When left unstimulated or in the presence of an antigen-negative cell line, inducible hIL-12 secretion was at or below our detection limit (Figure S5C). A marked increase in IFN-γ was observed in iIL-12 compared with control ieGFP CAR-T cells (Figure 4E), demonstrating the function of inducible hIL-12 as consistent with its endogenous role in inducing T_h responses and promoting IFN-γ secretion.

To test iIL-12-CAR-T cells in vivo, we first determined the efficacy of iIL-12-CAR-T cells at a dose of 0.5 million cells per mouse (Figure S5D). iIL-12-CAR-T cells were able to eliminate tumors, unlike control HER2 CAR-T cells lacking iIL-12, as demonstrated by imaging (Figure S5E), tumor volume (Figure S5F), and survival (Figure S5G). CD8 and CD4 T cells in peripheral blood persisted only in mice receiving iIL-12-CAR-T cells (Figure S5I). There were no signs of toxicity or detectable differences in weight (Figure S5J).

To demonstrate improved safety with respect to currently available constitutive IL-12 alternatives, we performed an experiment similar to the above (Figure 4F), which recapitulated improved efficacy data at a dose as low as 0.1 million cells per mouse (Figures 4G–4I). We found that 80% of mice receiving Uni-Vect ihIL-12+cHER2 CAR-T cells cleared tumors (Figures 4G and 4H) and survived (Figure 4I), while all mice with chIL-12 expression died owing to toxicity related to unregulated release of IL-12. In contrast, ihIL-12 did not cause observable toxicity or weight loss in surviving mice (Figure 4J). Although not significant, T cells in peripheral blood were higher in ihIL-12 compared with control EGFP CAR-T cells, and T cell counts were elevated in survival
chIL-12 compared with the inducible setting, which may have contributed to toxicity (Figure 4K).

Importantly, control iIL-12-CAR-T cells targeting an irrelevant antigen, CD20 (pASP36; iIL-12+cCD20-41BB CAR, Table S1), were unable to control HER2}\(^+\) tumor cell growth, indicating that xenoreactivity is not responsible for tumor clearance in our model (Figure S5K). To further address this possibility, we tested whether TCR knockout iIL-12-CAR-T cells were also able to control tumor growth. In the same model, edited TCR-knockout iIL-12-CAR-T cells were effective at a dose of 0.15 million CAR\(^+\) per mouse (Figure S5L). Together, these results demonstrate how potent immune modulators delivered with Uni-Vect fit within a tight therapeutic window, which is essential for their use in armored CAR-T cell therapies.

**Antigen-inducible secretion of tocilizumab-based scFv-Fc from CAR-T cells to ameliorate CRS in vivo**

Systemic administration of the IL-6R\(\alpha\) blocking antibody tocilizumab is the predominant clinical strategy to control CRS associated with CAR-T cell therapy.\(^{31}\) However, controlling neurological toxicity is difficult, because the blood-brain barrier restricts entry of tocilizumab into the central nervous system, in some cases necessitating invasive intrathecal administration.\(^{8,32}\) Therefore, we utilized Uni-Vect to develop CAR-T cells that autonomously deliver a tocilizumab-based antibody (Figure 5A).

First, we evaluated the functionality of Uni-Vect in the context of hematological malignancies, where treatment with CAR-T cells is associated with CRS. A CD20 CAR was integrated in the reverse architecture plasmid to achieve high levels of inducible expression, generating pASP28 (ieGFP+cCD20-41BB CAR, Table S1). Robust EGFP expression was concurrent with target cell lysis when pASP28-CAR-T cells were co-cultured with CD20-transduced K562, but not with the CD20\(^-\) parental line (Figures 5B and S6A).

We then designed an IL-6R\(\alpha\) blocking antibody by linking the tocilizumab-derived heavy and light chains\(^{33}\) in an scFv (single-chain variable fragment) format fused with human IgG1 Fc (fragment crystallizable) to obtain anti-IL6R\(\alpha\) scFv-Fc, referred to as iToci (Table S1). This molecule was cloned into the inducible module of pASP28 to generate pASP52 (iToci+cCD20-41BB CAR, Table S1). CAR-T cells expressed iToci in the presence of CD20 antigen (Figure 5C) and had similar phenotype compared with control ieGFP CAR-T cells following ex vivo expansion (Figure S6B). Next, we validated iToci-CAR-T cells in the context of a second hematological antigen system using a clinically applicable CD19 CAR,\(^{1,5}\) using the newly generated pASP97 (iToci+cCD19-41BB CAR, Table S1) and control pASP96 (ieGFP+cCD19-41BB CAR, Table S1). In primary T cells, both constructs lysed a CD19\(^+\) cell line with similar efficacies, concomitant with inducible antibody secretion (Figure 5D). Retention of CAR-mediated lysis demonstrated that secretion of iToci did not impair the cytotoxic activity of engineered cells in vitro. To demonstrate the functionality of the autonomously secreted IL-6R\(\alpha\)-blocking antibody from primary T cells, we investigated its binding capacity to human IL-6R\(\alpha\) expressed on the cell surface. Supernatant from iToci-producing, but not control, CAR-T cells stained IL-6R\(\alpha\)-expressing cells, demonstrating specific binding activity (Figure 5E).

Uni-Vect is modularly designed, which makes it amenable to customized design regarding the selection of the receptors and effectors. In addition, by exchanging the DNA response element, it can be engineered to sense customized inputs. Here, Uni-Vect was re-configured (Figures S6C and S6D) to develop a cell-based assay to interrogate IL-6 signaling and determine whether anti-IL6R\(\alpha\) scFv-Fc is bio logically active as an IL-6 blocking agent (see STAR Methods). Specific and dose-dependent blocking of IL-6 signaling was observed, albeit at a level less efficient than recombinant tocilizumab (Figure S6E). These results demonstrate that the engineered Fc-based IL-6R\(\alpha\) blocking antibody is biologically active.

Finally, we tested if iToci-CAR-T cells can ameliorate CRS in vivo in a humanized mouse model.\(^{34,35}\) Manufactured iToci (pASP97; iToci+cCD19-41BB CAR, Table S1) and control ieGFP (ieGFP+cCD19-41BB CAR, Table S1) CAR-T cells demonstrated similar in vitro expansion (Figure S6F). After verifying the engraftment of human hematopoietic stem cells in SGM3 mice, they were challenged with NALM6 cells (Figure 5F). Seven days later, iToci or control ieGFP CAR-T cells were injected, and the mice were monitored for CRS manifestation over the next 13 days. We found that both iToci and control IgG CAR-T cells fully controlled tumor growth (Figure 5G). In contrast to control CAR-T cells, CRS was ameliorated in the iToci group as indicated by only transient weight loss (Figure 5H) and survival of 100% compared with 37% in control mice (Figure 5I). Of note, although similar at day 4, T cell expansion was lower in iToci compared with the control group as CRS progressed at later time points (Figure 5J). Key inflammatory cytokines associated...
Figure 5. Antigen-inducible secretion of biologically active tocilizumab-based scFv-Fc from CAR-T cells

(A) Schematic of iToci integrated into Uni-Vect.

(B) pASP28-CAR-T cells were co-cultured with CD20+ or CD20− K562 cells at a 5:1 effector-to-target ratio for 24 h. Flow graph represents inducible EGFP, concomitant with target cell lysis. K562 cell lines (black rectangle) were stained for glycophorin A expression and appear as a glycophorin+EGFP− population. CAR-T cells appear in the glycophorin− population (green rectangle).

(C) pASP52-CAR-T cells were co-cultured with CD20+ or CD20− K562 cells at a 3:1 effector-to-target ratio for 72 h. Antigen-dependent secretion of iToci was measured (n = 3).
with CRS severity were significantly decreased in the iToci group on day 4 after infusion (Figure 5K). This includes IL-6, which counts clinical observations following tocilizumab administration. Notably, 2/8 instances of hemorrhages were observed with control CAR-T, while none were observed in the iToci group (Figure S6G). In one mouse with multifocal microhemorrhages, a parenchymal hemorrhage of the cerebellum (Figure S6G, left), and a parenchymal hemorrhage of the cortex (Figure S6G, right) were observed.

In a repeated experiment, the amelioration of CRS was tested in a more stringent model with rapid weight loss and higher levels of secreted cytokines. The explanation for such challenging conditions is not clear and may have to do with donor-intrinsic factors of the cord-blood hematopoietic stem cells (HSCs) or CAR-T cells themselves. Even under these challenging conditions, iToci was still effective at providing a significant advantage in terms of survival (Figure S6H) and decreased release of cytokines (Figure S6I). Overall, these data demonstrate that Uni-Vect can be implemented for autonomous antigen-triggered secretion of biologically active IL-6Rα blocking antibody by CAR-T cells in situ to ameliorate CRS.

Inducible transcription factors program relevant CAR-T cell states and improve expansion in vivo

Transcription factors are master regulators of T cell differentiation, expansion, fitness, and anti-tumor responses. These features, which we refer to as therapeutically relevant T cell states (TRTSs), are highly present in successful CAR-T therapies. Here, we explored the implementation of Uni-Vect to install the transcription factors FOXO1 and TCF7, which are associated with early differentiation states, T cell memory, and a functional phenotype, to modulate TRTSs. Using Uni-Vect, we combined NFAT-inducible TCF7 in the construct pASP72 (iTCF7+cHER2-41BB CAR, Table S1) or FOXO1 in construct pASP73 (iFOXO1-3A+cHER2-41BB CAR, Table S1) with a constitutive HER2-targeting CAR (Figure 6A). For the latter, we created a human FOXO1 version called FOXO1-3A that is resistant to AKT-mediated phosphorylation and hence insensitive to nuclear export.

In CAR-T cells exposed to repeated stimulations with HER2 
SKOV3 target cancer cells in vitro (Figure S7A, top), we found that antigen-dependent FOXO1-3A expression preserved a less differentiated phenotype, as determined by the increased frequency of CD62L+ CD45RA+ CAR-T cells compared with control cells. This population was CD95+, indicating a T memory stem cell phenotype. These effects were observed in CAR+ but not in CAR− populations (Figure 6B).

In experiments where sorted CAR+ iTF-CAR-T cells were restimulated multiple times with SKOV3 targets in vitro (Figure S7A, bottom), inducible expression of both TCF7 and FOXO1-3A enhanced antigen-dependent proliferation of iTF-CAR-T cells and decreased expression of the inhibitory marker Lag3 (Figure 6C). CyTOF (Cytometry by time of flight) analysis of iTF-CAR-T cells revealed that FOXO1-3A favorably affected T cell markers associated with effector function, including increased expression of IFN-γ and T-Bet, and a reduction in the inhibitory marker TIGIT (Figure 6D). Incorporation of the iTFs did not impair the cytolytic capacity of HER2-targeting CAR-T cells at the time of manufacturing or after the generated infusion product was subjected to several rounds of antigen exposure (Figure S7B).

To reduce the possibility that the transduction efficiency and/or levels of CAR expression would affect the results, prior to functional assays we normalized the frequency of CAR+ cells based on the initial transduction and monitored MFI as a measure of CAR expression. Although the transduction efficiency varied depending on the genetic construct, the MFI of CAR+ cells was similar among the groups for all in vitro and in vivo experiments (Figure S7C).

We then tested the in vivo activity of iTCF7 and iFOXO1-3A compared with control ieGFP-CAR-T cells. Mice harboring an established subcutaneous HER2+ SKOV3 ovarian tumor received 0.7 million CAR+ iTF-CAR-T cells (Figure 6E). At the dose administered, all three cellular products, with the exceptions of two mice in the ieGFP and one in the iFOXO1-3A group, cleared tumors, and the mice survived after rechallenge with SKOV3 (Figures S7D and S7E). We noticed minor differences in kinetics that in mice in the inFOXO1-3A group seemed to have the fastest response to rechallenges (Figure S7D). We simultaneously investigated how inducible TCF7 and FOXO1-3A affected CAR-T cell expansion and persistence in vivo. Expansion of T cells in peripheral blood on day 28 was significantly increased in the iFOXO1-3A-CAR-T cell group (14-fold) compared with control CAR-T cells, while the effect of activation-induced TCF7 expression was less prominent (Figures 6F and S7F for individual mice). Importantly, iFOXO1-3A CAR-T cell expansion was transient, and ultimately contracted after the tumor was cleared. Upon a second rechallenge on day 77, iFOXO1-3A CAR-T cells again expanded significantly more than control cells. We did not observe toxicity or weight loss in any responding mice even at the point of peak expansion in the iFOXO1-3A-CAR-T cell group (Figure S7G).

The improved in vitro expansion and accompanying differences in the phenotype of the manufactured iFOXO1-3A cellular product led us to investigate what differentiation states and phenotypes were associated with increased in vivo expansion. We analyzed the phenotype of CAR+ T cells from mice in different treatment cohorts (Figure S7H for gating strategy). A consistent increase in the effector/EMRA (effector memory T cells

(D) pASP97-CAR-T cells or control pASP96-CAR-T cells were co-cultured with the CD19+ NALM6 cell line for 48 h. Lysis was measured by luciferase assay (left) and concomitant secretion of iToci was measured by ELISA (right) (n = 3).

(E) pASP97-CAR-T cells or control pASP96-CAR-T cells were injected into humanized SGM3 mice engrafted with the CD19+ NALM6 cell line (n = 8 mice per group for iToci+cCD19 CAR and ieGFP+cCD19 CAR; n = 5 for untransduced group). The experiment was performed once.

(F) In vivo study design. pASP97-CAR-T cells or control pASP96-CAR-T cells were injected into humanized SGM3 mice engrafted with the CD19+ NALM6 cell line (n = 8 mice per group for iToci+cCD19 CAR and ieGFP+cCD19 CAR; n = 5 for untransduced group). The experiment was performed once.

(G–K) We monitored (G) tumor growth control, (H) weight loss (two-way ANOVA), (I) survival of mice (log-rank Mantel-Cox test), and (J) hCD45+CD3+ T cell numbers in peripheral blood (two-way ANOVA). (K) Inflammatory cytokines were measured in serum 4 days after CAR-T cells injection (two-way ANOVA). SN, supernatant; NS, not significant. Error bars are presented as mean ± SD in (C) and (D) and as mean ± SEM in (G), (H), (J), and (K). See also Figure S6 and Table S1.
**Article**

**Cancer Cell**

40, 1470–1487, December 12, 2022

---

**Figure A**

- CAR-T CELL
- IMMUNE RECEPTOR
- TARGET CELL
- UNI-VECT

**Legend**
- Autonomous antigen-induced expression
- IL-2: Enhanced anti-tumor efficacy
- TCR: Amelioration of CRS
- Transcription Factors: Improved T cell intrinsic properties

**Figure B**

- eGFP
- TCF7
- FOXO1-3A

**Figure C**

- CellTrace Violet
- Lys3

**Figure D**

- IFNγ
- T-Bet
- TIGIT

**Figure E**

- SKOV3 (Luc', GFP')
- SKOV3 (Luc', GFP')

**Figure F**

- CD45^+CD8^+ (CellRC)
- Time (days)

**Figure G**

- Time (days)

---

(legend on next page)
re-expressing CD45RA+ T cell compartment (CD62L- , CD45RA+) was observed in the iFOXO1-3A-CAR-T cell cohort compared with control CAR-T cells at the later stages of the experiment (Figure 6G). At the time of peak expansion on day 28 that was not the case, but we did observe a significant increase in the EM (Effector memory) compartment (CD62L+ , CD45RA-). Together these results demonstrate that inducible FOXO1-3A expression in CAR-T cells improves TRTS, which translated into improved antigen-dependent and transient expansion in vivo, while the favorable phenotypic profile may support improved fitness.

**DISCUSSION**

T cell-intrinsic dysfunctions and the immunosuppressive tumor microenvironment are key factors limiting the clinical efficacy of CAR-T immunotherapies. Even when efficacious, significant CAR-T cell toxicity can limit their therapeutic potential. These shortcomings emphasize a dire need for strategies to safely outfit T cells with critical attributes required for effective therapy. We addressed this challenge through development of the Uni-Vect platform: a single lentiviral vector that combines activation-induced production of an accessory molecule and constitutive expression of an immune receptor.

During development of the system, two distinct configurations emerged. A forward architecture demonstrated low leakage for tight control of potent immune modulators, and a reverse architecture achieved high levels of inducible expression. The observed increase in transcriptional activity of a non-activated inducible module in the reverse orientation (Figure S2B) is unlikely to be due to tonic signaling (Figure S2G) and may be explained by bidirectional activity of the EF1α promoter in a combination with PMIN. Indeed, bidirectional promoters have been previously developed and characterized in lentiviral vectors. The proximity of the EF1α promoter to the NFAT response element might also increase the accessibility of the transcriptional machinery to enhance inducible output in the reverse architecture.

The limitations in clinical activity and safety of CAR-T cells highlight the need to accessorize, or “armor,” CAR-T cells. However, current strategies to engineer T cells with inducible factors using activation-triggered promoters are at risk of dysregulated accessory molecule expression. Our approach integrates endogenous TCR knockout in a CAR-T cell manufacturing procedure similar to one used in clinical trials. Although TCR knockout results in a modest reduction in transduction efficiency, small numbers of TCR-knockout, IL-12-secreting CAR-T cells can safely mediate cancer regression (Figure S5L). Moreover, clinical trial results demonstrate that cancer remission can occur with transduction efficiency as low as 5%, and alternative protocols for TCR knockout may improve transduction efficiency.

Alternatively, endogenous TCRs can be edited by integration of a gene encoding an immune receptor into the TRAC locus utilizing CRISPR-Cas9 and either viral or non-viral donor template delivery. In contrast to Uni-Vect, these approaches tested only constitutive immune receptor expression. Another strategy employed TALEN technology with a virally delivered donor template to place a CAR into the TRAC locus and IL-12p70 into either IL-2Rα or PDCD1 genes. This approach enabled simultaneous TCR knockout and activation-inducible expression of an effector molecule but, in contrast to Uni-Vect, led to non-homogeneous modification of the cellular product. Integration of Uni-Vect into these genome targeting platforms could thus enable inducible effector molecule and constitutive immune receptor expression in one step for a homogeneous T cell product while knocking out the endogenous TCR or other molecules.

For potential therapeutic implementations of Uni-Vect, we featured three approaches to upgrade CAR-T cell functions. We developed iIL-12-CAR-T cells and demonstrated improved efficacy and safety compared with current alternatives. iIL-12, but not control ieGFP, CAR-T cells were capable of eradicating solid tumors in vivo at low doses. Importantly, iIL-12 did not cause lethal toxicity, as was the case when IL-12 was expressed constitutively (Figure 4). In addition to the transient nature of IL-12 secretion by iIL-12-CAR-T cells, a lower overall level of IL-12 may have contributed to the reduced toxicity in this preclinical model, compared with constitutive IL-12. Liu et al. recently developed a single-vector system in which inducible and constitutive modules were placed into face-to-face orientation and demonstrated that inducible IL-12 improved the anti-tumor activity of CAR-T cells. Alternatively, endogenous TCRs can be edited by integration of a gene encoding an immune receptor into the TRAC locus utilizing CRISPR-Cas9 and either viral or non-viral donor template delivery. In contrast to Uni-Vect, these approaches tested only constitutive immune receptor expression. Another strategy employed TALEN technology with a virally delivered donor template to place a CAR into the TRAC locus and IL-12p70 into either IL-2Rα or PDCD1 genes. This approach enabled simultaneous TCR knockout and activation-inducible expression of an effector molecule but, in contrast to Uni-Vect, led to non-homogeneous modification of the cellular product. Integration of Uni-Vect into these genome targeting platforms could thus enable inducible effector molecule and constitutive immune receptor expression in one step for a homogeneous T cell product while knocking out the endogenous TCR or other molecules.

For potential therapeutic implementations of Uni-Vect, we featured three approaches to upgrade CAR-T cell functions. We developed iIL-12-CAR-T cells and demonstrated improved efficacy and safety compared with current alternatives. iIL-12, but not control ieGFP, CAR-T cells were capable of eradicating solid tumors in vivo at low doses. Importantly, iIL-12 did not cause lethal toxicity, as was the case when IL-12 was expressed constitutively (Figure 4). In addition to the transient nature of IL-12 secretion by iIL-12-CAR-T cells, a lower overall level of IL-12 may have contributed to the reduced toxicity in this preclinical model, compared with constitutive IL-12. Liu et al. recently developed a single-vector system in which inducible and constitutive modules were placed into face-to-face orientation and demonstrated that inducible IL-12 improved the anti-tumor activity of CAR-T cells.

To study tumorigenesis in vivo after initial tumor clearance, the mice were rechallenged twice with SKOV3. Data are representative of two experiments performed.
systems may reveal new principles in engineering complex vector systems.

In our second therapeutic implementation, we addressed CAR-T cell toxicity through autonomous secretion of tocilizumab-based antibody to suppress CRS. To validate the biological activity of iToci, we adapted Uni-Vect to detect IL-6 signaling, which demonstrated modularity that enabled detection of customized signals (Figure S6C). We anticipate that this STAT3-responsive system can also be therapeutically applied in iToci-CAR-T cells to quantitatively respond to IL-6 levels characteristic of CRS. We demonstrated that iToci-CAR-T cells enabled autonomous in situ expression of iToci that ameliorated CRS-related toxicity in a humanized mouse model. A recent study demonstrated that constitutive expression of an anti-inflammatory IL-1 receptor antagonist in CAR-T cells protected mice from severe CRS.48 In contrast, iToci-CAR-T cells are capable of autoregulation to self-sufficiently alleviate CRS manifestations with tocilizumab-based antibody secretion. Interestingly, we observed lower in vivo expansion of iToci-CAR-T cells compared with control CAR-T cells (Figure S5J), implying that multiple factors may have contributed to CRS amelioration. IL-6 signaling blockade may have limited expansion at later time points by reducing the overall inflammatory milieu. This hypothesis is supported by the decreased levels of inflammatory cytokines on day 4 when T cell numbers in the blood were equivalent. Conversely, we cannot exclude that iToci-CAR-T cells can be qualitatively different compared with control CAR-T cells in function in addition to secreting iToci, although in vitro expansion, phenotype, and in vitro and in vivo cytototoxic activity was similar (Figures 5 and S6). Even though further studies are necessary to better understand the underlying mechanisms, we conclude that iToci-CAR-T cells were able to reach the right balance of in vivo expansion and cytokine secretion to be effective in tumor clearance without lethal toxicity (Figures 5G–5K).

Our third therapeutic implementation programs T cell differentiation states, functionalities, and expansion via inducible TF expression. Analysis of iTF-CAR-T cell function and phenotype revealed that antigen-inducible expression of a single TF can favorably affect TRTS (Figures 6B–6D). iFOXO1-3A-CAR-T cells demonstrated remarkably increased peak expansion in peripheral blood compared with control CAR-T cells (Figure 6F). iTF-CART cell expansion was transient due to Uni-Vect, and T cells ultimately contracted after tumors were cleared. This finding highlights the importance of inducible TF expression to reduce the risk of malignant transformation or autoimmunity. No antigen-independent proliferation or sustained growth was observed in vitro or in vivo. FOXO1-3A was recently tested in the context of cellular immunotherapies in which nanocarriers were used to deliver FOXO1-3A mRNA in vitro for transient expression during the manufacturing process to program CAR-T cells in a “hit-and-run” manner.41 Uni-Vect enables permanent genetic modification of the cellular product, while the expression of FOXO1-3A remains transient. This expands the activity of FOXO1-3A beyond T cell manufacturing to autonomously function after infusion. This was demonstrated through an in vitro rechallenge model, where iFOXO1-3A-CAR-T cells developed less differentiated T cell states, increased TSCM pools (Figure 6B), and also increased expression of molecules that indicate efficacy (Figure 6D). Induced differences in T cell states were aligned with improved in vitro and in vivo expansion. Unexpectedly, there was a slight shift to a phenotype associated with effector functions of iFOXO1-3A-CAR-T cells in vivo (Figure 6G). Further in-depth studies are needed to investigate the underlying mechanisms. In our models, inducible TCF7 resulted in modest phenotypic change and increased expansion. In a recent
study, TCF7 was identified as one of the lead candidates that increased T cell proliferation but not tumor clearance by TCR-engineered T cells. A comparison between inducible and constitutive expression of FOXO1-3A and TCF7 in CAR-T is an important next step. However, the potential for uncontrolled proliferation and malignant transformation due to constitutive TF expression may be an unnecessary risk given the improvements already evident when using Uni-Vect.

Engineered T cells expressing transgenic receptors have immense potential as therapeutic agents, but extensive characterization of potential immune receptors is necessary to increase therapeutic efficacy and reduce the risk of adverse events. Although testing receptors in primary T cells is indispensable, a Uni-Vect cell-based system that can assess NFAT activation as a readout of TCR signaling provides a rapid screening assay to qualify receptors for further studies. We utilized Uni-Vect to develop J\textsubscript{ASP90} or J\textsubscript{ASP89} and demonstrated how TCRs engineered into these reporter cell lines can be characterized for antigen specificity and avidity (Figure 3) with a pattern of recognition identical to T cells from which the receptors were isolated. Compared with other antigen-responsive reporter cell lines that use two-component systems, Uni-Vect enables homogeneous modification and straightforward selection of cells that eliminates the need for clonal selection and activation to reveal the signal. Furthermore, Uni-Vect allows for rapid query of cell line panels or tumors for processing and presentation of specific tumor antigens (Figure 3) and for screening of antigen expression on non-targeted tissues to avoid toxicity. This system may be complementary to other recently developed platforms. Uni-Vect lays the foundation for a single-vector system with fixed stoichiometry for researchers who need to characterize the activity of candidate immune receptors in either cell lines or primary cells.

Our findings demonstrate that Uni-Vect is a broadly applicable platform for upgrading T cells in a user-friendly plug-and-play manner (Figure 7) and enables the manufacturing of well-defined cell products with homogeneous transgene distribution. We demonstrate three therapeutic implementations of Uni-Vect in developing CAR-T cells with upgraded functionality. In addition, Uni-Vect can be utilized to screen and characterize antigen-specific immune receptors. While these implementations are significant and have therapeutic potential, the downstream applications of Uni-Vect extend well beyond what could be covered in this paper. As the field of immunotherapy progresses, we predict that the need for a system that efficiently couples inducible and constitutive gene expression will expand. With these contributions, we have established a foundation for more effective and clinically actionable next-generation cellular immunotherapies.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability

- **EXPERIMENTAL MODELS AND SUBJECT DETAILS**
  - Cell lines and primary human T Cells
  - Animal model details

- **METHOD DETAILS**
  - Design and molecular cloning of lentiviral constructs
  - Lentivirus production
  - Generation of CAR-T cells: T-Cell activation, transduction and expansion
  - TCR knockout in CAR-T cells using CRISPR/Cas9 RNPs
  - Characterization of T-cell receptors
  - In vitro analysis of CAR-T cell function
  - Flow cytometry
  - Fluorescent-activated cell sorting (FACS)
  - Fluorescence microscopy
  - Biological activity of iToci
  - Western blot
  - Mass cytometry staining and analysis
  - Animal studies
  - In vivo iToci-CAR-T cell study in humanized mice

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.ccell.2022.11.006.

**ACKNOWLEDGMENTS**

The authors thank the University of Pennsylvania (UPenn) Stem Cell and Xenograft Core, Small Animal Imaging Facility, Human Immunology Core, Flow Cytometry Core, and Wistar Institute Flow Cytometry for their technical support. The authors thank the UPenn CyTOF Core, specifically Takuya Ohtani, for running mass cytometry samples. The authors thank Saar Gill (UPenn) for the provision of tumor cell lines and CD19- and HER2-targeting CARs. We acknowledge John E. Wherry (UPenn), J. Joseph Melenhorst (UPenn), Robert Vonderheide (UPenn), Nicola Mason (UPenn), Saar Gill (UPenn), Mirosław Kozłowski (UPenn), Marco Ruella (UPenn), Olga Schestova (UPenn), Mauro Castellarin (UPenn), and Jie Wang (Duke University) for insights and feedback. We thank Dalia Omran (UPenn) for coordinating the distribution of samples. We thank Alfredo Tovar (NIBR) for help with molecular cloning of PASP4.2.1. We thank Incucyte Systems for Live-Cell Imaging and Analysis for providing test equipment. Funding was provided by the Parker Institute for Cancer Immunotherapy (PICI) (A.S., D.J.P., B.M.C., G.P.L., C.H.J.J.), R01EB026892 (D.J.P.), R21CA205794 (G.P.L.), and R01CA204261 (B.M.C., G.P.L.). A.B. received funding from a pharmacology T32 training grant (T32GM008076). M.C. received funding from EU H2020 (project CARAMBA). G.P.L. received funding from AIRC (IG 16458 and IG 20201024865) and the Italian Ministry of Research and University (PRIN 2017WC6489). C.B. and M.C. received funding from the Italian Ministry of Health (Ricerca Corrente CAR-T project RCR-2019-23669115), EU IMI (project T2Evolve), and AIRC (ARC 5 per Mille Rf. 22737). A.S. received funding from the Slovenian Research Agency (ARRS) for Project J3-3084 and Program P1-0245 and from Research fund of the National Institute of Biology for Project 10ICIGEN (ICI). Graphics were created with Inkscape and GIMP.

**AUTHOR CONTRIBUTIONS**

characterization study. M.C. and C.B. designed and led and C.M.B.C., and B.G. performed the experiments and analyzed data for the in vivo iToci study. F.S. performed histopathological analysis of the mouse brains for the iToci study. M.A.E. designed the CyTOF panel and conducted the experiments and analysis. M.A.E., A.S., A.B., and M.K. designed flow cytometry panels. T.B. assisted with TCR-knockout studies. A.S. and M.A.P., with the assistance of A.R.-G. and A.B., performed in vivo experiments. A.S., A.B., and D.J.P. wrote the manuscript with assistance from G.P.L., B.M.C., M.C., C.H.J., A.D.P., C.B., and M.A.E. All authors reviewed, discussed, and commented on the results and the manuscript.

DECLARATION OF INTERESTS
A.S., A.D.P., C.H.J., and D.J.P. are co-inventors on PCT International Patent Applications by The Trustees of the University of Pennsylvania, which incorporate discoveries and inventions described here. Tmunity Therapeutics has licensed non-exclusive rights to commercialize licensed cytokine-secreting products, such as CAR- or TCR-modified T cells engineered to express a transgene-encoded cytokine. C.H.J. is a co-scientific founder of Tmunity Therapeutics and Capstan Therapeutics and is a member of the scientific advisory boards of Alunous, BluesphereBio, Caballeta, Carisma, Cellares, Poseida, Verismo, and viTToria bio. C.H.J. is an inventor on patents and/or patent applications licensed to Novartis Institutes of Biomedical Research and Tmunity Therapeutics and receives license revenue from such licenses. D.J.P. is a member of the scientific advisory boards for Bellicium Pharmaceuticals and InstilBio. D.J.P. is inventor on patents and/or patent applications licensed to Prescient Therapeutics, Tmunity Therapeutics, and Mitterny Biotec and receives license revenue from those licenses.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

REFERENCES


Cancer Cell 40, 1470–1487, December 12, 2022


# STAR METHODS

## KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Myc tag (Alexa Fluor® 647 Conjugate)</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2233S</td>
</tr>
<tr>
<td>Goat polyclonal anti-human IgG</td>
<td>Sigma-Aldrich</td>
<td>Cat# 11886-2ML</td>
</tr>
<tr>
<td>CD4 Pacific Blue</td>
<td>BioLegend</td>
<td>Cat# 300521; RRID: AB_493098</td>
</tr>
<tr>
<td>CD45RA APC/7</td>
<td>BioLegend</td>
<td>Cat# 304128; RRID: AB_10708880</td>
</tr>
<tr>
<td>CD45RO BV570</td>
<td>BioLegend</td>
<td>Cat# 304226; RRID: AB_2563818</td>
</tr>
<tr>
<td>CD62L BV605</td>
<td>BD Biosciences</td>
<td>Cat# 562719; RRID: AB_2744441</td>
</tr>
<tr>
<td>CD95 PE</td>
<td>BioLegend</td>
<td>Cat# 305608; RRID: AB_314546</td>
</tr>
<tr>
<td>CD8 BV786</td>
<td>BD Biosciences</td>
<td>Cat# 563823; RRID: AB_2687487</td>
</tr>
<tr>
<td>anti-Myc-Tag (9B11) Mouse mAb HRP Conjugate</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2040S</td>
</tr>
<tr>
<td>anti-CD95 Clone DX2 164Dy</td>
<td>Fluidigm</td>
<td>Cat# 3164008B</td>
</tr>
<tr>
<td>anti-CTLA-4 Clone 14D3 170Er</td>
<td>Fluidigm</td>
<td>Cat# 3170005B; RRID: AB_2858238</td>
</tr>
<tr>
<td>anti-CD62L Clone DREG56 153Eu</td>
<td>Fluidigm</td>
<td>Cat# 3153004B; RRID: AB_2858235</td>
</tr>
<tr>
<td>anti-CD45RO Clone UCHL1 165Ho</td>
<td>Fluidigm</td>
<td>Cat# 3165011B; RRID: AB_2756423</td>
</tr>
<tr>
<td>anti-CD244 polyclonal 142Nd</td>
<td>Novus Biologicals</td>
<td>Cat# AF1039</td>
</tr>
<tr>
<td>anti-CD4 Clone RPA4 145Nd</td>
<td>Fluidigm</td>
<td>Cat# 3145001B</td>
</tr>
<tr>
<td>anti-CD8 Clone RPA8 146Nd</td>
<td>Fluidigm</td>
<td>Cat# 3146001B</td>
</tr>
<tr>
<td>anti-EOMES clone 644730 148Nd</td>
<td>Novus Biologicals</td>
<td>Cat# MAB6166</td>
</tr>
<tr>
<td>anti-Lag-3 Clone 11C3C65 150Nd</td>
<td>Fluidigm</td>
<td>Cat# 3150030B</td>
</tr>
<tr>
<td>anti-CD3 Clone UCHT1 170Er</td>
<td>Fluidigm</td>
<td>Cat# 3170001B; RRID: AB_2811085</td>
</tr>
<tr>
<td>anti-TIGIT Clone MBSA43 154Sm</td>
<td>Fluidigm</td>
<td>Cat# 3154016B; RRID: AB_2888926</td>
</tr>
<tr>
<td>anti-CCR7 Clone G043H7 159Tb</td>
<td>Fluidigm</td>
<td>Cat# 3159003A</td>
</tr>
<tr>
<td>anti-CD45 Clone HI30 89Y</td>
<td>Fluidigm</td>
<td>Cat# 3089003B</td>
</tr>
<tr>
<td>anti-Granzyme B Clone GB11 171Yb</td>
<td>Fluidigm</td>
<td>Cat# 3171002B</td>
</tr>
<tr>
<td>anti-CD57 Clone HCD57 172Yb</td>
<td>Fluidigm</td>
<td>Cat# 3172009B; RRID: AB_2888930</td>
</tr>
<tr>
<td>T-cell receptor mimic (TCRm) monoclonal antibody</td>
<td>Kim et al.58</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Biological samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary T cells</td>
<td>University of Pennsylvania Human Immunology Core.</td>
<td>NA</td>
</tr>
<tr>
<td>CD34+ Cells</td>
<td>Lonza</td>
<td>Product number “Dn39444” for the experiment in Figures 5F–5K,0 “CB21/0017/35” for the experiment in Figures S6H and S6I</td>
</tr>
<tr>
<td><strong>Chemicals, peptides, and recombinant proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD3/CD28 dynabeads</td>
<td>Invitrogen</td>
<td>Cat# 402.03D</td>
</tr>
<tr>
<td>anti-biotin microbeads</td>
<td>Miltenyi</td>
<td>Cat# 130-105-637</td>
</tr>
<tr>
<td>HER2 beads</td>
<td>Acro Biosystems</td>
<td>Cat# K006</td>
</tr>
<tr>
<td>PMA + ionomycin</td>
<td>eBioscience</td>
<td>Cat# 00-4970-93</td>
</tr>
<tr>
<td>Live/Dead Aqua</td>
<td>Thermo-Fischer Scientific</td>
<td>Cat# L-34966</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>GIBCO</td>
<td>Cat# 72400-047</td>
</tr>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td>VWR</td>
<td>Cat# S170G</td>
</tr>
<tr>
<td>Peptides</td>
<td>This manuscript</td>
<td>Please see Table S2</td>
</tr>
<tr>
<td>Custom p/HLA multimers</td>
<td>Proimmune, Sarasota, FL</td>
<td>NA</td>
</tr>
</tbody>
</table>

(Continued on next page)
Continued

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE SOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Critical commercial assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amaxa P3 Primary Cell kit and protocol</td>
<td>Lonza</td>
<td>Cat# V4XP-3012</td>
</tr>
<tr>
<td><strong>Experimental models: Cell lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK 293T</td>
<td>ATCC</td>
<td>CRL-3216</td>
</tr>
<tr>
<td>Jurkat E6.1</td>
<td>ATCC</td>
<td>TIB-152</td>
</tr>
<tr>
<td>CD19+ NALM6 human acute lymphoblastic leukemia</td>
<td>ATCC</td>
<td>CRL-3273</td>
</tr>
<tr>
<td>Engineered CBG + GFP+ NALM6 human acute lymphoblastic leukemia</td>
<td>University of Pennsylvania, Dr. Saar Gill</td>
<td>NA</td>
</tr>
<tr>
<td>Parental human HER2+ ovarian cancer cell line SKOV3</td>
<td>ATCC</td>
<td>HBT-77</td>
</tr>
<tr>
<td>Engineered CBG + GFP+ human HER2+ ovarian cancer cell line SKOV3</td>
<td>University of Pennsylvania, Dr. Saar Gill</td>
<td>NA</td>
</tr>
<tr>
<td>Parental HER2+ breast cancer cell line MDA-MB-468</td>
<td>ATCC</td>
<td>HBT-132</td>
</tr>
<tr>
<td>Engineered CBG + GFP+ HER2+ breast cancer cell line MDA-MB-468</td>
<td>University of Pennsylvania, Dr. Saar Gill</td>
<td>NA</td>
</tr>
<tr>
<td>CD20+ Raji Burkitt’s lymphoma</td>
<td>ATCC</td>
<td>CCL-86</td>
</tr>
<tr>
<td>Engineered CBG + GFP+ Raji Burkitt’s lymphoma</td>
<td>University of Pennsylvania, Dr. Saar Gill</td>
<td>NA</td>
</tr>
<tr>
<td>K562 erythro-megakaryoblastic leukemia cell line</td>
<td>ATCC</td>
<td>CRL-3343</td>
</tr>
<tr>
<td>Engineered CD20+ K562 erythro-megakaryoblastic leukemia cell line</td>
<td>University of Pennsylvania, Dr. Avery D. Posey, Jr.; Panjwani et al.54</td>
<td>NA</td>
</tr>
<tr>
<td>XL1-Blue Supercompetent Cells</td>
<td>Agilent Technologies</td>
<td>Cat# 200236</td>
</tr>
<tr>
<td><strong>Experimental models: Organisms/strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD/scid/IL2rg</td>
<td>University of Pennsylvania Stem Cell and Xenograft Core</td>
<td>RRID:IMSR_JAX:005557</td>
</tr>
<tr>
<td>SGM3 (NOD.Cg-PrkdcscidIl2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ)</td>
<td>The Jackson Laboratory</td>
<td>RRID:IMSR_JAX:013062</td>
</tr>
<tr>
<td><strong>Recombinant DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentiviral construct pASP4.2</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP4.2.1</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP5</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP7</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP8</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP9</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP18</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP26</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP28</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP30</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP36</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP38</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP52</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP59</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP72</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP73</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP75</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP85</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP89</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP90</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP96</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
<tr>
<td>Lentiviral construct pASP97</td>
<td>This manuscript</td>
<td>Please see Table S1</td>
</tr>
</tbody>
</table>

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel J. Powell, Jr. (poda@pennmedicine.upenn.edu).

Materials availability
All genetic constructs and sequences are listed in the Table S1. Plasmids generated in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell lines and primary human T Cells
Human CAR-T cells were generated from normal donor T cells provided by the University of Pennsylvania Human Immunology Core. All primary T cell studies were covered through approval by the University of Pennsylvania Institutional Review Board (IRB). All de-identified donors signed approved consent forms. Cells were cultured in complete medium (CM) comprising of RPMI 1640 (GIBCO) supplemented with 10% FBS (VWR), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C and 5% CO2. All cell lines were routinely tested for mycoplasma. Details are listed in method details (Cell lines section). For the in vivo iToci study, the human primary samples provided are human cord blood CD34+ cells. Cells were purchased from a commercially available vendor (Lonza; product number “Dn39444” for the experiment in Figures 5F–5K, “CB21/0017/35” for the experiment in Figures S6H and S6I). Normal donor T cells: T cells were isolated from healthy volunteers who previously signed an informed consent, which authorized use for general research purposes. Samples were centrally collected and sent to the laboratory for processing after complete anonymization. The leukemia cell line NALM-6 was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 (BioWhittaker) supplemented with 10% FBS (Lonza), 100 IU/mL penicillin/streptomycin and glutamine. The following cell lines were gift from the Gill lab at the University of Pennsylvania: Parental and engineered CBG+GFP+ human HER2+ ovarian cancer cell line SKOV3, parental and engineered CBG+GFP+ human HER2+ breast cancer cell line MDA-MB-468, CD20+ and engineered CBG+GFP+ Raji Burkitt’s lymphoma and CD19+ and engineered CBG+GFP+ NALM6 human acute lymphoblastic leukemia. These cells were further sorted for uniform protein expression using fluorescence-activated cell sorting (FACS). Parental and engineered CD20+ K562 erythro-megakaryoblastic leukemia cell lines were a gift from the Posey lab at the University of Pennsylvania. HEK 293T and Jurkat cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in complete medium (CM) comprising of RPMI 1640 (GIBCO) supplemented with 10% FBS (VWR), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C and 5% CO2. All cell lines were routinely tested for mycoplasma.

Animal model details
NOD/SCID/IL2rgnull (NSG) mice were purchased from the Stem Cell and Xenograft Core (University of Pennsylvania). Female mice (6–12 weeks old) were kept in a pathogen-free environment within individually ventilated cages. Animal studies were performed in full accordance with the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC protocol n. 805773). The specific designs of in vivo studies are indicated at each experiment in the manuscript.

The iToci study: All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of IRCSS San Raffaele Scientific Institute and by the Italian Governmental Health Institute (Rome, Italy). Mice were kept in a specific-pathogen-free (SPF) facility within individually ventilated cages and were given irradiated food and water ad libitum. All experiments were approved...
by the Institutional Animal Care and Use Committee (IACUC 646) of IRCSS San Raffaele Scientific Institute and by the Italian Governmental Health Institute (Rome, Italy). 6 to 8-week-old SGM3 (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/M-loySzJ; The Jackson Laboratory) were used for experiments. Both male and female animals were employed in all experiments.

METHOD DETAILS

Design and molecular cloning of lentiviral constructs
The lentiviral constructs designed and used in this study are listed and explained in detail in Table S1 including their architecture and annotated sequences of relevant elements. The basic Uni-Vect genetic cassettes comprise NFAT-inducible eGFP and constitutive mCherry modules in the single lentiviral transfer plasmid either in forward (pASP4.2) or reverse (pASP5) orientation (Table S1). The Uni-Vect cassette was synthesized (GeneArt) and cloned into 3rd generation self-inactivating (SIN) lentiviral transfer vector pELNS55 or pTRPE56 that The University of Pennsylvania uses for clinical CAR-T cell manufacturing. In the forward architecture design, antigen-inducible eGFP and downstream constitutive EF1α-driven mCherry modules are placed in the forward 5′-3′ orientation relative to the 5′LTR. The inducible module is composed of a synthetic NFAT response element that comprises 6 repetitions of the consensus NFAT binding site derived from the pGL4.30 (Promega) (PNFAT; Table S1) placed directly upstream of the previous validated57 minimal promoter Pmin, which is derived from the pGL4.23 (Promega). A linker region placed downstream of the Pmin creates the optimized distance between transcription and translation start sites, followed by the Kozak sequence and a start codon for eGFP. To further characterize the system, we investigated if an upstream ATG start codon between the Pmin and the linker in pASP4.2 influenced system performance and demonstrated no significant difference compared to pASP4.2.1 where ATG was absent (Figure S2A). Priority was given to the version of Uni-Vect without upstream ATG. The reverse architecture is composed of the same modules as in the forward architecture but placed in the back-to-back orientation. The inducible module was therefore placed in a reverse 3′-5′ orientation relative to the 5′LTR. Hl-12 fusion protein, iToci (anti-hIL6Rα sFc-Fc-Myc tag), hIL-6Rα, TCF7 and FOXO1-3A were synthesized (GeneArt) and cloned into the Uni-Vect as described in Table S1. All genetic constructs were prepared using standard digest and ligate restriction enzymes-based molecular cloning techniques and were sequence verified. All cloning and plasmid propagation steps were performed in XL1-Blue Supercompetent Cells (Agilent Technologies, Cat# 200236) to maintain integrity of lentiviral transfer plasmids.

Lentivirus production
The human embryonic kidney (HEK) 293 T cell line (American Type Culture Collection, Manassas, VA, USA) were grown to 70% confluence in CM at 37°C and 5% CO2. Uni-Vect transfer plasmid and lentiviral packaging plasmids pRSV.REV (Rev expression vector), pMDLg/p.RRE (Gag/Pol expression plasmid) and pVSV-G (VSV glycoprotein expression vector) were added to Opti-MEM (Thermo Cat. 31985088) at a 15:18:18:7 mass-unit ratio. This mixture was added to a 10:1 Opti-MEM and Lipofectamine 2000 (Thermo Cat# 11668019) mixture at 1:1 vol ratio and let incubate at RT for 10 min before addition to HEK 293T cells in fresh culturing media. Supernatants containing the lentivirus were collected at 24 and 48 h and passed through 0.45 μm filters. Filtered lentivirus product was concentrated by ultracentrifugation at 25,000 rpm for 2.5 h and stored at –80°C. The viral titer was determined by transducing HEK 293T cells and expressed as Infection Units per mL (IU/mL).

Generation of CAR-T cells: T-Cell activation, transduction and expansion
Healthy donor primary human T cells were purchased from the Human Immunology Core (University of Pennsylvania). CD4+ and CD8+ T cells were combined at 1:1 ratio and stimulated with anti-CD3/CD28 dynabeads (Invitrogen) in a 3:1 ratio on Day 0. After 24 h (Day 1), lentivirus was added at a multiplicity of infection (MOI) of 0.3–10 (depending on the experiment). CM volume was doubled daily until Day 6 when Dynabeads were removed by magnetic separation. Cells were then counted daily by Coulter Counter (Beckman Coulter) and maintained at 0.75 × 106 cells/mL by addition of fresh IL-2 (100 IU/mL, Prometheus Therapeutics and Diagnostics) supplemented CM until Day 10 when media was switched to non-supplemented CM. Cells were either frozen or used experimentally once the cell volume reached 280–330 IL at around Day 14. In some experiments as stated in the manuscript, CAR-T cells were sorted for CAR+ using FACS. During manufacturing, T cells were maintained at 37°C and 5% CO2. The transduction efficiency was determined by flow cytometry for the expression of relevant gene (mCherry, eGFP or immune receptor). For experiments with Jurkat cell line, cells were transduced with Uni-Vect lentiviruses at various MOIs as stated in the manuscript and used in experiments 2–5 days later. For evaluation of the Uni-Vect in primary human T cells they were transduced with constructs at MOIs indicated in the figures and expanded according to the standard protocol described above. Rested cells were stimulated with phorbol 12-myristate 13-acetate (PMA) + ionomycin (Cell Stimulation Cocktail, eBioscience™ Cat# 00-4970-93), anti-CD3/CD28 dynabeads, HER2 beads (Acro Biosystems Cat# K006) or with HER2+ SKOV3 ovarian cancer and HER2- MDA468 cell lines at effector to target (E:T) ratios indicated in the figures with or without dasatinib (Sigma-Aldrich). Inducible eGFP was monitored using flow cytometry. Expression of reporter genes was monitored by flow cytometry and quantified as MFI after 24 h and by fluorescence microscopy. In reversibility experiments, transduced Jurkat cells were monitored by flow cytometry and quantified as MFI after 24 h and by fluorescence microscopy. In reversibility experiments, transduced Jurkat cells were FACS sorted for mCherry+.

To compare the functionalities of Uni-Vect with a two-component lentiviral system, we benchmarked pASP30 (ieGFP + cHER2-41BB CAR, Table S1) against a system with modules split into two lentiviral vectors pASP5 (ieGFP + cmCherry, Table S1) and pP7 (constitutive HER2-41BB CAR, Table S1). We transduced primary T cells with either pASP30 or a combination of pASP5 and pP7 viruses, each at MOI3. In principle, the number of integrated inducible and constitutive modules should be similar between Uni-Vect and the two-component system but with the total amount of virus added being two times higher in the latter.
TCR knockout in CAR-T cells using CRISPR/Cas9 RNPs

Healthy donor primary human T cells were purchased from the Human Immunology Core (University of Pennsylvania). CD4+ and CD8+ T cells were combined at 1:1 number ratio. On Day 0 T-cells were centrifuged and resuspended in fresh CM supplemented with IL-7 and IL-15 (25 ng/mL each, R&D Systems). On Day 1 T-cells were washed 1× with PBS. 5 μg TRAC (5'-TGTGTAGACAT-GAGTGCTA-3') and 5 μg TRBC (5'-GGAGAATGACGAGTGGACC-3') sgRNA per sample were incubated with 10 μg TrueCut Cas9 (Thermo Cat# A36498) for 10 min. PBS was aspirated from washed T-cells and the 50e6/mL (5e6 in 100 μl) cells were electroporated using the Amaxa P3 Primary Cell kit and protocol (Lonza Cat# V4XP-3012) with a Lonza 4-D Nucleofector electroporation device (Cat# AAF-1002B model). After electroporation, cells were resuspended in IL-7/IL-15 (25 ng/mL each) supplemented CM for 48 h before use in the CAR-T cell production protocol as described in “Generation of CAR-T cells: T-Cell activation, transduction and expansion”. Residual TCR expression following knockout was sufficient for activation by Dynabeads in the CAR-T cell production protocol. Following expansion, edited TCR+ CAR-T cell products were purified by negative selection with anti-biotin microbeads (Miltenyi Cat# 130-105-637) and biotin conjugated anti-CD3 (Biolegend, Cat# 317320) on a MACS separation column (Miltenyi, Cat# 130-042-201) where the remaining CD3 positive, and therefore TCR+, cells were removed. Flow through containing TCR- cells was collected and cells were placed in fresh CM. This pipeline is schematically represented in Figure S3A.

Characterization of T-cell receptors

A Jurkat TCRα/β negative cell line was generated from the Jurkat E6.1 cell line using Cas9 protein and TRAC and TRBC1/TRBC2 sgRNA as described above. This TCR α/β negative Jurkat cell line was transduced with human CD8αβ heterodimer and pASP89 or pASP90 Uni-Vect/mCherry constructs to generate JASP89 or JASP90 reporter lines. JASP89 or JASP90 reporter cells were flow cytometry sorted to 99% purity based on TCR negative (anti-human TCRαβ antibody clone IP26, Biolegend, San Diego, CA), CD8 positive (anti-CD8 antibody, ThermoFisher Scientific, Waltham, MA), mCherry positive expression and low basal NFAT (eGFP+) signal. Melanoma neoantigen-specific TCRs (Table S2) were cloned into pTRPE, and produced lentivirus was used for transduction of JASP89 or JASP90 reporter cells. Neoantigen-specific TCR-expressing cells were sorted using custom p/HLA multimers (Proimmune, Sarasota, FL) and expanded for use in functional assays. For antigen recognition assays, titrated concentrations of mutated or wild-type peptide (10 μM–1 PM) were used to pulse monoallelic HLA-A*02:01-expressing K562 cells for 1 h at 37°C. TCR signaling via NFAT activation was monitored by eGFP expression. CD69 (anti-CD69 antibody, Biolegend, San Diego, CA) expression was monitored after stimulation by cell surface staining. Culture supernatants were collected and cells were placed in fresh CM. This pipeline is schematically represented in Figure S3A.

In vitro analysis of CAR-T cell function

Cytotoxicity assays

Lytic function of CAR-T cells was measured using the LucScreen Extended-Glow Luciferase Reporter Gene Assay System (Applied Biosystems). Depending on the experiment, Uni-Vect-engineered CAR-T cells and tumor cells expressing click beetle green luciferase (CBG) were combined at 3:1, 1:1, 0.5:1 and 0.3:1 Effector to Target (E:T) ratios in a total of 200 μL CM in an white opaque walled 96-well plate and co-cultured overnight at 37°C and 5% CO2. Plates were then centrifuged at 1200 rpm for 5 min, 100 μL of medium was removed from each well for further analysis, and luciferase buffer was added directly into the plate according to the manufacturer’s protocol. Luciferase readings were obtained using a microplate reader (BioTek Synergy H4). Cytotoxicity was calculated using the following equation where: (1 - luminescence(CAR-T cells + target cells)/luminescence(Target cells)) x 100. Cytokine release. CAR-T cells were stimulated according to specific experiment either with PMA + ionomycin or through co-culture with target cells. Cytokine levels were measured by a sandwich ELISA according to the manufacturer’s instructions (BioLegend) as follows: human hIFN-γ was detected by hIFN-γ (BioLegend, Cat# 430104), human hIL-2 was detected by hIL-2 (BioLegend, Cat# 431804) and hIL-12 was hIL-12 (BioLegend, Cat# 431704). Live-cell imaging. Jurkat cells were transduced with pASP4.2 or pASP5 and sorted 5 days later for mCherry+. Next day the cells were plated in 96-well plates and stimulated with PMA + ionomycin for 24 h and then washed, left unstimulated for 120 h, and then re-stimulated. eGFP was monitored in a real-time manner using IncuCyte live-cell imaging (Incucyte® Systems for Live-Cell Imaging and Analysis).

Flow cytometry

Samples were spun down at 1300 RPM, then washed once with staining buffer. Samples collected from mice had 2 mL of Ack Lysis buffer added for 5 min to lyse red blood cells then washed once with PBS. Samples were stained for CAR surface expression for 30 min at room temperature with 100 μL solution containing the goat polyclonal anti-human IgG (Sigma-Aldrich, Cat# 11886-2ML) conjugated with Lightning-Link APC (Expedeon). After CAR staining, samples were washed three times with staining buffer and then incubated at 4°C for 30 min in 50 μL of an antibody cocktail to label human surface markers. The following anti-human antibodies...
were used: CD4 Pacific Blue (BioLegend Cat# 300521), CD45RA APCy7 (BioLegend Cat# 304128), CD45RO BV570 (BioLegend Cat# 304226), CD62L BV605 (BD Biosciences Cat# 562719), CD95 PE (BioLegend Cat# 305608), CD8 BV786 (BD Biosciences Cat# 563823). Samples were washed three times and incubated with Live/Dead Aqua (Thermo-Fischer Scientific Cat# L-34966) for 10 min to discriminate live and dead cells. Samples were washed twice to remove Live/Dead Aqua before being run on a Fortessa.

**Fluorescent-activated cell sorting (FACS)**
Samples were spun down at 1300 RPM, then washed once with staining buffer (phosphate-buffered saline, 2% fetal bovine serum). Samples were stained for CAR surface expression for 30 min at room temperature with 100 µL solution containing the goat polyclonal anti-human IgG (Sigma-Aldrich, Cat#11886-2ML) conjugated with Lightning-Link APC (Expedeon). After CAR staining, samples were washed three times with staining buffer then washed with Live/Dead Aqua (Thermo-Fischer Scientific Cat# L-34966) for 10 min to discriminate live and dead cells. Samples were washed twice to remove Live/Dead Aqua. Samples were sent to the The Penn Cytomics and Cell Sorting Resource Laboratory at UPenn for fluorescent-activated cell sorting (FACS) on an Aria to sort for CAR positive cells. For sorting parameters, singlets were detected using FSC-H versus FSC-A followed by SSC-H versus SSC-A. Cells negative for Live/Dead Aqua were identified as live cells.

**Fluorescence microscopy**
Uni-Vect-engineered CAR-T cells or Jurkat cells were stimulated according to specific experiment either with PMA + ionomycin or target cells. To directly observe Uni-Vect performance, eGFP and mCherry were visualized using EVOS Cell Imaging System (ThermoFisher Scientific).

**Biological activity of iToci**
To analyze biological activity of iToci (anti-hIL6R scFv-Fc-Myc tag), we first developed a cell-based reporter assay to interrogate IL-6 signaling. We adapted the reverse architecture of Uni-Vect by exchanging NFAT with the STAT3 response element and integrating IL-6Rx as a constitutive module to generate pASP59 (Table S1). To validate the reporter system, HEK 293T cells were transduced with pASP59 and stimulated with increasing amounts of IL-6 and eGFP expression was monitored via flow cytometry.

To produce iToci, pASP52-CAR-T cells (iToci + cCD20-41BB CAR) were co-cultured with CD20+ cell line for 72 h to induce antigen-dependent expression and secretion. iToci was also expressed in a constitutive manner by transducing HEK 293T cells with the construct pASP75 (EF1a–iToci). Supernatants from cell cultures were collected and iToci levels were measured using human IgG1 Platinum ELISA (eBioscience, Cat# BMS2092TWO). First, supernatants from activated iToci-CAR-T cells were added to the HEK 293T cells engineered to express hIL-6Rx and incubated for 1 h. Binding iToci was detected by secondary stain against Myc tag (Cell Signaling Technology, Cat#2233S). Second, the biological activity of the anti-hIL6Rx scFv-Fc (iToci) was further analyzed by competitive inhibition of IL-6 signaling. To that end, Uni-Vect was reconfigured to develop a cell-based assay system to interrogate IL-6 signaling. We adapted the reverse architecture of Uni-Vect by exchanging NFAT with the STAT3 response element and integrating IL-6Rx into a constitutive module to generate pASP59 (STAT3-eGFP + chIL-6Rx, Figure S6C left and Table S1). To demonstrate functionality of this reporter system, HEK 293T cells were transduced with pASP59 and exposed to increasing amounts of human IL-6 which triggered a dose dependent response observed by eGFP MFI (Figure S6C right). To avoid a need of stimulating iToci-CAR-T cells to produce iToci, which would interfere with the developed cell-based assay because of factors that activate the STAT3-responsive promoter, we constitutively expressed iToci in HEK 293T cells and verified its molecular mass (Figure S6D). Supernatant containing constitutively expressed iToci or control antibody from HEK 293T cells was added to the generated IL-6-responsive reporter cell line 15 min before stimulation with hIL-6 (7.5 ng/mL, R&D Systems Cat# 206-IL-050/CF). After 24 h, the molar ratio-dependent decrease in eGFP expression was monitored via flow cytometry. Recombinant tocilizumab (Actemra) was used as a control.

**Western blot**
Supernatant (30 µL) from constitutively expressed iToci (anti-hIL6R scFv-Fc-Myc tag) was mixed with loading buffer (Lammelli buffer; BioRad) with or without 5% β-mercaptoethanol (BioRad), boiled for 5 min at 95°C and centrifuged at 14000 g for 3 min. Samples were resolved on 4–15% MiniGels protein TGX (BioRad) and run at 150 V for 1 h. A protein ladder (BioRad) was run along with the samples. Protein samples were transferred to a PVD membrane (Millipore) at 100 V for 1 h. The membranes were washed with TBST (1% Tween; BioRad) and incubated overnight at 4°C with Myc-Tag (9B11) Mouse mAb HRP Conjugate (1:1000) (Cell Signaling Technology, Cat# 2040S). Membranes were washed four times in between the primary and secondary antibody incubation steps. Membranes were developed using the ECL prime Western blotting detection reagent (GE Healthcare, RPN2236) and imaged (GE ImageQuant LAS 4000 series imaging system). Irrelevant constitutively expressed protein was used as a control.

**Mass cytotmetry staining and analysis**
All mass cytotmetry reagents were either bought from Fluidigm or conjugated in house using MAXPAR kits (Fluidigm). Antibody information is in key resources table. Cell identifier stain Iridium191/193, identifier 127Idu, and Cisplatin 195 were purchased from Fluidigm. All antibodies were titrated to determine optimal concentrations for staining samples. For downstream intracellular effector molecule detection, samples were treated with GolgiStop (BD Biosciences) 4–5 h before staining. For staining, single-cell suspensions of iegFP (pASP90), iTCF7 (pASP72), and iFOXO1-3A (pASP73) CAR-T cells were spun down, and incubated with 127IdU (5-Iodo-2’-deoxyuridine) and cisplatin 195 in PBS for 10 min at room temperature to identify live/dead cells. Next, cells were washed
in the staining buffer provided by Fluidigm and incubated for 30 min at room temperature with an antibody cocktail containing all surface antibodies. Following surface marker incubation, cells were washed three times in staining buffer. To detect intracellular markers, cells were fixed using 30 min with nuclear antigen staining buffer (Fluidigm), washed twice with Maxpar Nuclear Antigen Staining Perm (Fluidigm), and then incubated with the intracellular staining cocktail for 2 hrs at room temperature. Cells were washed three times before being fixed in 1.6% PFA with 125nM Iridium 191/193 overnight at 4C. The next day, cells were washed 2× in PBS, then 1× in dH2O. Data acquisition was performed on a CyTOF Helios (Fluidigm) by the CyTOF Mass Cytometer Core at the University of Pennsylvania. The CyTOF core performed bead-based normalization for all samples. To perform traditional biaxial analysis, Flowjo version 10 software was used. Intact single cells were identified using Iridium 191/193 and event-length. Live cells were identified using 127dU and cisplatin, where dead cells are positive for cisplatin. CD3 and CD45 positivity identified T-cells. High-dimensional analysis was performed using viSNE from cyt a tool written in Matlab (R2016b). Single, live, CD3∗CD45− exported FCS data from ieGFP (pASP90), iTCF7 (pASP72), and iFOXO1-3A (pASP73) Uni-Vect samples were imported into cyt, arcsinh5-transformed, randomly subsampled by cyt for 25,000 cells per sample, then bh-SNE mapped as described.59 Bh-SNE mapping used the following parameters: CD95, CTLA-4, CD62L, PD-1, CD45RO, CD244, CD4, CD8, EOMES, Lag-3, TIGIT, CCR7, Granzyme B, CD57. All viSNE plots were created using cyt.

Animal studies

NOD/SCID/IL2rgnull (NSG) mice were purchased from the Stem Cell and Xenograft Core (University of Pennsylvania). Female mice (6–12 weeks old) were kept in a pathogen-free environment within individually ventilated cages following protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC protocol n. 805,773). The specific designs of in vivo studies are indicated at each experiment in the manuscript. For solid human HER2+ ovarian cancer tumor models, mice were subcutaneously (s.c.) injected in the flank with engineered CBG+GFP+ human HER2+ ovarian cancer cell line SKOV3 (numbers used in specific experiments are indicated in the figure). After 4 days when tumors were established and confirmed by imaging, CAR+ T cells targeting HER2 at doses indicated in the specific experiments in the figure, were injected intraperitoneally (i.p.). For the iTF-CAR-T cell study, mice were re-challenged with the same cell line at doses and times indicated in the figure. Tumor progression/clearance was measured via (i) bioluminescence imaging and quantified as the total flux per second and (ii) tumor volume as determined by caliper measurement using the formula volume = 3.14/6 × (length × width2), with length being the longest diameter and width being the shortest diameter. Mice were sacrificed upon losing 20% body weight or reaching more than 20 mm in diameter. Blood samples were collected via retro-orbital bleeds. Number of peripheral blood T cells was quantified using Trucount Tubes following the provided protocol (BD Biosciences). The staining panel consisted of anti-human CD3-brilliant violet 605 (BioLegend), anti-human CD45-PE (eBiosciences), and anti-human CD8 APC-H7 (BD Biosciences).

In vivo iToci-CAR-T cell study in humanized mice

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of IRCSS San Raffaele Scientific Institute and by the Italian Governmental Health Institute (Rome, Italy). Mice were kept in a specific-pathogen-free (SPF) facility within individually ventilated cages. 6 to 8-week-old female and male SGM3 (NOD.Cg-Pkdcscid il2rgtm1Wjl/Tg(CMV-IL3,CSF2,KITLG)1Eav/MiloSzj; The Jackson Laboratory) mice were used. Upon hematopoietic reconstitution, mice were injected intravenously (i.v.) with 0.5x106 NALM6 acute lymphoblastic leukemia cells expressing a secreted luciferase and the LNGFR marker gene. Seven days later, mice received 1x107 ieGFP + cCD19 CAR-T cells, iToci + cCD19 CAR-T cells or untransduced T cells by i.v. infusion. Tumor growth was monitored by bioluminescence assay using the QUANTI-Luc detection reagent (InvivoGen, rep-qlc1) and expressed as relative light units (RLUs). Circulating human T cells were analyzed by FACs and counted using Flow-Count Fluospheres (Beckman Coulter). For evaluating CRS toxicity, mice were daily monitored for weight loss and analyzed for human cytokine concentrations at day 4 by LegendPLEX (Biolegend). Mice were euthanized when RLU was \( \geq \)1.6x106 or when signs of inhumane suffering manifested. For evaluating signs of neurotoxicity, brains from the experimental mice were collected at necropsy, fixed in 4% buffered formalin, embedded in paraffin, cut and stained. Haematoxylin and eosin stained 3-μm paraffin sections were examined for histopathological analysis, blindly and independently by two pathologists. Sections were acquired by a digital scanner (Aperio Scanscope and photomicrographs were taken using Aperio Image Scope v10.2.1.2315.

QUANTIFICATION AND STATISTICAL ANALYSIS

Information on specific statistical tests used is provided in the figure legends and/or method details. Statistical analysis was performed in GraphPad Prism 8.0 (GraphPad). Each figure legend denotes the statistical test used. All central tendencies indicate the mean, and all error bars indicate standard deviation unless otherwise indicated. Statistical analysis and tests performed included 2-tailed Student t test, two-way ANOVA or ANOVA multiple-comparison with p values generated using Tukey’s or Welch multiple-comparisons test. For non-parametric comparisons, we used Kruskal-Wallis tests. All t-tests were two-sided. In figures, p values are shown. Log Rank Mantel-Cox test was used for survival analysis. Technical replicates were assumed to be normally distributed, otherwise no assumptions of normality were made. Statistical details of all experiments can be found in figure legends.