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Rapid parallel reconstruction and specificity screening of hundreds of T cell receptors

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Abstract

The ability to screen the reactivity of T cell receptors (TCRs) is essential to understanding how antigen-specific T cells drive productive or dysfunctional immune responses during infections, cancer and autoimmune diseases. Methods to profile large numbers of TCRs are critical for characterizing immune responses sustained by diverse T cell clones. Here we provide a medium throughput approach to reconstruct dozens to hundreds of TCRs in parallel, which can be simultaneously screened against primary human tissues and broad curated panels of antigenic targets. Using Gibson assembly and miniaturized lentiviral transduction, individual TCRs are rapidly cloned and expressed in T cells; prior to screening, TCR-cell lines undergo combinatorial labelling with dilutions of 3 fluorescent dyes, which allows the retrieval of the identity of individual T cell effectors when they are organized and tested in pools using flow cytometry. Upon incubation with target cells, we measure the upregulation of CD137 on T cells as a readout of TCR activation. This approach is scalable and simultaneously captures the reactivity of pooled TCR-cell lines, whose activation can be deconvoluted in real-time, thus providing a path for screening the reactivity of dozens of TCRs against broad panels of synthetic antigens or against cellular targets, such as human tumor cells. We applied this pipeline to systematically deconvolute the antitumoral and antiviral reactivity and antigenic specificity of TCRs from human tumor-infiltrating lymphocytes. This protocol takes approximately 2 months, from experimental design to data analysis, and requires standard expertise in cloning, cell culture and flow cytometry.

Editorial Summary:

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Author contributions

G.O. conceived and designed the protocol. G.O. and C.J.W. directed the overall study. G.O. and A.A. performed experimental and data analysis and discussed the results. All the authors wrote the manuscript.

Supplementary Materials are available for this paper at the end of the document or at linked Excel files and include:

Supplementary Figures 1–3

Supplementary Tables 1–5

This protocol outlines a medium-throughput strategy based on combinatorial dye staining of pools of effector T cells to screen in parallel the reactivity of up to hundreds of T cell receptors against patient primary tissues or panels of antigens.

Introduction:

T cells play a central role within the immune system, as they can control and eradicate non-self-antigens during infections[1–3], but they can also give rise to dysfunctional immune responses across a wide variety of pathologies, including cancers[4–7] and autoimmune diseases[8–10]. The potential of T cells relies on the expression of an arsenal of immune receptors, the T cell receptors (TCRs), which are generated through somatic recombination of TCRα and β-chain loci and are shaped and selected by central tolerance, antigen presentation and recognition[11]. The intrinsic diversity of the TCR repertoire grants the adaptive immune system the ability to recognize an extremely diverse array of antigens presented on the surface of target cells in the context of major histocompatibility complexes (MHCs); however, such extensive clonality makes assessing the specificity of any one TCR a complex problem. Advances in single-cell profiling have provided the ability to capture the TCR sequences of T cells sustaining an immune response[12–14]. The challenge is now the generation of efficient and straight-forward strategies for assessing the specificities of TCRs in order to investigate the mechanisms underlying the elicitation of T cell responses in a variety of physiologic or pathologic contexts.

Deconvoluting the specificities driving T cell responses requires a methodology with the capacity to test many candidate TCRs in parallel against a diverse set of potentially relevant antigens. The available TCR screening platforms have focused either on identifying the antigen-specificity of a small set of TCRs expressed *in vitro* against a large library of antigens[15,16] (e.g. using target cells gene-modified with antigen libraries), or on testing polyclonal TCR repertoires isolated *ex vivo* from primary samples and/or reconstructed in TCR libraries against a small pre-defined set of antigens[17–20] (e.g. using HLA-multimer staining, sequencing and/or screening of TCR libraries, Fig. 1). While these experimental approaches can be adapted to interrogate a significant portion of the landscape of TCRs or antigens, they often require repetitive rounds of screening of TCR-transduced T cells and the laborious isolation and sequencing of responding TCRs or antigen-expressing targets. As a consequence, experiments are time-consuming and are unfeasible when effector T cells and/or target cells are derived from limited human clinical samples. Therefore, we sought to establish a system to reconstruct and screen the reactivity of multiple TCRs against primary samples and curated panels of target antigens in an efficient and feasible manner that has enabled the collection of results in a real-time fashion.

Here, we present such a strategy for rapid reconstruction and reactivity screening of dozens to hundreds of TCRs, which can be tested in parallel against a wide range of antigens, including primary cells from patient tissues and autologous antigen presenting cells (APCs) pulsed with broad panels of antigen peptides. By screening TCR reactivity against curated collections of peptides defined based on specific scientific questions, we enable the discovery of T cell specificity for broad categories of antigens. The protocol we

describe has the potential to generate results in a short time frame (2 months), is scalable and can be performed with standard laboratory techniques, as it leverages cloning, lentiviral transduction of T cells and flow cytometry-based reactivity screening. Thus, this protocol can be efficiently integrated into immune studies, to gain insights into T cell specificities elicited in an adaptive immune response, as we have done by coupling T cell profiling and TCR reactivity screening for the characterization of tumor-infiltrating T cells in cancer patients[4,21–23].

Development of the protocol

We have developed a protocol that enables the parallel cloning, expression in T cells and specificity screening of 10–100 TCRs identified from bulk or single-cell TCR-sequencing data.

The first goal was to rapidly and efficiently clone a large number of TCR constructs in parallel. To achieve this, we took advantage of Gibson Assembly[24] as it ensures high efficiency of the cloning reactions in a single step (Fig. 2, Part 1). To increase the throughput of cloning, we directly grew transformed bacteria in antibiotic-selection media, without the traditional selection and validation of single colonies of bacteria with discrete plasmids. Despite this, the high efficiency of the cloning reactions was evident in the resulting high proportional predominance of plasmids with the correct integration of the TCR constructs following DNA extraction from unselected bacteria, a finding which can be confirmed by standard sequencing (see Anticipated Results). Contaminant plasmids (with failed TCR-gene insertion or with frameshift mutations) are not expressed as stable TCRs once transduced in effector cells, and therefore they do not hamper the efficiency of the protocol. These steps were designed to generate a large number of plasmids over the course of two days, allowing for reliable, efficient and cost-effective cloning of TCR constructs.

The second challenge was to generate a high number of effector cell lines (~100) in parallel, each transduced with a different TCR. To this end, we have optimized and miniaturized the production of lentiviral particles and the transduction of effector cells in a 96-well plate format (Fig. 2, Part 2). With the small amount of virus produced in 96-well plates, steps to concentrate and filter viral particles become infeasible and were therefore not considered further. Instead, we attempted to increase the transduction efficiency of effectors through repeated consecutive exposure to virus-containing supernatant over a longer timeframe (5 days). We established transfection conditions to maximize the production of lentiviral particles at the expense of cell viability of packaging Lenti-X 293T cells; by doing so, we also minimized the risk of contamination of effector cell culture with Lenti-X 293T cells, which could otherwise be transferred because of the lack of filtration of viral supernatant.

The third challenge was to enable the simultaneous screening of the reactivity of individual TCR-transduced cell lines. To achieve this, we took advantage of 3 cell-trace dyes (CellTrace Far Red, Violet, and CarboxyFluorescein Succinimidyl Ester [CFSE]) to label the cytoplasm of T cells, thus providing a fluorescent signal that can be used to distinguish each effector by flow cytometry once T cells are pooled together. We designed a protocol to color each TCR-transduced cell line with a different combination of dilutions of the 3 dyes (Fig. 2, Part 3), which makes it possible to distinguish up to 64 TCR-transduced lines. This

combinatorial cytoplasmatic staining can be then paired with flow cytometric detection of markers that are upregulated by CD8+ or CD4+ T cells upon transduction of TCR signals, such as CD137 for primary T cells[25] or CD69 for Jurkat cells[26,27].

Of note, the analysis of TCR reactivity requires that each tested TCR is stably expressed and detected on the surface of the TCR-transduced cell line. This can be readily achieved in T cell lines devoid of endogenous TCR chains (e.g. TCRα/β deficient Jurkat cells[28–30]) generated through gene knock-out. Conversely, gene-modification of primary T cells with TCRs requires the use of TCR sequence designs that favor the correct pairing of the exogenous TCRα/β chains and that can be easily distinguishable from the endogenous TCRs. To achieve this, we have incorporated previously described modifications of TCR constant chains, which include murinization of the TRAC-TRBC domains, insertion of cysteines to create additional disulfide bonds and amino acid changes that favor hydrophobicity of the intermembrane TRAC portion[31–34]. In this manner, the tested TCRs can be stably detected by flow cytometry on the surface of both primary T and Jurkat TCR-transduced cell lines, using a fluorochrome-conjugated antibody specific for the murine TRBC portion. This allowed us to compare the performances of TCRs in the two most common *in vitro* cellular systems that are widely utilized to screen reactivity (TCR-KO Jurkat or primary T cells), to assess the conditions that favor the detection of TCR reactivity even in the presence of low levels of cognate antigens.

Altogether, these strategies have been aimed at balancing efficiency and feasibility of individual steps in order to rapidly generate multiple TCR-transduced cell lines that can be organized in pools and screened against a potentially wide array of antigenic targets. By miniaturizing the TCR cloning and expression steps and optimizing the simultaneous reactivity screening of many TCRs, this protocol offers a versatile medium-throughput approach that can be reiterated multiple times within a reasonable time frame or can be scaled and adapted to diverse experimental contexts.

Comparison to other methods

This protocol is optimized to screen the specificity of hundreds of TCRs in a relatively short time period (~2 months). Our strategy simplifies TCR cloning, enables the expression of multiple individual TCRs in TCR-transduced cell lines and provides a feasible strategy to screen TCR reactivity against multiple targets with minimal consumption of cellular material. Compared to other approaches, this protocol is designed to test around 24–200 TCRs per patient, against a curated library of 100–200 antigenic targets (including pooled or single peptides, Fig 1) designed to cover primary cellular targets and multiple classes of antigens, including neoantigens, tumor-associated antigens, or viral epitopes. The same procedure can be easily reiterated multiple times, to test more TCRs or to screen a higher number of antigens, and it can be adapted to measure TCR reactivity against cellular targets in different formats, including cell lines or APCs pulsed with peptides or modified to express libraries or antigens in alternative formats (e.g. tandem minigenes, TMGs).

This protocol differs from other approaches of TCR specificity screening in two major ways: i) the high-throughput of screened TCRs, and ii) the intermediate-throughput of screened antigens. The screening of a higher number of TCRs (>1000) can be accomplished by

repeating this protocol multiple times. Alternatively, thousands of TCRs sequenced from primary or *ex vivo*-cultured T cells can be organized in a gene-library and transduced in effectors as a pool[20]. Such an option would require optimized transduction conditions to achieve expression of one TCR per cell (to avoid potential mispairing of chains derived from two or more TCRs). In addition, pooled TCRs would need to be screened only though sorting of reactive T cells and sequencing of their TCRs, which in turn would increase the experimental time and costs while preventing the possibility to obtain results in real-time. Recently developed molecular biology approaches enable the cloning of native TCR repertoires sequenced upon *in vitro* enrichment of antigen-specific T cells into reporter cell lines (TCR-KO Jurkat cells), thus allowing the discovery of TCRs reactive against specific immunogenic epitopes[20]. Strategies exploiting TCR libraries can be adapted to screen the specificity of primary polyclonal T cells, including peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes (TILs). The reactivity of primary T cells can also be directly assessed upon antigen stimulation, but the TCR signal might be limited by the functional state of effector cells *in vivo*. Indeed, the poor functionality of exhausted TILs may hamper the detection of T cell activation upon stimulation with targets[35], while making infeasible the use of universal markers to measure the reactivity of T cells with specialized function, such as CD4+ T regulatory cells (Tregs)[36]. While the transfer of TCR libraries in reporter cell lines can overcome this issue, the competition between different TCRs expressed in pools might preclude the detection of reactivity of low affinity and/or lowly represented TCR-clonotypes. Conversely, when sufficient numbers of primary T cells are not available, our protocol grants the possibility of transferring a substantial proportion of the TCR repertoire into reporter T cell lines, to test the performance of the TCRs in functional effectors, in the absence of confounding phenotypic factors.

Multiple strategies have been designed to maximize the coverage of antigens in TCR reactivity screening. DNA-[15,37] or RNA-encoded[16,38] antigen libraries[39] have been expressed in APCs engineered with reporter genes to identify cognate antigens[16,40]. These systems enable the screening of thousands of antigens, with greater coverage of potential antigens than our approach, but require isolation of responding cells and sequencing of their antigen-cassette to identify the immunogenic targets and thus preclude rapid follow-up experiments. Furthermore, such techniques require autologous APCs that are not immortalized (by EBV infection, as used in this protocol) or APCs engineered to express the HLA alleles of interest, which are either limited in number or time-consuming to produce, respectively. Our approach is best suited for identifying TCR reactivity against a broad set of targets, selected on the basis of a scientific rationale (e.g. investigation of specific classes of viral antigens, tumor antigens, etc.) and designed on the basis available datasets (e.g. peptides from HLA-binding immunopeptidome analysis, neoantigens or tumor-associated antigens detected from RNA/DNA sequencing). The throughput of the protocol can be further increased using pooled peptides or APCs engineered to express libraries of antigens, but this would require deconvolution of TCR reactivity deriving from individual TCR-antigen pairs. Furthermore, among the strategies discussed here, this protocol uniquely makes feasible the testing of TCR reactivity against primary dissociated and viable tissue samples, whose availability is often limited in the context of human studies.

Overview of the procedure

This protocol consists of three distinct phases: 1) parallel cloning of multiple (10–100) TCR α/β-chains, 2) parallel transduction of TCRs into individual effectors and expansion of TCR-transduced cell lines, and 3) combinatorial labeling of TCR-transduced cell lines and simultaneous reactivity screening of pools of effectors against panels of targets (Fig. 2). Although the protocol is designed to express TCRs in primary T cells and screen their reactivity through surface upregulation of CD137, the steps can be adjusted to test TCR reactivity in TCR-KO Jurkat cells, or to measure T cell activation through other surface or intracellular activation markers, including CD69, CD107a/b or IFNγ. Here, we first include criteria for selection of TCR sequences from sequencing data and design of constructs for TCR expression in effector cell lines. Of note, we report the procedures required to clone up to 96 TCRs per experiment (**Part 1, Steps 1–39**), but it is possible to repeat or scale up the experiment to reconstruct hundreds of TCRs. Second, we describe how to set up lentiviral transduction to generate multiple TCR-transduced cell lines (**Part 2, Steps 40–107**). Finally, we provide steps for labeling the TCR-transduced cell lines, organizing them in pools of effectors and screening their reactivity against panels of antigens (**Part 3, Steps 108–140**). Strategies for the preparation of target cells are outlined in Box 1. We discuss how to analyze the flow cytometry data and interpret the results, providing gating strategies and representative results (Fig. 3).

Application of the method

This protocol can be applied to reconstruct TCRs detected in sequencing data, in order to screen their reactivity, discover their cognate antigens and measure their functional avidities. By measuring the functional potential of TCRs *in vitro*, it is possible to infer the behavior of T cells expressing those TCRs in *in vivo* systems, as TCRs transduce the primary signal affecting T cells survival, proliferation and cytotoxicity[41]. This protocol can be highly informative if coupled with single-cell profiling of T cells, since it complements phenotypic characterization of T cells with functional insights into their reactivity, shedding light on the T cell populations involved in pathologic contexts. This is particularly helpful in human studies, where the paucity of viable specimens often precludes the isolation of *ex vivo* antigen-specific T cell lines or the analysis of their reactivity against cellular targets such as tumor or normal cells dissociated from primary tissues. Indeed, this protocol allows for the transfer of a portion of the T cell repertoire into functional T cell lines, which can be tested simultaneously against viable cells, thus minimizing the amount of target cells required during immune assay interrogation. We have applied this protocol to investigate the link between phenotype, antigen specificity and avidity in T cells with *in vitro*-verified antitumor specificity: starting from single-cell RNA/TCR-seq data, we selected more than 300 dominant CD4+ or CD8+ T cell clonotypes comprising 10–40% of total CD3+ TILs detected within the tumor microenvironment of 4 patients with melanoma[4,21]. Over 6 months, we repeated the protocol several times to screen: i) the antitumor reactivity of TCRs, by measuring their transduction signal in the presence of autologous patient-derived melanoma cell lines or non-tumor controls; ii) the cognate antigens (melanoma-associated antigens, neoantigens or viral antigens) recognized by the TCRs; iii) the avidity of recognition for the validated peptide-HLA complexes. Using this protocol, we defined the

portion of the intratumoral T cell repertoire dedicated to anti-tumor recognition and we subsequently deorphanized the specificity of 25–75% of antitumor TCRs from patients. Such a rate of discovery of TCR-antigen pairs was favored by the design of a curated antigen library covering known categories of melanoma antigens, selected based on their detection within patient tumors and/or reported to be immunogenic in the literature. As in our protocol the composition of the target library is guided by the knowledge and characterization of potentially immunogenic epitopes, we expect that the rate of TCR deorphanization could vary in different experimental settings, according to the rationale and data supporting the selection of TCRs and antigens.

This protocol can be adapted to test TCR reactivity against any type of viable cell line, primary target or APC that can be isolated or expanded from patient blood, such as PBMCs, monocyte-derived dendritic cells (DCs) or immortalized B cells. Of note, the isolation of targets or APCs from the same subject in which the TCRs were detected ensures that TCR recognition is specific and not driven by alloreactivity derived from MHC-mismatches. Conversely, when using allogeneic APCs matched with single MHCs, proper controls are required to assess the degree of TCR alloreactivity against unstimulated target. Cognate antigens of TCRs can be assessed through APCs pulsed with synthetic peptides, such as those encoding for viral, self or tumor antigens reported in public databases or predicted to be immunogenic with standard computational pipelines (NetMHCPan[42,43], HLAthena[44]). In addition, APCs can be pulsed with cell lysates, which offer the opportunity to investigate the indirect (mediated by APCs) presentation of cognate antigens. These strategies can be utilized to investigate important T cell populations across diverse physiologic or pathologic states, such as those observed in cancer, infectious diseases, or autoimmune diseases.

This protocol also offers the potential to study or validate the reactivity of TCRs sequenced in T cell populations of known antigenic specificity, as in the case of T cells sorted based on binding to MHC-peptide complexes or *in vitro* recognition of selected antigens. For example, we have used FACS-sorting of CD4+ T cells binding fluorochrome-conjugated peptide-MHC-class II tetramers to characterize CD4+ neoantigen-specific T cells circulating in cancer patients after vaccination[45]. After sequencing of isolated T cells, our protocol was applied to test the specificity of 58 TCRs: 46 of them (79%) exhibited *in vitro* recognition of patient APCs pulsed with the predicted neoantigen peptide. Similarly, we have employed this protocol to assess the antitumor recognition of more than 400 TCRs sequenced among CD8+ T cells isolated from PBMCs based on degranulation and cytokine secretion upon *in vitro* challenge with autologous melanoma cell lines. We documented a specific antitumor recognition for 52% of tested TCRs, finding that a high proportion of TCRs circulating among PBMCs often contaminates antigen-specific T cell populations isolated *in vitro*[4]. Therefore, this protocol is an important tool to validate the reactivity of representative TCRs isolated from T cells with predicted antigenic specificity.

Experimental Design

Selection of TCRs—As this protocol allows for reconstruction and testing of dozens to hundreds of TCRs per iteration, it is essential to carefully select TCRs from a given dataset, using criteria that ensure reliable TCR sequences and favor the discovery of cognate

antigen-TCR pairs. Because of their heterodimeric nature, identification of TCRs requires the targeted sequencing of two loci (TCR α and β-chains) and the assignment of the correct chain pairing on a cellular basis. As a consequence, pairs of TCR α and β-chains can be unambiguously established using any approach of single-cell TCR-seq[12,14]. To ensure the reliability of tested TCRs, it is important to select TCR α and β-chains that are consistently detected with identical sequences (V, D, J genes and CDR3s) and repeatedly paired together, as detected in multiple single T cells (at least 2 cells). In the case of testing the specificity of T cell clones expressing multiple TCRα or β-chains (two TCRα-chains and one TCRβ chain, or vice versa), each TCR α/β-pairing must be reconstructed and screened in parallel. In these settings, it is highly likely that only one configuration will display a relevant reactivity. Finally, it is recommended to select TCRs with a clear costimulatory restriction (CD4 or CD8), as defined through scRNA-seq, CITE-seq or flow cytometry, as such knowledge is pivotal for analyzing the reactivity of TCRs once expressed in effector cell lines with CD4 and/or CD8 expression.

Choice of the expression system: promoter of the TCR gene—T cell reactivity relies on the stable expression of TCRs on the surface of effector cells. However, expression of TCRs in gene-modified T cells may fluctuate as a result of a variable transduction efficiency. Therefore, for high-throughput reconstruction of hundreds of TCRs in parallel, it is important that TCRs are encoded under the control of promoters that provide a stable expression level, possibly independent of the number of viral particles transduced per cell (i.e. vector copy number, VCN). To achieve reliable TCR reactivity despite variable transduction efficiencies, we have evaluated the suitability of two well-characterized constitutive promoters, pPGK[46] and pEF-1α[47], to drive the level of TCR expression (Fig. 4A). To test this, we cloned a TCR specific for an HLA-A*02:01-restricted Flu antigen under the control of these two promoters. The two constructs were used to produce lentiviruses for transduction of TCRα/β KO Jurkat cells (hereafter referred to TCR-KO Jurkat) at increasing concentrations of viral particles. The surface assembly of the exogenous TCR was monitored by flow cytometry, through surface expression of CD3 molecules; a VCN of 1 was defined by a transduction efficiency of ~10%. While TCR expression driven by the PGK promoter was highly dependent on the level of transduction, high levels of CD3 were observed in TCR-KO Jurkat cells transduced with pEF1a-FluTCR, independent of the transduction efficiency (Fig. 4A). To assess whether different levels of TCR expression could affect the functional performances of TCR-transduced cells, we evaluated the ability of TCR-transduced CD3+ Jurkat cells to bind cognate peptide-HLA complexes, at both high VCN or low (≅1) VCN. Although TCRs expressed under the control of both pPGK and pEF-1α could efficiently bind cognate antigen when expressed at high VCN, only TCRs cloned with pEF-1α conferred efficient binding of HLA-peptide complexes at a VCN of 1 as nearly all CD3+ (TCR-transduced) Jurkat cells could bind antigen. Conversely, only 11% of CD3+ cells with a pPGK-driven TCR exhibited peptide-HLA binding (Fig. 4B). Importantly, the signal transduction of TCRs expressed under pPGK could be affected by the levels of TCR expression, as assessed by measuring upregulation of CD69 on the surface of TCR-transduced (CD3+) Jurkat cells stimulated with HLA-A*02:01+ APCs pulsed with increasing doses of cognate peptide. We found that only the high and stable TCR expression controlled by pEF-1α provided consistent TCR reactivity

at both high and low VCN, even at low doses of cognate antigens (Fig. 4C). Thus, pEF-1α confers levels of TCR expression and functionality that are independent of transduction efficiency and VCN, and this promoter is thus preferable for driving TCR expression in the context of reactivity screenings.

Selection of effector cells: TCR-KO Jurkat cells versus primary T cells—TCR transduction signal is associated with a cascade of events that can be measured in cells as a readout of TCR reactivity[25,27]. As such events rely on the functional capabilities of a cellular system, the selection of the effector cells that are optimal for TCR expression and reactivity screening is a central consideration. One such system is TCR-KO Jurkat cell lines engineered to express the CD8A/B costimulatory molecules and fluorescence reporter genes under the control of response elements for immune transcription factors (e.g. NFAT-GFP or NFkB-mCherry)[48]. In this protocol, the fluorescence of reporter genes would interfere with the signal provided by the intracellular dyes used to label and distinguish individual TCR-cell lines in pools of effectors (Fig. 2C). This hurdle can be easily overcome by assessing TCR-induced cell activation via upregulation of CD69 molecules on the surface of TCR-transduced Jurkat cells after overnight stimulation through flow cytometry[49]. TCR-KO Jurkat cells represent a model system that can be easily manipulated *in vitro* to efficiently express TCRs of interest with an unlimited availability of TCR-transduced effectors. Furthermore, in TCR-KO Jurkat cells, the expression of a transgenic TCR can be easily detected by flow cytometry using a fluorescently-conjugated antibody against CD3, as the assembled TCR associates with CD3 complexes on the surface of cells.

TCRs can also be expressed and screened in primary T cells: in this system, an exogenous TCR can take advantage of the activity of the physiologic TCR signaling pathways that trigger T cell activation and effector functions. Indeed, response to TCR stimulation can be reliably assessed in primary T cells through flow cytometry, by measuring the upregulation of CD137, CD107a/b or IFNγ, thus making this system compatible with the proposed combinatorial labeling of effector cell lines. As previously discussed, a number of strategies have been developed to ensure the stable pairing of exogenous TCR α/β-chains and to allow their differentiation from endogenous ones. In our hands, the use of murinized TCR constant chains has provided a reliable tool to measure the surface expression of the transduced TCRs on the surface of primary T cells through an antibody against the murinized TRBC (Fig. 3, Supplementary Fig. 1). This procedure has circumvented the need for knockout of endogenous TCR α/β-chains, which would not be a feasible option when dozens or hundreds of TCR-transduced cell lines are generated in parallel.

To assess the sensitivity of these cellular systems, we compared the performance of a set of TCRs with known antigen specificity when expressed in CD8+CD4+ TCR-KO Jurkat cells or in primary T cells. We selected 6 TCRs with known specificity for viral epitopes or cancer neoantigens[23,45], with wide range of TCR avidities and diverse restrictions for HLA-class I or II alleles (Fig. 5A, Supplementary Table 1). Each TCR was murinized, cloned and expressed in TCR-KO Jurkat cells or primary T cells from a healthy donor. When transduced in primary T cells, these TCRs exhibited a high expression of the murinized exogenous chains, which could compete with surface assembly of the human endogenous TCR chains resulting in the detection of transduced cells (mTRBC+) with or without co-expression

of endogenous human TCRα/β-chains (Supplementary Fig. 2). We tested the reactivity of these transduced effectors after overnight incubation with HLA-matched Epstein-Barr virus (EBV)-immortalized B cells (EBV-lymphoblastoid cell lines, EBV-LCLs)[50], pulsed with increasing doses of cognate peptides (Fig. 5B). We opted to monitor TCR transduction signal through flow cytometry, by detecting CD69 surface upregulation on TCR-transduced (CD3+) CD4+CD8+ TCR-KO Jurkat cells or by measuring CD137 upregulation on TCR-transduced (mTRBC+) CD4+ or CD8+ primary T cells. The results of this experiment showed that for all the tested TCRs, upregulation of CD137 was detectable on transduced primary CD4+ or CD8+ T cells at levels of cognate antigen that were lower compared to those that were able to trigger a comparable CD69 upregulation on Jurkat cells transduced with the same TCR (Fig. 5B). Quantification of functional TCR avidity measured in these two experimental settings demonstrated that primary T cells were much more sensitive to low levels of cognate peptide than Jurkat cells, with an EC50 15-fold lower in T cells than in TCR-KO Jurkat cells (range 8–633 fold, $p = 0.0059$, Fig. 5C). Importantly, primary T cells transduced with an EBV-specific TCR were reactive to cognate EBV antigens endogenously expressed by EBV-LCLs, regardless of the concentration of pulsed exogenous peptide (Fig. 5B, EBV-TCR). Conversely, TCR-KO Jurkat cells did not respond to physiologic levels of cognate antigen presented endogenously by HLA-matched EBV-LCLs; CD69 upregulation could, however, be elicited on EBV-TCR-transduced Jurkats in the presence of high doses ($\geq 10^4$ pg/mL) of cognate antigen provided by stimulation with exogenous peptide. To further validate this observation, we tested the ability of TCR-transduced Jurkat cells or primary T cells to transduce TCR signals in response to endogenous antigens. We generated tandem minigenes (TMGs) encoding a viral Flu antigen together with mutated neoantigens (NeoAg) or their wildtype counterparts; these constructs were lentivirally transduced in HLA-matched EBV-LCLs to generate epitopes that could be endogenously processed and presented by APCs. Under these conditions, primary T cells transduced with viral or NeoAg-specific TCRs readily recognized endogenously expressed cognate antigens, including: i) EBV antigens presented in all EBV-LCLs targets; ii) Flu antigens encoded within NeoAg or WT-TMGs and iii) NeoAgs specifically expressed by NeoAg-TMG and not WT-TMGs (Fig. 5D). Two CD8-TCRs (NeoAg#2-TCR and NeoAg#3-TCR) were not able to induce high or detectable upregulation of CD137 on transduced CD8+ T cells, likely due to their low avidity for their cognate antigens (Fig. 5C, 5D-**top**). Conversely, TCR-KO Jurkat cells transduced with the same TCRs exhibited lower or absent reactivity to endogenously processed cognate antigens: only Flu-TCR conferred moderate reactivity to HLA-matched EBV-LCLs transduced with FluAg containing-TMGs (Fig. 5D-**bottom**).

Together, these results demonstrate that primary T cells are a highly sensitive cellular system that allows detection of TCR reactivity (measured as CD137 upregulation) even in the presence of low levels of antigens, such as those that are physiologically expressed within the HLA-binding immunopeptidome of target cells (e.g. virus-infected cells or tumor cells). In TCR-transduced Jurkat cells, TCR reactivity (monitored as CD69 upregulation) can be efficiently detected only in the presence of high doses of cognate antigen, such as those reproduced *in vitro* by pulsing APCs with high amounts of peptides. Therefore, we suggest using TCR-transduced Jurkat cells to screen TCR reactivity preferentially against APCs pulsed with high doses of peptides and we recommend testing the sensitivity of any tested

reporter gene prior to its use in TCR-KO Jurkat cells. Conversely, we selected primary T cells transduced with murinized TCRs as the optimal sensitive cellular system for screening TCRs reactivity against both peptide pulsed and un-pulsed targets. Of note, in this scenario, the reactivity screenings are performed in T cells with 2 TCRs (the exogenous and the endogenous ones): we thus investigated whether the endogenous TCRs could potentially compete with exogenous murinized TCRs, limiting their expression and reactivity. We selected and transduced 8 MLANA-specific TCRs in primary T cells (Supplementary Table 1)[4] with different patterns of expression (low vs high): flow-cytometric co-staining of both exogenous (murinized) and endogenous (human) TCR chains demonstrated that lowly-expressed TCRs were preferentially co-expressed with endogenous human TCRs (Fig 5E-**left**). Conversely, highly-expressed TCRs could outcompete the endogenous chains, resulting in both mTRBC+hTCR- and mTRBC+hTCR+ populations (Fig 5E-**right**), likely corresponding to cells with dominant or weak endogenous TCRs, respectively[51]. Therefore, transduction of different exogenous murinized TCRs in primary T cells could result in diverse patterns of surface expression and competition with endogenous human chains. However, the level of TCR reactivity was not systematically affected by such different expression patterns: when we measured CD137 upregulation on CD8+ T cells transduced (mTRBC+) with highly or lowly-expressed MLANA-specific TCRs, we observed that both TCR categories had a wide range of sensitivities for APCs pulsed with increasing doses of cognate $MLANA_{27–35}$ peptide (Fig. 5F). In summary, in primary T cells, the reactivity and avidity of exogenous murinized TCRs does not correlate with the strength of their surface expression and with the level of competition with the endogenous TCRs, but rather depends on the intrinsic properties of each TCR.

Screening and combinatorial staining of TCR-transduced effectors—After transduction of TCR constructs, the reactivity of cell lines can be assessed. TCR-transduced Jurkat cells can be tested as soon as they achieve stable growth in culture; conversely, TCR-transduced T cells can only be tested starting from 2 weeks after their stimulation, as initial activation by CD3/CD28 stimulating beads (required to transduce T cells) drives high background expression of CD137 during the first week of activation. Importantly, the composition of TCR-transduced T cells generally changes after T cell activation and expansion, as early phases (week 1–2) are dominated by CD4+ T cells, while late T cell cultures (week 3) are characterized by predominance of CD8+ T cells. Therefore, when using primary T cells, it is important to establish the costimulatory restriction of the tested TCRs: CD4-specificities should be screened at week 2 after activation, while testing of CD8-TCRs is preferred on week 3.

To simultaneously test multiple TCR-transduced cell lines, we developed a staining strategy using combinations of dilutions of 3 dyes to distinguish each effector once cells are organized in pools and analyzed by flow cytometry. By staining effectors using up to 4 concentrations (3 dilutions and a negative staining) for each of the 3 dyes (CellTrace Far Red (FR), Violet, and CFSE), it is possible to label, combine and distinguish by flow cytometry up to 64 TCR-transduced cell lines (Fig. 2C, 3-**Middle**). The staining can be easily adjusted to combine fewer effectors (Supplementary Table 1): as an example, in Fig. 3 we report staining of 33 effector cell lines that were stained with 4 dilutions of CellTrace Violet and 3

dilutions each of CellTrace Far-Red and CFSE. Of note, when a high number of TCRs are reconstructed and expressed in effectors, the staining strategy can be repeated to organize the effectors in multiple pools. For example, TCR constructs can be cloned in 96 well-plates and transduced into effectors to generate 96 TCR-transduced cell lines, which can be divided into 3 groups of 32 effectors, stained with a 4/3/3 combinatorial dilution of 3 dyes (Fig 3, Supplementary Table 2) and organized in 3 discrete pools. We recommend including internal negative controls in each pool, such as untransduced effectors and/or cell lines transduced with an irrelevant TCR (Fig. 3), which will provide the background reactivity of the effectors. Finally, we avoid the use of unstained effectors (triple negative condition: FR-neg, CFSE-neg, Violet-neg, Fig. 3) as CD8- (CD4+) effectors cannot be discriminated from target cells using flow cytometry.

Selection and preparation of target conditions—Because this combinatorial staining allows the simultaneously screening of multiple effectors against the same target cells, it is possible to test the reactivity of TCR-transduced cell lines even against primary targets with limited availability. Indeed, this protocol can be applied to test the reactivity of numerous TCRs against patient-derived or HLA-matched primary cells. For instance, it is possible to determine whether TCRs can directly recognize epitopes presented by patient-derived tumor cell lines or primary tumor cells isolated from patient biopsies. To foster antigen presentation, target cells can be cultured with high doses of inflammatory stimuli (i.e. IFNγ) to induce upregulation of peptide-MHC complexes. The specificity of such recognition can be assessed by testing the reactivity of TCRs against non-tumor controls, such as peripheral blood cells, fibroblasts or cell cultures generated from the patient's healthy tissues. Specificity for viral antigens can be established by testing TCR reactivity against HLA-matched cell lines infected with viral particles. Furthermore, it is possible to mimic indirect recognition, by testing TCR reactivity against APCs pulsed with cell lysates obtained from the targets of interest (e.g. tumor cell lines or virus-infected cell lines). It is important to note that this experimental modality favors the presentation of peptides captured from the extracellular environment within the MHC class II molecules, and therefore is expected to elicit recognition of CD4-restricted TCRs.

The optimization and miniaturization of the TCR screening assay allows for scaling up of the number of tested target conditions, enabling the experimental assessment of TCR reactivity against hundreds of different individual peptides or peptide pools pulsed on HLA-matched APCs. As hundreds of targets can be screened in one day, screening of TCRs can become sustainable and less time-consuming. This requires the availability of high numbers of APCs, and therefore EBV-LCLs are a suitable choice, since they constitute a virtually infinite source of HLA-matched APCs. Of note, the presence of epitopes processed from viral proteins can trigger the reactivity of EBV-specific TCRs regardless of the presence of pulsed peptides (Fig. 3, 5D). When available in sufficient quantities, autologous or HLA-matched PBMCs, in vitro cultured B cells[5,7,39] or dendritic cells[52] can be used as APCs. Finally, when HLA-matched APCs are unavailable, it is possible to use HLA class I-negative cells lines (e.g. B721.221, K562, Cos7) transfected to express patient-specific HLA alleles[44,53]. Monoallelic HLA lines can be used in parallel or in pools to test TCR

reactivity against the full set of patient HLA alleles and to identify the HLA restriction recognized by deorphanized TCRs.

Analysis of data—As discussed in previous sections, TCR reactivity can be monitored through flow cytometry by measuring CD137 or CD69 upregulation on TCR-transduced primary T cells or Jurkat cells, respectively. As depicted in Fig. 3, analysis of flow cytometry data consists of: 1) identification of singlets and lymphocytes using the forward- and side-scatter channels; 2) selection of CD8+ or CD8-(CD4+) T cells (or CD8+CD4+ Jurkat cells); 3) identification of single effector subpopulations with discrete combinations of CellTrace dyes; 4) selection of TCR-transduced cells (mTRBC+ for primary T cells or mTRBC/CD3+ for Jurkat cells); and 5) evaluation of CD137 or CD69 expression. The level of CD137/CD69 upregulation measured in each effector line is assessed in the absence of targets or in presence of activation agents (Phytohemagglutinin L (PHA) and phorbol 12-myristate 13-acetate (PMA)+Ionomycin) as negative or positive controls, respectively. Importantly, the ability to test effectors simultaneously allows the inclusion of internal controls (untransduced effectors or cells transduced with an irrelevant TCR, Fig. 3-**Internal Control**) within the same pool of effectors; these controls are pivotal for defining the background TCR reactivity, which can be subtracted from CD137 expression detected in corresponding conditions. When using primary T cells, these controls are necessary to assess the background reactivity of the endogenous TCR repertoire. When upregulation of CD137/CD69 is observed for selected TCRs cultured with peptide-pulsed APCs, the specificity of reactivity can be assessed by monitoring the same parameters in the presence of APCs pulsed with solvent or unrelated peptides (Fig. 3-**TCR2**). Strong antigen reactivity also drives downregulation of TCR surface expression, which can provide further evidence of recognition of the cognate antigen (Fig 3-**TCR2, right**). Finally, when EBV-LCLs are used as APCs, TCRs might show reactivity to all patient EBV-LCL conditions, independently of the tested peptide: in these cases, we infer reactivity against EBV-derived antigens (Fig. 3-**TCR1**). Specificity can be further validated by testing for the absence of TCR reactivity against patient B cells not transformed with EBV (see Anticipated results), as we have shown previously[4].

Advantages and limitations

Our approach allows for the investigation of TCR antigen reactivity in biological contexts where hundreds of TCRs can be tested against hundreds of antigens. This strategy provides the key advantage of minimizing the use of often-limited human tissue samples, including tumors, through pooled testing of TCR reactivity. It also allows for testing of a curated library of peptides or peptide pools covering important classes of antigens, each determined by a specific disease context and designed to answer specific hypothesis. While such an approach may limit the coverage of the entire patient TCR repertoire or potential antigen space, application of this method with precise biological questions and antigenic candidates defined by data can lead to the successful identification of TCR specificity against tissues and antigens (see Anticipated results).

The major limitation of our approach is cost: both DNA gene-blocks encoding TCRs and libraries of peptides can be expensive. By cloning and testing multiple TCRs in

parallel, it is possible to partially reduce the costs of TCR inserts, since vendors can offer discounted prices ($150–200 per gene-blocks) when purchasing a high volume of TCR inserts. Expansion of this assay to include thousands of TCRs or peptides, while technically straightforward, is thus constrained by reagent costs. We hope the development of high-throughput technologies for the production of these reagents will lead to a reduction of cost, which will increase the applicability of this protocol to uncover TCR specificities in diverse disease contexts.

Materials:

Biological materials

!CAUTION Any experiments using human material must conform to relevant institutional and national regulations, and informed consent must be obtained. Experiments shown in this manuscript were conducted using T cells isolated from peripheral blood collected from healthy donors under DF/HCC study ID T0191 approved by the Dana-Farber/Harvard Cancer Center Institutional review board (IRB). Reactivity of TCR-transduced T cells is usually tested against cells isolated from patients (e.g. tumor, PBMCs, APCs) or antigen peptides identified from genomic analyses (e.g. whole exome sequencing, bulk RNA-seq) of patient samples. In such cases, all patients were required to provide written informed consent for the collection of tissue and blood samples for research and genomic profiling, as approved by an IRB. For experiments shown in “Anticipated results”, approvals were obtained from Dana-Farber/Harvard Cancer Center IRB, 14–362[23].

- NEB 5-alpha Competent E. coli and SOC medium (NEB, Plate (**Step 24**): cat. no. C2987P, Tubes (**Step 19**): C2987H)
- Lenti-X 293T cells (Takara Bio, cat. no. 632180)

  !CAUTION: Lentiviral work should be performed wearing protective gloves and clothing in BL2 containment. All waste should be treated with a 10% bleach solution for at least 20 minutes. In this protocol, the lentiviral particles are produced using 2nd generation plasmids designed to minimize the chance of generating replication competent virus, but caution is still advised.
- Peripheral blood mononuclear cells harvested from healthy donors
- ΔTCRαβ CD8+ Jurkat cells (as generated by other groups[6,22,54])

Reagents

General Reagents

- Ficoll Paque Plus (Cytiva, cat. no. GE17-1440-02)
- ACK Lysing Buffer (ThermoFisher,, cat. no. A1049201)
- rCutSmart Buffer (NEB, cat. no. B6004S)
- BamHI-HF (NEB, cat. no. R3136L)
- SalI-HF (NEB, cat. no. R3138L)

- UltraPure DNase/RNase-Free Distilled Water (ThermoFisher, cat. no. 10977023)
- UltraPure Low Melting Point Agarose (ThermoFisher, cat. no. 16520100)
- UltraPure TAE Buffer, 10X (ThermoFisher, cat. no. 15558026)
- UltraPure Ethidium Bromide, 10 mg/mL (ThermoFisher, cat. no. 15585011)

  !CAUTION: Ethidium Bromide is a powerful mutagen. Be sure to wear proper PPE to avoid skin exposure, including gloves, laboratory coat, and closed toed shoes. When possible, work in a chemical hood, and always dispose of liquid waste in a clearly labeled waste container.
- DNA Gel Loading Dye (6X) (ThermoFisher, cat. no. R0611)
- QIAquick Gel Extraction Kit (QIAGEN, cat. no. 28706)
- QIAGEN Plasmid Plus 96 Miniprep Kit (QIAGEN, cat. no. 16181)
- QIAGEN Plasmid Plus Midi Kit (QIAGEN, cat. no. 12943)
- QIAGEN EndoFree Plasmid Maxi Kit (QIAGEN, cat. no. 12362)
- Gibson Assembly Master Mix (NEB cat. no. E2611L)
- LB Broth (Fisher Scientific, cat. no. BP9723–500)
- LB Agar, Lennox (Fisher Scientific, cat. no. DF0401–17)
- Ampicillin (Fisher Scientific, cat. no. ICN19014805)
- Glycerol (Molecular Biology) (Fisher Scientific, cat. no. BP229–1)
- Phosphate-buffer saline (PBS, 1X), sterile-filtered (Corning, 21–040-CV)
- Sodium azide (ThermoFisher, cat. no. 190380050) !CAUTION: Fatal if swallowed, in contact with skin or if inhaled. May cause damage to organs through prolonged or repeated exposure. Very toxic to aquatic life with long lasting effects. Wear protective gloves and clothing and only work in a well-ventilated area. Avoid release into the environment.
- Hybri-Max DMSO (Sigma Aldrich, D2650–100mL) !CAUTION: Combustible liquid. Keep away from heat and flames; wear protective gloves.

Plasmids:

- Plasmid backbone: Lentiviral vector backbone for transduction of a gene cassette with EF1a promoter, a gene of interest (GFP inactivated by stop codons) between two restriction sites and terminal sequence WPRE (Addgene #212009 or similar)
- Plasmid psPAX2: 2nd generation lentiviral packaging plasmid encoding for HIV-1 Pol and Gag (Addgene #12260 or similar)
- Plasmid pMD2.G (VSV-G): plasmid encoding for envelope proteins for lentiviral production (Addgene #12259 or similar)

Cell Culture Reagents

- Dulbecco's modified Eagle medium (DMEM), high glucose, pyruvate (ThermoFisher, cat. No. 11995065)
- RPMI Medium 1640 (ThermoFisher, cat. no. 11875119)
- Trypsin-EDTA (0.05%), phenol red (Life Technologies, cat. no. 25300120)
- Fetal Bovine Serum (FBS) (ThermoFisher, cat. no. 10437028)
- Penicillin/Streptomycin (P/S), 10,000 U/mL (ThermoFisher, cat. No. 15140122)
- Recombinant Human IL-2 (ThermoFisher, cat. no. 202IL010)
- Recombinant Human IL-7 (Peprotech cat. no. 200-07-100UG)
- Recombinant Human IL-15 (Peprotech cat. no. 200-15-100UG)
- Opti-MEM (Life Technologies, cat. no. 31985062)
- TransIT-LT1 Transfection Reagent (Mirus Bio, cat. no. MIR2300)
- Pan T Cell Isolation Kit (Miltenyi Biotec, cat. no. 130-096-535)
- CD3/CD28 Dynabeads (ThermoFisher, cat. no. 11132D or Biolegend, cat No. 422603)
- Polybrene Infection/Transfection Reagent (ThermoFisher, cat. no. TR1003G)
- PMA (Sigma Aldrich, cat. no. P1585–1MG) !CAUTION: Fatal if swallowed, in contact with skin or if inhaled. Causes severe skin burns and eye damage. May cause an allergic skin reaction. May cause allergy or asthma symptoms or breathing difficulties if inhaled. Suspected of causing cancer. Handle only in a fume hood and wear protective gloves and clothing.
- Ionomycin (Sigma Aldrich, cat. no. I0634–1MG) !CAUTION: Harmful if swallowed. Handle with gloves in a fume hood.
- PHA-L (Sigma Aldrich, cat. no. 11249738001) !CAUTION: May form combustible dust particles in air. Handle with gloves in a fume hood.
- Dimethyl Sulfoxide (DMSO, for freezing medium) (ThermoFisher, cat. no. BP231–100) !CAUTION: Combustible liquid. Keep away from heat and open flames, and wear protective gloves.

Antibodies

- Anti-Human CD3:PE/Cyanine7 Antibody (BioLegend Cat# 317334, https://scicrunch.org/resolver/AB_2561451)
- Anti-Human CD4:Pacific Blue Antibody (BioLegend Cat# 317429, https://scicrunch.org/resolver/AB_1595438)
- Anti-Human CD8a (referred to as CD8 hereafter):Brilliant Violet 785 Antibody (BioLegend, cat. no. 301046, RRID: AB_2563264)

- Anti-Human CD137:PE Antibody (BioLegend Cat# 309804, https://scicrunch.org/resolver/AB_314782)
- Anti-Human CD69:PE Antibody (BioLegend Cat# 310906, https://scicrunch.org/resolver/AB_314840)
- Anti-Murine TCR Beta:PE-Cyanine7 Antibody (Thermo Fisher Scientific Cat# 25-5961-82, https://scicrunch.org/resolver/AB_2573507 or Biolegend Cat# 109222, https://scicrunch.org/resolver/AB_893627)
- Anti-Murine TCR Beta:PE Antibody (Thermo Fisher Scientific Cat# 12-5961-82, https://scicrunch.org/resolver/AB_466066 or Biolegend Cat# 109208, https://scicrunch.org/resolver/AB_313430)

Dyes

- Zombie Aqua Fixable Viability Kit (BioLegend, cat. no. 423101)
- CellTrace Violet Cell Proliferation Kit (Life Technology, cat. no. C34557)
- CellTrace Far Red Cell Proliferation Kit (Life Technology, cat. no. C34564)
- CellTrace CFSE Cell Proliferation Kit (Life Technologies, cat. no. C34554)
- Rainbow Calibration Particles, 8 peaks (Spherotech, cat. no. RCP-30–5A)

Primers for TCR plasmid sequencing (Integrated DNA Technologies (IDT))

Oligonucleotide	Description	Sequence (5’–3’)	Step
Primer 1	End-pEF-1a Forward Primer	GTGGGTGGAGACTGAAGTTAGGCC	**37**
Primer 2	TRBC Forward Primer	CCTGACCATGTGGAACTGAGCTG	**37**
Primer 3	P2A Reverse Primer	GTCCAGGATTTTCCTCCACATCTCCG	**37**
Primer 4	WPRE Reverse Primer	GAGGAGAAAATGAAAGCCATACGGGAAGC	**37**

Equipment

- Finnpipette F2 Multichannel Pipette (P200, 30–300 μL) (ThermoFisher, cat. no. P4662070); use with 200 μL barrier pipette tips (Genesee Scientific, cat. no. 26–412)
- Finnpipette F2 Multichannel Pipette (P10, 1–10 μL) (ThermoFisher, cat. no. P4662080); use with 10 μL pipette tips; use with 10 μL barrier pipette tips (Thomas Scientific cat. no. 1159M41)
- Finnpipette F2 Multichannel Pipette (P20, 5–50 μL) (ThermoFisher, cat. no. P4662090); use with 20 μL barrier pipette tips (Genesee Scientific, cat. no. 26–404)
- Finpipette Multistepper 8 Channel P1000 (ThermoFisher, cat. no. P4540500, or comparable pipette with min. 1000 μL capacity); use with compatible 1000 μL tips (USA Scientific, cat. no. 1111–2721)

- twin.tec PCR Plate 96 LoBind, semi-skirted (ThermoFisher, cat. no. E0030129504)
- Bacteriological Petri Dish, 100 mm × 15 mm (Corning, cat. no. 351029)
- 24-well Tissue Culture Plate (CellTreat, cat. no. 229123)
- 48-well deep-well plates (Axygen, cat. no. P-5ML-48-C-S)
- 96-well round bottom plate (Corning, cat. no. 353077)
- 96-well flat-bottom plate (Corning, cat. no. 353072)
- 96-well V-bottom plate (Corning, cat. no. 3894)
- G-Rex 24 Well Plate (Wilson Wolf, cat. no. 80192M)
- 175 cm^2 U-shaped Cell Culture Flask with Vent Cap (Corning, cat. no. 431080)
- 1.5 mL microcentrifuge tube (ThermoFisher, cat. no. 0540296)
- Falcon, 50 mL centrifuge tube (Corning, cat. no. 352070)
- Microseal ‘B’ PCR Plate Sealing Film (Bio-Rad, cat. no. MSB1001)
- Tissue culture plate sealing film, AeraSeal (MedSupply Partners, cat. no. 16-BS25)
- 70-μm cell strainer (Corning, cat. no. 352350)
- QIAvac 96 vacuum manifold (QIAGEN, 19504)
- DynaMag-2 Magnet (ThermoFisher, cat. no. 12321D)
- BD LSRFortessa Cell Analyzer (BD Biosciences) or similar spectral flow cytometers

  !CRITICAL: It is preferred to use flow cytometers equipped with blue, yellow, red and violet lasers or to use spectral flow cytometers. This will allow for clear separation and detection of signals from dyes (CellTrace CFSE, FarRed and Violet) and from antibodies conjugated with PE, PE-Cy7 and Brilliant Violet fluorochromes.
- NanoDrop ND-1000 Spectrophotometer (ThermoFisher)
- 37°C, 5% CO_2 cell culture incubator (any brand)
- Bacterial incubator with shaker (any brand, at 200–250 rpm)
- Microscope (any brand with the capacity to observe 96-well plates both under bright field and fluorescent light)
- Centrifuge (any brand, with a rotor to centrifuge 50 mL tubes at 500*g*, swinging bucket rotor to centrifuge 96-well plates at 590*g* at 37°C and deep-well 48-well plates at 2100*g* at 4°C)

Software

- Snapgene software version 7.0.1 or higher (www.snapgene.com)
- FlowJo software version 10.9 or higher (https://www.flowjo.com/solutions/flowjo/downloads)

Data files

- TCR-seq data with paired TCRα and TCRβ chains

!CRITICAL: TCR-seq can be obtained from multiple sources, including plate- or droplet-based single-cell TCR-seq identifying paired TCRα and TCRβ chains (Box 2). TCRs can be sequenced directly from primary materials or from T cells expanded *in vitro* upon antigen-stimulation and/or sorted based on activation markers.

Reagent Setup

LB-Amp Broth—Prepare LB-Amp Broth according to the manufacturer's instructions by adding 25g of LB Broth to 1 L of purified water (such as that generated by Milli-Q Ultrapure Water System, Millipore Sigma), mixing, and autoclaving for 15 minutes. LB Broth without antibiotics can be stored at room temperature (RT, ~20°C) for up to 2 months, as long as there is no evidence of bacterial growth. Ampicillin is added at a concentration of 100 μg/mL just before bacterial culture.

Amp-Agar Plates—Prepare LB-Agar Plates according to manufacturer's instructions by adding 35g of LB agar to 1 L of purified water (Mill-Q Ultrapure Water System, or similar), mixing, and autoclaving for 15 minutes. The autoclaved agar should then be cooled to roughly 60°C, before adding Ampicllin to a final concentration of 100 μg/mL. The agar can then be poured or pipetted into 100 mm × 15 mm petri dishes and allowed to harden for at least 30 minutes at RT, and ideally overnight to dry before storage at 4°C for up to three months.

Agarose Gel for separation of linearized plasmid—Prepare a 1% (wt/vol) UltraPure Low Melting Point Agarose gel in 1x TAE buffer (prepared by diluting 10x TAE buffer stock in water) and dissolve by heating the solution for several minutes, then mixing until the agarose is completely dissolved. Add Ethidium Bromide (stock: 10mg/mL) at a dilution of 1:10,000 under a chemical fume hood, mix the solution and let it rest briefly to remove air bubbles. Pour the solution into an appropriate gel tray, with combs sufficiently large to accommodate 50–80 μL of sample. Use immediately once the gel has hardened sufficiently.

Complete RPMI medium—Supplement RPMI Medium with 10% FBS (vol/vol) and 100 U/mL penicillin-streptomycin. This can be stored at 4°C for up to 2 months.

Complete DMEM medium—Supplement DMEM medium with 10% FBS (vol/vol) and 100 U/mL penicillin-streptomycin. This can be stored at 4°C for up to 2 months.

Freezing medium—Supplement FBS with 10% DMSO (vol/vol). This can be stored at 4°C for up to 2 months.

Transfection medium—Supplement DMEM with 10% (vol/vol) FBS inactivated at 50°C for 30 minutes; do not add penicillin-streptomycin. This can be stored at 4°C for up to 2 months.

Complete T cell medium—Supplement RPMI Medium 1640 with 10% (vol/vol) heat-inactivated FBS (30 minutes at 50°C), 100 U/mL penicillin-streptomycin, 5 ng/mL Recombinant Human IL-7 and 5 ng/mL of Recombinant Human IL-15. This can be stored at 4°C for up to 3 weeks.

FACS buffer—Supplement 500 mL of PBS supplemented 2% (vol/vol) FBS and 0.05% (vol/vol) Sodium Azide. This can be stored at 4°C for up to 1 year.

CellTrace CFSE, Far Red, and Violet Dyes—Reconstitute CellTrace dyes in 50 μL of DMSO per tube, immediately before the preparation of dye dilutions (**Step 118–120**). Reconstituted dyes should be used on the day of staining, but in our experience can be stored at 4°C and used for up to three months after reconstitution.

Production of common plasmids—Extract lentiviral packaging plasmids (psPAX2 and pMD2.G) and the backbone plasmid from transformed bacteria using the EndoFree Plasmid Maxi Kit, according to manufacturer's instructions. Expand transformed bacteria overnight in LB-Amp Broth at 37°C, shaking at 200–250 rpm. Elute plasmids in UltraPure water and dilute to a concentration of 100ng/μL, as verified by quantification with Nanodrop.

Lenti-X 293T cells—Before the start of transfection, ensure Lenti-X 293T cells have been passaged at least 2 times (passage 3 and 4, **Steps 40–44**) and expand them to ensure the availability of the number of cells that are needed to produce the virus used during multiple rounds of transduction (see Box 3).

Peripheral blood mononuclear cells (PBMCs)

1. Collect PBMCs from healthy donors, to be used as source of primary T cells. Harvest blood collars from healthy donors and purify PBMCs through gradient separation using Ficoll Paque Plus, following manufacturer instructions.
2. Resuspend PBMCs in 5 mL ACK Lysing Buffer and perform lysis of red blood cells by incubating the cell suspension at RT for 5 minutes. Block the reaction with 10 mL of Complete RPMI Medium.
3. Add PBS to a final volume of 50mL and centrifuge for 15 min at low speed (200g) at RT to separate residual platelets. Aspirate the supernatant and resuspend the PBMCs in 50 mL PBS. Determine cell number.
4. Freeze PBMCs aliquots. Centrifuge cells for 10 min at 500g at RT, Aspirate the supernatant and resuspend at 100×10^6 cells/mL in Freezing medium. Freeze aliquots of 1 mL (100×10^6 cells) and store long-term in liquid nitrogen.

TCR-KO Jurkat cells—Grow TCR-KO Jurkat cells in Complete RPMI medium in suspension in flasks (see **Step 43** for the number of cells needed). Split Jurkat cells when

they reach a concentration of 1–2×10^6 cells/mL by seeding T175 flasks with a minimum of $1/10^{th}$ of the culture.

Antigen-presenting cells—Prepare antigen-presenting cells for TCR specificity screens prior to commencing transduction steps. Options for cellular targets are described in Box 1.

Equipment setup

Flow cytometer compensation

1. Create experimental protocols with parameters for the detection of signals from fluorochrome-conjugated antibodies or dyes, as used to assess TCR transduction efficiency (**Steps 97–107**) and to screen TCR specificity (**Steps 128–136**).

2. For each flow cytometry panel (**Steps 100–101**, **118 and 131**), prepare controls for compensation. These include unstained cells, single-stained cells for each fluorochrome-conjugated antibody or CellTrace dye, and cells stained with all used fluorochrome-conjugated antibodies and dyes. We recommend labeling cells with dyes the day before the flow cytometric acquisition Set up voltages and establish a compensation to ensure the signals from single-color controls show a level of positivity that is distinct from the signal leaked from other fluorochromes into the same channel. For each color, confirm these voltages allow for distinct positive and negative populations in both single and fully stained samples to be detected.

   !CRITICAL STEP: Ensure that the fluorochrome conjugated antibodies allow for clear distinction of positive and negative populations within the stained cells. To achieve this, we recommend using PBMCs stained with antibodies specific for lineage markers (e.g. CD3, CD8, and CD4) conjugated with the required fluorochromes as compensation controls.

3. Assess the performance of the instrument by acquiring Rainbow Calibration Particles. Before each acquisition, calibrate the performance of the instruments using Rainbow Calibration beads to ensure the voltages of the instrument produce the same level of signal as was used for compensation.

Procedure

Part 1. Cloning of the TCR fragment into the plasmid backbone (Timing: ~3 weeks)

Selection of TCRs and design of cloning inserts (Timing: 3 weeks)

1. Select pairs of TCR α and β-chains from available data (see Box 2).

2. Prepare sequences required for TCR construct design and annotate the output of TCR sequencing and the nucleotides of the CDR3 regions. If the TCR is designed from scTCR-seq data, we recommend selecting one cell expressing the TCR of interest and extracting its sequencing data using its cellular barcode. Identify and annotate the CDR3 nucleotide sequences (Supplementary Table 3, black and bolded). For both TCR α/β-chains, we recommend retrieving

sequences covering at least 20–30 bp upstream and downstream of the CDR3 regions. (TCR Sequencing Outputs, Supplementary Table 3).

3. Identify canonical TRAV/TRBV gene sequences expressed by each TCR. Download the full TRAV and TRBV transcript sequences from Ensembl[55] (or other comparable sources of TRAV/TRBV genes): these will include the first nucleotides of the CDR3 sequences (Canonical TRAV/TRBV sequences, Supplementary Table 3).

4. In each TRAV/TRBV Canonical sequence, identify and mark the "TRAV/TRBV regions", starting with the ATG translation start codon and ending 6 bp (2 amino acids) before the start of the CDR3 nucleotide sequence (Supplementary Table 3, TRAV/TRBV regions, underlined). The start of the CDR3 can be identified by matching the last TRAV/TRBV nucleotides with the CDR3 sequences annotated in **Step 2.**

   !CRITICAL STEP: The last few amino acids prior to the CDR3 sequence often vary from TCR clonotype to TCR clonotype, and are thus best taken from sequencing output.

5. In the TCR sequencing output, identify and mark the start of the TRAC and TRBC sequences ("TRAC/TRBC overlap", the first 6 bp overlapping the murinized constant chains, see example in Supplementary Table 3).

6. In the TCR sequencing output, identify and mark the end of the canonical TRAV and TRBV sequences ("TRAV/TRBV overlap", the last 6 bp overlapping with the TRAV/TRBV regions established in **Step 4**, see example in Supplementary Table 3).

7. In the TCR sequencing output, identify the "CDR3A flanking region" or "CDR3B flanking region", starting at the end of "TRAV/TRBV overlap" and ending at the beginning "TRAC/TRBC overlap". (see example in Supplementary Table 3, underlined).

8. Using Snapgene, arrange the "TCR block" sequence as follows: 1) the "TRBV region" from **Step 4**; 2) the "CDR3B flanking region" from **Step 7**; 3) the murinized TRBC sequence (Murinized TRBC, Supplementary Table 3); 4) a Furin Site-SGSG-P2A linker (Supplementary Table 3); 5) the "TRAV region" from **Step 4**; 6) the "CDR3A flanking region" from **Step 7**; 7) the murinized TRAC sequence culminating in a stop codon (Murinized TRAC, Supplementary Table 3)

9. At the 5' and 3' end of the TCR block, add the sequences that are required for Gibson Assembly ("5'/3' Gibson assembly overlap", Supplementary Table 3), overlapping with the ends of the linearized plasmid used as backbone (example included for Addgene plasmid #212009, Supplementary Table 3).

   !CRITICAL STEP: It is essential that all components of the TCR block are in frame with the canonical start codon. If sequences are arranged correctly, one continuous reading frame should extend across the entirety of this sequence,

including the expected amino acid sequences of both the CDR3B and CDR3A, until the stop codon at the end of murinized TRAC.

10. For each TCR, check for the presence of sequence patterns (DNA hairpins, repetitions, GC or G rich areas) that might cause problems during the synthesis of the gene inserts. An online tool to predict the complexity of the gene insert (via a complexity score) is available on the Integrated DNA Technologies (IDT) website: https://www.idtdna.com/site/order/gblockentry. To address repetitive regions or otherwise problematic regions, modify nucleotide bases in codons where possible without affecting the amino acid sequence of TCR chains. We recommend minimizing the complexity score as much as possible.

11. Order gBlock gene fragments: we recommend ordering an amount of insert that is sufficient for at least 2 cloning attempts (200 ng), diluted in UltraPure RNAse/DNAse-Free Distilled Water at a concentration of 25 ng/μL. Alternatively, gene blocks can be purchased in a dry format and reconstituted prior to use.

    !CRITICAL STEP: It is also possible to subcontract cloning to different vendors, by ordering the cloning of the designed TCR inserts directly into expression plasmids. If this option is employed, the user can forgo **Steps 12–39**. This solution would save ~2 days of work for 96 TCR constructs but would significantly increase the costs of production of the TCR plasmids.

TCR cloning (Timing: 4 days)—!CRITICAL: It is important to minimize the persistence of circular plasmid (undigested), since the cloning procedure will be performed without selection of colonies, and bacteria transformed with undigested plasmid could survive in LB selection broth. For this reason, the digestion is performed overnight, in 3 steps (**Steps 12–14**).

12. Linearization of the backbone plasmid. Excise the coding region of a gene flanked by sites recognized by 1 or 2 restriction enzymes from the backbone. In our backbone plasmid, the coding region (inactivated GFP) is flanked by restriction sites recognized by BamHI and SalI. We recommend linearizing 60 μg of backbone plasmid (corresponding to 12 of the reactions shown below), as this should yield sufficient material to clone 96 TCR constructs (1 plate, ~10 μg). Assemble reactions in individual PCR tubes as follows, and incubate for 2 h at 37°C.

Reagent	**Per reaction**
Plasmid	5 μg
rCutSmart Buffer (10X)	10 μL
BamHI-HF (20,000 U/mL)	40 U (2 μL)
SalI-HF (20,000 U/mL)	40 U (2 μL)
UltraPure Dnase/Rnase-Free Distilled Water	up to 100 μL
Total Volume	*100 μL*

13. After 2 hours, add another aliquot of restriction enzyme (40 units (2 μL) per reaction for each restriction enzyme) and incubate overnight at 37°C (14–20 h).

14. Following overnight incubation, add a further aliquot of restriction enzyme (40 units (2 μL) per tube for each restriction enzyme), and incubate for 2 h at 37°C.

    PAUSE POINT: Digested plasmid can be stored at −20°C for months.

15. Gel extraction of linearized plasmid. Prepare a 1% (wt/vol) UltraPure Low Melting Point Agarose gel with Ethidium Bromide (1:10,000) for DNA visualization. Dilute samples using 6x DNA loading dye prior to gel loading. Load samples and run the gel (voltage: 140V, 1.5 h) to separate the linearized plasmid from insert. Visualize DNA under UV light and excise the gel-band corresponding to the expected linearized size of the backbone plasmid (~7750 base pairs).

    ?TROUBLESHOOTING

16. Purify the band using the QIAquick Gel Extraction Kit, following manufacturer's instructions. Elute linearized DNA with the minimum volume of water per column (25–30 μL) to achieve a goal concentration of >20–25 ng/μL. Based on our experience, the total expected yield is ~1.5 μg (~30–40%) of DNA per reaction, from an initial input of 5 μg per reaction.

    PAUSE POINT: Linearized plasmid can be stored at −20°C for months.

17. Test the efficiency of linearization. Prepare two individual Gibson assembly reactions in 0.2 mL PCR tubes, as follows.

Reagent	**Per TCR cloned**
Linearized Plasmid	100 ng
Gibson Assembly Master Mix (2X)	10 μL
Insert (25 ng/μL) or water	5 μL
UltraPure DNase/RNase-Free Distilled Water	up to 20 μL
Total Volume	*20 μL*

    For the first reaction use 5 μL of a representative TCR gBlock (100–125 ng), for the second one use 5 μL of water as a blank reaction (negative control).

18. Incubate the tubes for 30 min at 50°C, followed by cooling to −20°C to halt the reaction.

19. Transform 2.5 μL of these two products using NEB 5-alpha Competent *E.*coli, according to manufacturer's instructions.

20. Plate 100 μL of transformed bacteria on LB-Amp Agar plates, spread the bacteria on the surface and incubate the plates overnight at 37°C. After incubation, observe the growth present on each plate. If results are satisfactory,

proceed to **Steps 21–39** (Gibson Assembly of TCR constructs in 96-well format).

!CRITICAL STEP: Successful linearization of the plasmid should lead to a high number (>100) of Ampicillin-resistant colonies in the TCR-plate, and very few colonies (0–10) in the negative control plate.

?TROUBLESHOOTING

21. Gibson Assembly of TCR constructs in 96-well format. Prepare a cloning master mix on ice for the assembly of TCR gene blocks into the linearized backbone, as follows.

Cloning master mix

Reagent	**Per TCR cloned**
Linearized Plasmid	100 ng
Gibson Assembly Master Mix	10 µL
UltraPure DNase/RNase-Free Distilled Water	up to 15 µL
Total Volume	*15 µL*

Dispense 15 µL of the cloning master mix per well in a 96-well PCR plate.

22. Add 5 µL of each TCR insert (~100–125 ng) to each well and seal the plate with a clear adhesive plate cover. Spin down the plate to remove bubbles from wells. Confirm visually that each well has similar volume of reagents (20 µL).

23. Incubate the Gibson Assembly plate for 30 min at 50°C, followed by cooling to −20°C to halt the reaction.

PAUSE POINT: The product of this Gibson Assembly reaction can be stored at −20°C for months and used for subsequent bacterial transformations.

24. Bacterial transformation to expand the assembled plasmids. Thaw a 96-well plate (or fractions of a plate) of NEB 5-alpha Competent *E. coli* on ice.

25. Add 2.5 µL of the product of one Gibson Assembly reaction into each *E. coli* well.

26. Incubate the bacterial plate on ice for 20 min.

27. Incubate the plate at 42°C for 20 s, and immediately put it back on ice for 5 min (heat shock).

28. Add 180 µL/well of room temperature SOC medium, mixing thoroughly using a P200 multichannel pipette, seal the plate with a clear Microseal ‘B’ PCR sealing film, and incubate at 37°C for 1 h.

29. Prepare ~600 mL LB-Amp Broth with 100 µg/mL Ampicillin.

30. In four 48-well deep-well plates, add 3 mL of LB-Amp Broth per well.

!CRITICAL STEP: We recommend doubling the number of 48-well plates (four 48-well plates for 96 TCRs) to increase the amount of growing bacteria per TCR (2 wells per cloned TCR) and thus the yield of subsequent plasmid extraction.

31. After incubation (**Step 28**), inoculate 35 μL of each bacterial preparation in each of the corresponding wells of 48-well deep-well plates containing LB-Amp Broth. Cover plates with AeraSeal aerated covers, seal carefully and shake overnight (>16h) at 37°C at 200–250 rpm.

!CRITICAL STEP: Shaking vigorously is recommended, to increase bacterial growth. However, in this deep well format, increased shaking speed increases the risk of contamination across adjacent wells. Ensure plates are properly secured and level prior to commencing the overnight incubation.

?TROUBLESHOOTING

32. Centrifuge the 48-well plates at 2100*g* at 4°C for 15 min.

If two wells of transformed bacteria per TCR cloning were established (as recommended), these should now be combined: discard half of the media without disturbing the pellet, and resuspend the bacterial pellet using multichannel pipettes. Combine both wells with bacteria transformed with the same Gibson Assembly reaction product.

!CRITICAL STEP: This step is labor-intensive; we strongly recommend using a multichannel pipette with ≥1mL capacity to transfer liquids and resuspend bacteria.

33. (Optional) Store an aliquot of each transformed bacteria. Dispense 50 μL of autoclaved glycerol into each well of a 96-well PCR plate. For each well, add 100 μL of the corresponding TCR bacterial culture and resuspend by pipetting. This glycerol stock can be used to re-expand bacteria to reproduce TCR plasmids or to select individual pure bacterial colonies.

PAUSE POINT: A glycerol stock of TCR-transformed bacteria can be stored at −80°C for months.

34. Pellet the bacteria (2100*g* at 4°C for 15 min) before proceeding to plasmid extraction. Discard supernatant by inverting the plates and dry the edges of the wells with paper towels.

35. Purify plasmids in a 96-well plate format using the QIAGEN Plasmid Plus 96 Miniprep Kit, following manufacturer's instructions. In the final steps, elute TCR plasmids in plate-columns with 150 μL of sterile water per well. This constitutes the TCR-plasmid stock.

!CRITICAL STEP: This step is labor-intensive, and we strongly recommend using multichannel pipettes with tip volume capacities of 30–300μL and ≥1mL to efficiently transfer and mix solutions.

36. Quantify the concentration of each plasmid using a Nanodrop, or equivalent device for the measurement of DNA concentration.

37. Sequence each TCR plasmid using primers for reactions that fully cover the inserted fragment (for our construct: Primers 1–4). Sequencing will ensure that insertion of the gene block occurred in the correct orientation, and that no major mutations have been introduced through cloning. Compare the sequence to the one of the expected plasmid (assembled in silico using Snapgene, **Steps 1–10**) and ensure that each base is correctly sequenced in at least one reaction.

!CRITICAL STEP: In this methodology, TCR-plasmids are produced in bulk, and plasmids without insert or with incorrect TCR blocks may be present in a mixture. The cloning reaction has been set up to efficiently produce plasmids with the correct insert, as >50–70% of the sequenced product contains the correct TCR gene inserts. The efficiency strongly depends on the quality of the purchased TCR gene-block. Sequencing is thus essential to ensure that plasmids with the proper insertion are the most abundant construct within the mixture of cloned plasmids. Sequencing reactions starting from nucleotides located outside the coding region (example: Primers 1 and 4) are the most informative to assess purity of the plasmid product, as such primers can generate reactions even in plasmids that do not contain the insert.

?TROUBLESHOOTING

38. (Optional) For TCR-plasmids that fail to generate sequencing results confirming the expected insertion as the most abundant plasmid product, we suggest proceeding with standard screening of individual bacterial colonies of *E. coli*, grown on LB-Amp Agar plates, as described in **Step 20**. Individual colonies can then be selected and expanded in LB-Amp Broth for plasmid extraction using the QIAGEN Plasmid Plus Midi Kit according to manufacturer's instructions, with sequencing to confirm which colonies contain the selected plasmid with correct integration of the TCR gene block.

39. In a 96-well PCR plate, prepare an aliquot (100–200 μL) of each TCR plasmid diluted in sterile water to a final concentration of 25–30 ng/μL and store in a 96-well PCR plate (Supplementary Table 4, diluted TCR plasmid plate). After dilution, ensure that plasmid concentration is within the suggested range through measurement with a Nanodrop. Further adjust and measure the concentration until all plasmids are resuspended at 25–30 ng/μL.

!CRITICAL STEP: In the very rare case a TCR is unable to be cloned (after multiple attempts), we recommend leaving a gap in the diluted TCR plasmid plate. This removes the need to change the position of a large number of TCR plasmids, and provides a negative control of a non-transduced T cell line.

PAUSE POINT: Both diluted and TCR-stock plasmids can be stored at −20°C for months and used for production of lentivirus.

Part 2. Parallel production of lentiviruses and T cell transduction (Timing: 21–27 d (primary T cells) / >17 d (Jurkat cells))

Expansion of Lenti-X 293-T cells for transfection (Timing: 5 d)

40. At the start of this procedure: At least five days before transfection, thaw an aliquot of Lenti-X 293T cells (Box 3, **Step 7**) and plate in a 175 cm^2 flask in complete DMEM medium. Grow in an incubator at 37°C, 5% CO_2 (passage 3).

41. Split the cells when they reach 70–80% confluency (Box 3, **Steps 3–6**). Plate 0.9×10^6 Lenti-X 293T cells per 175 cm^2 flask for expansion to 70–80% confluency over 3 days (passage 4). After 3 days, each flask should contain roughly ~$10–20\times10^6$ Lenti-X 293T cells. Calculate the number of Lenti-X 293T cells needed for the experiment based on **Step 43** to determine the required number of flasks.

!CRITICAL STEP: T cell transduction efficiency relies heavily on the concentration and amount of lentiviral particles, which in turn depends on the quality of Lenti-X 293T cells during these stages. To ensure high quality Lenti-X 293T cells, minimize exposure to Trypsin during detachment, make sure to seed a single-cell suspension of cells, and split cells when their confluency is <80–90%. Work fast and with warm reagents (37°C) to minimize the time in which cells are exposed to room temperature outside the incubator.

!CRITICAL STEP: While Lenti-293T cells can be used after many passages, we recommend generating lentivirus with Lenti-X 293T no later than passages 6–8. For each transfection, it is best to start from aliquots frozen at early passages (passage 2, Box 3, **Step 7**).

Transfection of Lenti-X 293T cells (1 d)

42. Calculate the required number of wells for effector cell transduction. We report here representative amounts of cells for transduction of 96 TCRs, with the aim of obtaining ~10^7 effector cells per TCR after 2 weeks of expansion. To generate enough T cells or Jurkat cells for TCR specificity testing, we recommend setting up TCR-transduction of effector cells in quadruplicate (four wells of a 96-well plate), plating 0.1×10^6 cells per well. Thus, the recommended number of effector cells is 0.4×10^6 cells per TCR or control condition.

43. Calculate the required number of wells for Lenti-X 293T cell transfection for transduction of 96 TCRs, (Supplementary Table 4).

- For primary T cell transduction: Lenti-X 293T cells are used to produce lentivirus-containing supernatant across 3 consecutive days, and thus 3 wells of Lenti-X 293T cells are required per well of T cells, for a total of 12 wells of Lenti-X 293T cells per TCR (1 set of 4 wells to be harvested on each transduction day).
- For transduction of Jurkat cells: Lentiviral supernatants are used on 2 consecutive days: this would require the preparation of a total of 8 wells per transduced TCR.

- As Lenti-X 293T cells are plated at a concentration of 2.25×10^4 cells/well, each TCR transduction requires 2.7×10^4 (for primary T cell effectors) or 1.8×10^4 (for Jurkat effectors) Lenti-X 293T cells. For other experimental conditions, these numbers can be scaled accordingly.

44. Detach Lenti-X 293T cells at the end of passage 4, using 0.05% Trypsin-EDTA as previously described (Box 3, **Steps 3–6**). Resuspend cells in Transfection medium, count cells and adjust to a concentration of 2.25×10^6 cells per mL of Transfection medium.

45. Using a multichannel pipette, plate 100 µL per well (2.25×10^4 cells Lenti-X 293T) of cell suspension in a flat-bottom 96-well plate (**Day −1**) and incubate overnight at 37°C, 5% CO_2 (16–20h). Plate Lenti-X 293T cells as needed according to the calculations outlined in **Step 43**, including cells required to produce lentiviruses for control conditions (untransduced or transduced with an irrelevant TCR). As an example, production of lentiviruses for 96 TCR constructs would require the preparation of 3 96-well plates (12 wells per TCR) or 2 plates (8 wells per TCR) of Lenti-X 293T cells when primary T cells or Jurkat cells are transduced, respectively (see Supplementary Table 4, Lenti-X 293-T transfection plates). We recommend plating Lenti-X 293T cells early in the afternoon (2–4 pm) to achieve a confluency of roughly 50–80% by 9 am the following morning.

!CRITICAL STEP: To ensure high production of viral particles, it is important to accurately count Lenti-X 293T cells using a hematocytometer or similar counting technique and plate them at a proper density.

46. After the overnight incubation (**Day 0**), check that the Lenti-X 293T plated in 96-well plates have reached a confluency of 50–80% or wait until cells reach such concentration.

47. Prepare and assemble packaging plasmids for Lenti-X 293T cell transfection. According to the type of effector cell used (primary T cells or Jurkat cells, see **Step 43**), prepare reagents for 12 or 8 wells of Lenti-X 293T cells per TCR/control, as follows:

Transfection master mix

Reagent	**Amount per well of Lenti-X 293T cells**
psPAX2 (100 ng/µL)	150 ng (1.5 µL/well)
pMD2.G (100 ng/µL)	60 ng (0.6 µL/well)
Opti-MEM (Room Temperature)	10.9 µL/well
Total Volume	*13 µL/well*

Scale up the volume based on the number of wells of plated Lenti-X 293T cells (**Step 43**); calculate an excess of 10% of wells to ensure enough master mix is available despite loss of volume occurring through pipetting.

48. In a 96-well round bottom plate (Supplementary Table 4, transfection plasmid mix plate), transfer 162.5 µL (*13 µL* × 12.5 for primary T cells) or 119.5 µL (*13 µL* × 8.5 for Jurkat cells) of the transfection master mix into one well per TCR or control to be transduced. These amounts are calculated based on the number of wells with Lenti-X 293T cells previously plated for each TCR/control (12 or 8 for primary T cells or Jurkat cells, respectively), with an excess of 0.5 wells to account for liquid loss with pipetting.

49. In each well of the transfection plasmid mix plate, add the corresponding TCR plasmid stored at a concentration of 25–30 ng/µL in a 96 well plate (diluted TCR plasmid plate, **Step 39**). Add 12.5 µL (for 12.5 wells) for primary T cells or 8.5 µL (for 8.5 wells) for Jurkat cells, corresponding to 25 ng of TCR plasmid/ Lenti-X 293T well.

50. Similarly, add 25 ng of plasmid per Lenti-X 293T well to establish control conditions. We recommend the following controls: 1) untransduced control (no plasmid, add an equivalent volume of Opti-MEM); 2) control plasmid containing a GFP insert (transduction efficiency control); 3) two unrelated TCRs (control TCRs to assess background reactivity), which can be selected based on their CD4 or CD8 restriction. Unrelated TCRs might exert alloreactivity against tested APCs, therefore we recommend producing lentivirus for at least 2 unrelated TCRs, to maximize the chances of generating a TCR-transduced control with low background reactivity.

51. Prepare the following master mix:

Transit master mix

Reagent	Amount per well of Lenti-X 293T cells
Opti-MEM (Room Temperature)	9.4 µL
*Trans*IT-LT1 Transfection Reagent	0.6 µL

Scale up the volume based on the number of wells of plated Lenti-X 293T cells (**Step 43**); calculate an excess of 10% of wells. Gently add *Trans*IT-LT1 Transfection Reagent to the Opti-MEM, mix by pipetting slowly 2–3 times, and incubate this master mix at room temperature for 5 min.

52. Dispense 125 µL (for primary T cells) or 85 µL (for Jurkat cells) of the Transit master mix in each well of the transfection plasmid mix plate, changing tips for every well. Gently mix by pipetting up and down 2 times. Incubate for 20–25 minutes at room temperature to allow for formation of DNA:*Trans*IT-LT1 complexes. This constitutes the final transfection master mix.

53. Add 24 µL of the final transfection master mix (13 µL of transfection master mix; 1 µL of TCR plasmid, 10 µL of transit master mix) in each of the corresponding Lenti-X 293T cell wells designated for each TCR. Incubate the

96-well flat-bottom plates with Lenti-X 293T overnight in a 37°C incubator, 5% CO_2.

!CRITICAL STEP: Add the transfection mix gently against the wall of the wells to avoid mechanically detaching the Lenti-X 293T cells.

!CRITICAL STEP: This step is labor-intensive, as the same transfection mix is added across two/three plates of Lenti-X 293T cells (Supplementary Table 4, Lenti-X 293-T transfection plates). Use a multichannel pipette and label plates well to avoid incorrect transfer.

54. After overnight incubation of Lenti-X 293T cells with the transfection reagents, visually confirm the presence of GFP+ Lenti-X 293T cells in the GFP control wells (recommended in **Step 50**) using a fluorescent microscope (**Day 1**).

?TROUBLESHOOTING

Preparation of primary T cells for transduction (Timing: 2 d)—!CRITICAL: For transduction of TCRs in primary T cells from healthy donors, plate T cells following **Steps 55–79**. For transduction of TCRs in TCR-KO Jurkat cells, plate cells following **Steps 80–96**.

55. Preparation of T cells from primary PBMCs. When Lenti-X 293T cells are plated (**Day −1**), thaw previously collected and processed human donor PBMCs (Reagent Setup, Peripheral blood mononuclear cells) and resuspend them in Complete RPMI medium at a concentration of 2–4 × 10^6 cells/mL. To favor T cell recovery and survival, add Recombinant Human IL-2 at low concentration (20 IU/mL), transfer the cell suspension on a 75 or 175 cm^2 cell culture flask and incubate overnight at 37°C. Thaw as many PBMCs as needed, based on the total number of T cells required for the experiment (as discussed in **Step 42**) and taking into account the following factors: 1) overnight recovery will cause loss of 20% of PBMCs; 2) T cells usually constitute 60% of PBMCs; 3) T cell selection usually yields ≥ 50% of expected T cells. Therefore, we recommend thawing approximatively 50 × 10^6 PBMCs for every 10 × 10^6 T cells required at day 0.

!CRITICAL STEP When possible, avoid the use of T cells from healthy donors with HLA alleles overlapping with those of the patients (source of TCRs and in many cases APCs). This minimizes the chance of presentation of donor antigens in the context of matched HLAs, which could give rise to high T cell background reactivity. Due to the polymorphic nature of HLA loci, it is uncommon to find patient-donor pairs with multiple matched HLA alleles. However, we recommend verifying that healthy donors and patients do not share the most common HLA allele in the Caucasian population (HLA-A*02:01). This can be easily assessed by flow cytometry, by staining donor and patients PBMCs with a fluorochrome conjugated anti-HLA-A2 antibodies.

!CRITICAL STEP It is preferred to use PBMCs frozen within 2 years, to ensure high viability of thawed cells.

56. After transfection of Lenti-X 293T cells (**Day 0**, **Step 53**), use a 70-μm cell strainer to filter the cell suspension of PBMCs into a 50 mL tube. This will remove any aggregates and cellular debris formed during overnight recovery.

57. Enrich for T cells using the Pan T Cell Isolation Kit according to the manufacturer's instructions. Centrifuge T cells (7 min, 500g, RT), discard the supernatant and resuspend in 5 mL Complete T cell medium. Determine cell concentration and take a volume corresponding to the required number of T cells (**Step 42**) with a 5% excess.

?TROUBLESHOOTING

58. In a 1.5 mL microcentrifuge tube, prepare the CD3/CD28 Dynabeads required for activation of T cells. Pipette 50 μL beads per 10^6 T cells (corresponding to a ratio of 2 beads per 1 T cell) and wash the beads 3 times with 1 mL of PBS using a magnetic tube rack. Resuspend beads in 1–2 mL of Complete T cell medium.

59. Add beads to the T cell suspension and adjust its volume with Complete T cell medium to reach a concentration of 0.7×10^6 cells/mL.

60. Mix the bead-T cell suspension thoroughly. Using a multichannel pipette, plate 150 μL of T cells (roughly $1{\times}10^5$ cells) per well in a 96-well flat-bottom plate. For each TCR/control condition, plate 4 wells of T cells (see **Step 42**, Supplementary Table 4, TCR-Transduction effector plates)

61. Incubate T cells overnight at 37°C, 5% CO_2.

62. After overnight incubation, examine the morphology of the plated T cells to assess cellular health visually. T cells should start to grow in large clumps around the activation beads (**Day 1**).

?TROUBLESHOOTING

Parallel transduction of multiple TCRs in primary T cells in a 96-well plate format (Timing: 14–20 d)

63. Lentiviral transduction of activated T cells. Following overnight incubation of both Lenti-X 293T cells and T cells (**Day 1**), prepare an aliquot (500 μL per plate) of Complete T cell medium supplemented with 100 ng/mL each of IL-7 and IL-15 (final concentration of 105 ng/mL). Prepare 96-well V-bottom plates following the same layout of plates with activated T cells (**Step 60**, Supplementary Table 4, TCR-Transduction effector plates); using a multichannel pipette, dispense 5 μL of the media supplemented with 105 ng/mL of IL-7 and 15 at the bottom of each well.

64. For each transduction condition (TCR or control), transfer the viral supernatant (roughly 120 μL) produced in 4 of the 12 wells with Lenti-X 293T transfected with corresponding plasmids (from Step 53). Transfer the supernatant into corresponding wells of the prepared 96-well V-bottom plates, following the layout of plates with activated T cells (**Step 60**, Supplementary Table 4, TCR-Transduction effector plates). Use a multichannel pipette and discard

tips when aspirating supernatants between different TCR-encoding viruses. The supplemented cytokines will be diluted in viral supernatant to a final concentration of ~5 ng/mL.

65. Centrifuge both the V-bottom plate loaded with viral supernatant and the T cell plate at 500*g* for 3 min at RT. The centrifugation is performed to precipitate any transferred Lenti-X 293T cells to the bottom of the V-bottom well.

?TROUBLESHOOTING

66. Gently remove and discard 110 μL of supernatant from each well of T cells; to avoid removal of T cells, gently aspirate media from the walls without touching the bottom of wells.

67. Using a multichannel pipette, aspirate viral supernatant from the ridge of the V-bottom plate (above the conical "V" portion of the well, to avoid transferring Lenti-X 293T cells into T cell culture) and transfer it to the corresponding wells with activated T cells. For T cells, this constitutes the first day of transduction (**Day 1**); incubate the T cells overnight at 37°C.

!CRITICAL STEP: Centrifugation should concentrate cells on the right edges for wells in plate columns 7–12, and on the left edges for wells in columns 1–6. Aspirate supernatant from the opposite well edges (right edges for plate columns 1–6 and left edges for columns 7–12) to avoid transfer of residual Lenti-X 293T cells to the T cell plate. This step is designed to bypass the filtering of lentiviral supernatant.

68. The following day (**Day 2**), prepare an aliquot (1 mL per plate) of Complete T cell medium supplemented with 100 ng/mL of IL-7 and IL-15, and 100 μg/mL of polybrene. Aliquot 10 μL per well in a 96-well V-bottom plate. Following the layout of conditions marked on the T cell plate (**Step 60**, Supplementary Table 4, TCR-Transduction effector plates), repeat the transfer the lentiviral supernatant from 4 wells of Lenti-X 293T cells transformed with corresponding TCR/control constructs, as outlined in **Steps 64–67**. After dilution in viral supernatant (roughly 120 μL), polybrene will reach a final concentration of 8 μg/mL.

?TROUBLESHOOTING

69. Perform a lentiviral spinfection. Centrifuge the plates with T cells in viral supernatant at 37°C at 590*g* for 2 hours, setting a low deceleration speed (3 or lower). After that, incubate the plates overnight at 37°C, 5% CO_2.

70. The following day (**Day 3**), repeat **Steps 64–67**. This time, add 10 μL/well of T cell medium with high concentration of cytokines (105 ng/mL IL-7 and 15) in a 96-well V-bottom plate. This will provide enough cytokines to support the growth of T cells for 3 days. Use viral supernatant harvested from the remaining 4 wells of transfected Lenti-X 293T cells per condition. After transfer of viral supernatant, resuspend the T cells completely with a multichannel pipette, by

pipetting up and down. Change tips before pipetting T cells transduced with different lentiviral supernatants.

?TROUBLESHOOTING

71. In wells located at plate edges, replenish Complete T cell medium as needed to account for media evaporation. Place the plate in the incubator and let the T cells expand for 3 days in a 37°C, 5% CO_2.

72. Dynabead removal (**Day 6**). Six days after T cell activation (corresponding to 3 days after last transduction, **Steps 70–71**), centrifuge the T cell plates at 500*g* for 3 min at RT to concentrate the T cells at the bottom of the wells.

!CRITICAL STEP: **Steps 73–78** are labor-intensive, as they require repeated transfer and mixing of each TCR-transduced cell line. Label all plates and tubes clearly, and use a P200 multichannel pipette to facilitate transfers of media.

73. Aspirate 110 μL of media from each well and replace it with 100 μL of warm Complete T cell medium. Resuspend cells by pipetting up and down with a multichannel pipette.

74. Pool the T cell-bead suspension from each set of 4 wells containing T cells transduced with the same lentivirus (encoding for a specific TCR/control) into a properly labelled 1.5 mL microcentrifuge tube (total of ~400–500 μL).

!CRITICAL STEP We recommend performing this procedure in small batches of 8–16 transduced TCRs or controls, to avoid errors. Repeat **Steps 74–77** for each batch of transduced T cells.

75. To collect residual beads and T cells, add 100 μL of Complete T cell medium into each well of the T cell plates, mix by pipetting, and transfer the medium into the corresponding 1.5 mL microcentrifuge tubes containing T cells and beads, previously labeled (**Step 74**). The T cell/bead suspension in each microcentrifuge tube will reach a volume of ~900–1000 μL.

76. Using a P1000 pipette, mix the T cell-bead suspension in each microcentrifuge tube vigorously (>30 times) to ensure detachment of the Dynabeads from the T cells. Place each tube in a magnetic tube rack and allow the beads to adhere against the side of the tube close to the magnet for at least 3 min.

77. Carefully pipette the medium containing the T cells detached from the Dynabeads out of the microcentrifuge tubes, without disturbing the layer of beads formed against the wall of the tube close to the magnet. Aspirate from the opposite side, without removing the tubes from the magnetic rack. Transfer the cell suspension into individual wells of a G-Rex 24 Well Plate. Supplement the T cells with 1 mL of Complete T cell medium and incubate at 37°C with 5% CO_2.

78. For the following days (**Day 7–15**), add Complete T cell medium when the culture has visibly yellowed due to cellular expansion. Volume of medium per well should not exceed 4–5 mL. As necessary, aspirate at least 50% of medium

with a pipette and replace it with an equal amount of Complete T cell medium without disturbing the cells at the bottom of the wells.

?TROUBLESHOOTING

79. Two days before labeling the cells for specificity screening (**Steps 108–136**), maintain the cell culture by using Complete RPMI medium without cytokines. Cytokine starvation is performed to preserve a low background level of activation and proliferation, which is necessary for screening of TCR reactivity.

Preparation of Jurkat cells for transduction and parallel transduction of multiple TCRs in Jurkat cells in a 96-well plate format (Timing: >10 d)

80. On the day of transfection (**Day 0**), passage the Jurkat cells to reach a concentration of roughly 0.1–0.4×10^6 cells per mL (Box 3, **Steps 2–6**). Culture cells in Complete RPMI medium in a 175 cm^2 flask; make sure to plate at least 50% of the cells required on **Day 2** for the experiment (see calculation in **Step 43**).

81. Forty-eight hours after transfection (**Day 2**), harvest Jurkat cells and pellet the cell suspension at 500g for 5 minutes at RT. Resuspend Jurkat cells in Complete RPMI medium at a concentration of 4×10^6 cells/mL.

82. Prepare a set of flat-bottom 96-well plates. Plate 25 μL (1×10^5 Jurkat cells) of cell suspension per well with 4 wells per transduced TCR/control (plate map established in **Step 43**, Supplementary Table 4, TCR-Transduction effector plates). Store the plates in an incubator (37°C, 5% CO_2).

83. Prepare 2 sets of 96-well V-bottom plates with the same layout as the Jurkat cell plates (Supplementary Table 4, TCR-Transduction effector plates).

84. For each transduction condition (TCR or control), remove the viral supernatant (roughly 120 μL) produced in 4 of the 8 wells with Lenti-X 293T transfected with corresponding plasmids (from **Step 53**). Transfer the supernatant into the corresponding wells of the first set of 96-well V-bottom plates. Use a multichannel pipette and change tips when aspirating supernatants containing different TCR-encoding viruses.

85. Centrifuge the V-bottom plates loaded with viral supernatant at 500*g* for 3 min at RT. This centrifugation is performed to precipitate any transferred Lenti-X 293T cells to the bottom of the V-bottom well.

?TROUBLESHOOTING

86. Using a multichannel pipette, aspirate the viral supernatant from the ridge of the V-bottom plate (above the conical “V” portion of the well, to avoid transferring Lenti-X 293T cells into T cell culture) and transfer it to the corresponding wells of the second set of 96-well V-bottom plates.

87. Repeat **Step 85**. For each condition, aspirate the viral supernatant as described in **Step 86** and transfer it (roughly 100–120 μL) into the corresponding wells of the flat bottom-96 well plates with Jurkat cells (**Step 82**).

!CRITICAL STEP: Centrifugation should concentrate cells on the right edges for wells in plate columns 7–12, and on the left edges for wells in columns 1–6. Aspirate supernatant from the opposite well edges (right edges for plate columns 1–6 and left edges for columns 7–12) to avoid transfer of residual Lenti-X 293T cells to the subsequent plate. This step is designed to bypass the filtering of lentiviral supernatant.

88. Perform a lentiviral spinfection. Centrifuge the plates with Jurkat cells in viral supernatant at 37°C at 590*g* for 2 hours, setting a low deceleration speed (3 or lower). After that, incubate the plates overnight at a 37°C, 5% CO_2.

89. The following day (**Day 3**), prepare two sets of 96-well V-bottom plates following the layout of TCR/control conditions in the plates with Jurkat cells (**Step 82**). Repeat the transfer of lentiviral supernatant from 4 wells of Lenti-X 293T cells transformed with corresponding TCR/control constructs, as outlined in **Steps 84–87**. After transfer of the viral supernatant, resuspend completely the Jurkat cells with a multichannel pipette, by pipetting up and down. Change tips before pipetting Jurkat cells transduced with different lentiviral supernatants.

?TROUBLESHOOTING

90. In wells located at plate edges, replenish Complete RPMI medium as needed to account for media evaporation. Place the plates in the incubator and let the Jurkat cells expand in viral supernatant for 3 days in a 37°C, 5% CO_2.

91. After 3 days (**Day 6**), centrifuge the plates with Jurkat cells in viral supernatant at 500*g* for 3 min at RT.

92. Aspirate 110 μL of viral supernatant from each well and replace it with 100 μL of warm Complete RPMI medium. Resuspend cells by pipetting up and down with a multichannel pipette.

93. Pool the Jurkat cell suspension from each set of 4 wells transduced with the same lentivirus (encoding for a specific TCR/control) into a properly labelled well of a 24-well tissue culture plate.

94. To collect residual Jurkat cells, wash the 96-well plates with 100 μL of Complete RPMI medium; after pipetting up and down, transfer the medium into the corresponding well of the previously established 24-well plates (**Step 93**). Add Complete RPMI medium to reach a volume of ~2 mL in each well of the 24-well plate.

95. For the subsequent days, expand Jurkat cells in multiple wells or in flasks. Maintain a concentration of ~$0.25–1\times10^6$ cells/mL of Complete RPMI medium.

96. Two days before screening TCR reactivity of TCR-transduced Jurkat cells (**Steps 108–136**), passage the cells in Complete RPMI medium to reach a concentration of 0.25×10^6 cells/mL.

Evaluation of transduction efficiency by flow cytometry (Timing: 3 h)

97. Eight days after the start of transduction (**Day 8**), resuspend each TCR-transduced cell line (Jurkat cells or T cells) and transfer roughly 150 µL of cell suspension into the corresponding wells of a round-bottom 96-well plate.

98. Centrifuge the 96-well plate at 500*g* for 3 min at RT. Invert the plate to discard the supernatant.

99. Prior to staining, wash the residual media in the wells with 200 µL of FACS buffer and centrifuge the plate at 500*g* for 3 minutes at RT. Discard the buffer by inverting the plate.

100. Prepare Zombie Aqua Fixable Viability Kit (Zombie Aqua), previously resuspended in DMSO according to manufacturer's instructions. Dilute stock aliquots 1:20 in PBS. Stain each TCR-transduced cell line with 2.5 µL per well of the diluted Zombie Aqua. Incubate for 10 min at RT, in the dark.

101. During this 10-minute incubation, prepare the following master mix, using these or analogous fluorophores.

Flow cytometry staining master mix

Antibody/Reagent	Volume per well (dilution factor)
FACS Buffer	50 µL
Anti-Murine TCR Beta:PE Antibody	2 µL (1:25)
Anti-Human CD4:Pacific Blue Antibody	2 µL (1:25)
Anti-Human CD3:PE/Cyanine7 Antibody	2 µL (1:25)
Anti-Human CD8:Brilliant Violet 785 Antibody	1 µL (1:50)
Total	57 µL

Add 50 µL of the master mix per well and incubate for 18 minutes in the dark.

102. Wash each well with 150 µL of FACS Buffer. Centrifuge cells at 500*g* for 3 min at RT and discard the supernatant through inversion of the 96-well plate. Repeat this step two additional times by washing each well with 200 µL of FACS Buffer.

103. After the last wash, resuspend the T cells in 150–200 µL of FACS Buffer. Transfer a 50 µL aliquot of the untransduced cells into a 5 mL Round Bottom Polystyrene Test Tube. This sample will be used to confirm physical parameters within the compensation established in Equipment Setup, Flow cytometer compensation.

104. Using a flow cytometer, acquire and analyze untransduced cells. As described in Equipment Setup, Flow cytometer compensation, use Rainbow Calibration

Particles to establish analogous voltages to the previously established compensation. Run untransduced cells first to properly set the physical parameters (FSC and SSC) during acquisition of cellular events. Using the gating strategy laid out in Supplemental Figure 1, confirm that the untransduced T cells show 0% detection of mTRBC:PE.

?TROUBLESHOOTING

105. Using the plate-reading mode of a BD-Fortessa instrument (or comparable flow cytometer capable of detecting the fluorophores included in the Flow cytometry staining master mix), collect data from roughly 50 μL (or 10,000 viable Zombie Aqua- CD3+ T cells) from each well with a distinct TCR-transduced cell line or control.

106. Analyze the data to determine TCR transduction efficiency for each TCR-transduced T cell line (outlined in Supplementary Fig. 1). Using FlowJo or a comparable software, select for singlets based on a linear relationship between FSC-H and FSC-A values (Singlets).

107. Determine the overall transduction efficiency as the percentage of mTRBC+CD3+ cells among Zombie Aqua- lymphocytes. Transduction efficiency can be further assessed separately on CD4 or CD8 T cells by analyzing mTRBC+CD3+ expression among Zombie Aqua- CD8+ or CD4+ T cells (see Supplementary Fig. 1).

!CRITICAL STEP: If a GFP plasmid was used as a control for positive transduction, the GFP fluorescence is not compatible with the signal from Zombie Aqua due to spectral overlap between the two signals. In this condition alone, evaluate GFP expression by gating on cells with physical parameters (side and forward scatters) of lymphocytes.

!CRITICAL STEP: For a well-controlled experiment, the untransduced well should have 0% transduction, the GFP well should have a positivity of 60–100%.

?TROUBLESHOOTING

Part 3: Flow cytometric screening of T cell specificity (Timing: 2 d)

CRITICAL: The same procedure is used for screening TCR-transduced primary T cells and Jurkat cells.

108. Define the experimental organization of TCR-transduced effector cell lines in pools. Based on the effector cell lines to be tested, organize TCR-transduced cell lines in multiple pools of up to 26, 35 or 63 effectors each, as depicted in Supplementary Table 2 (Effector pool plates). Each pool should contain control subpopulations, including untransduced effectors and cell lines transduced with irrelevant TCRs (Control 1, Control 2). For example, we divide 96 TCR-transduced cell lines in 3 pools of 32 effectors and 3 controls (35 effectors). For each pool, organize the layout of the required number of 48-well deep well plates

(one or two plates per pool depending on the number of effector populations per pool, see Supplementary Table 2)

!CRITICAL STEP: For TCR-transduced primary T cells, we recommend using effectors for specificity screening 14–21 days after T cell activation. Since T cell cultures enrich for CD8+ T cells over time, it is preferrable to test T cell lines transduced with CD4-restricted TCRs on days 14–16. Reactivity of TCR-transduced Jurkat cells can be assessed any time after assessment of transduction (**Steps 97–107**).

109. Determine the number of conditions that need to be tested for each pool of TCRs. Each condition will require 0.25×10^6 dye-labeled cells for each pool of effectors (see Preparation of target cells).

110. Calculate the total number of effector cells per pool required for the experiment, as follows: Total effectors per pool = (0.25×10^6) * (# of conditions)

111. Calculate the number of cells needed from each individual TCR-transduced cell line, as follows:

Individual Effectors = [(Total effectors per pool) / (# of TCR-transduced cell lines per pool)] + excess (50%)

Example: Screening of 96 TCR-transduced cell lines (organized in 3 pools of 35 subpopulations) against 200 target conditions requires at least 0.25×10^6 * 200 = 50×10^6 effectors per pool. For this number of effectors, 50×10^6 / 35 = 1.43×10^6 cells for each TCR transduced cell line are required. We recommend staining 1.43×10^6 + 50% * (1.43×10^6) = 2.14 $\times10^6$ cells for each TCR transduced cell line.

!CRITICAL STEP: The number of effectors taken per TCR-transduced cell line should be in excess of the number needed, to account for loss of cells during the staining due to washing or dye-toxicity.

112. Labelling of TCR-transduced effectors (Jurkat or primary T cells) with Cell-trace CFSE, Far-Red and Violet dyes. Resuspend the cultures of TCR-transduced cell lines through pipetting. Determine the average concentration of cells in culture by counting representative effectors.

!CRITICAL STEP: Ensure that enough representative effectors (5–10 cell lines) are used to achieve an accurate average cell concentration.

113. In one to two 48-well deep well plates per pool (depending on the number of TCRs per pool, see scheme in Supplementary Table 2), transfer the calculated number of T cells needed per TCR-transduced cell line into individual wells (Stained Individual Effectors, **Step 120**). These plates will constitute the effector pool plates. We recommend leaving the well corresponding to triple-negative dye staining (see scheme in Supplementary Table 2) empty, as such staining will be indistinguishable from target cells during flow cytometric acquisition.

!CRITICAL STEP: Excess TCR-transduced T cells can be kept in culture for one additional week (up to 3 weeks after initial activation with anti-CD3/CD28 beads, **Steps 58–60**) in case TCR reactivity assays need to be repeated. If repetition of the readout is not possible within this timeframe, we recommend freezing TCR-transduced cell lines (range 1–50×10^6) in 1 mL of Freezing medium, and storing them in liquid nitrogen. These cells can then be thawed in Complete RPMI medium, rested overnight and used for reactivity testing starting at **Step 113**.

114. Cover the 48-well plate with an AeraSeal cover and replace removed media with Complete RPMI medium in the deep-well 24 well plates containing TCR-transduced cells.

115. Wash effector pool plates by filling each well with PBS (up to a total volume of 4 mL per well). Cover the 48-well plate with an AeraSeal cover and centrifuge the effector pool plates at 500*g* for 6 min at RT. Carefully pour out the media by inversion and dry the inverted plates on a paper towel to obtain a dry pellet of cells, for ~10 seconds.

116. Resuspend each well in a volume of PBS to reach a concentration of 4 million cells/mL (referred to as 1 volume), and incubate plates at 37°C while performing **Steps 117–119**.

Example: if 2.5×10 effectors have been distributed in each well, resuspend in 625 µL (1 volume) of PBS.

117. Determine the number of volumes needed for each dilution of dye. Based on the plate scheme reported in Supplementary Table 2, determine how many wells will be stained with each dye dilution. Calculate the total amount of dye needed for each dilution considering that each effector population will be stained with 1 volume of each of the corresponding dilutions.

118. Dilute CellTrace dyes. As described in Reagent Setup, CellTrace CFSE, Far Red, and Violet Dyes, reconstitute tubes of dyes provided by the manufacturer with 50 µL of DMSO. Based on the established volumes in **Step 117**, prepare the highest dilution (High) of each dye in PBS and perform serial dilutions (Medium/Low) in PBS in the dark, as outlined in the following scheme:

- 3×3×3 Dilution Scheme (26 TCRs/pool):

Dye (in 50uL DMSO)	µL of dye/mL PBS (High)	Dilutions
CellTrace Far Red Cell Proliferation Kit	3.3 µL	Medium: 1:10 of High
CellTrace CFSE Cell Proliferation Kit	3.3 µL	Medium: 1:10 of High
CellTrace Violet Cell Proliferation Kit	3.0 µL	Medium: 1:10 of High

- 3×3×4 Dilution Scheme (recommended when testing 96 TCRs, 35 TCRs/pool):

Dye (in 50uL DMSO)	µL of dye/mL PBS (High)	Dilutions
CellTrace Far Red Cell Proliferation Kit	3.3 µL	Medium: 1:10 of High
CellTrace CFSE Cell Proliferation Kit	3.3 µL	Medium: 1:10 of High
CellTrace Violet Cell Proliferation Kit	3.0 µL	Medium: 1:6 of High; Low: 1:6 of Medium

- 4×4×4 Dilution Scheme (63 TCRs):

Dye (in 50uL DMSO)	µL of dye/mL PBS (High)	Dilutions
CellTrace Far Red Cell Proliferation Kit	4 µL	Medium: 1:6 of High; Low: 1:6 of Medium
CellTrace CFSE Cell Proliferation Kit	4 µL	Medium: 1:6 of High; Low: 1:6 of Medium
CellTrace Violet Cell Proliferation Kit	3.0 µL	Medium: 1:6 of High; Low: 1:6 of Medium

119. In a deep-well 48-well plate, using one of the schemes provided in Supplemental Table 2, add 1 volume of each of the corresponding diluted dyes, leaving the triple negative well empty. Add one PBS volume in each well assigned a dye-negative dilution. Each well should have a total of 3 volumes (one for each dye). This will constitute the dye plate; one set of dye plates is required for each pool of effectors.

!CRITICAL STEP: This step is labor-intensive and requires special attention to properly combine the dyes and subsequently mix them with to the corresponding TCR-transduced effector cell line, following the scheme reported in Supplementary Table 2.

120. Working in dark conditions with a multichannel pipette (ideally with a tip volume of ≥1 mL, P1000), transfer each well of mixed dyes (from dye plates, **Step 119**) to the corresponding wells of the effector pool plates in PBS (**Step 116**), such that each cell line is at a final concentration of 1×10^6 cells/mL (in 4 total volumes), and thoroughly resuspend the T cells by pipetting up and down. Stain for 20 min at 37°C in the dark.

!CRITICAL STEP: It is important that for the wells of each plate, the staining starts at approximately the same time. Work fast and avoid spillage during transfer to avoid contaminations across the wells. If multiple plates are dyed at the same time, ensure that each plate is stained for 20 minutes, staggering staining if necessary. Uneven staining times can cause unwanted signal overlap between different populations during flow cytometric analysis.

121. Wash each well by adding Complete RPMI medium to reach a total volume of ~4 mL per well. Centrifuge the plate for 6 min at 500*g* rpm at RT. Discard the supernatant by inversion and dry the inverted plate on a paper towel. Ensure visually that pellets stained with CFSE and Far Red dye have the highest green and blue coloration, respectively.

?TROUBLESHOOTING

122. Add 3 mL of Complete RPMI medium per well, cover with aeroseal covers and incubate the 48-well deep well plates at 37°C for 0.5–2 h to allow release of excess dye from cells.

123. After this incubation, centrifuge each plate for 6 min at 500*g* at RT, and pour out all media through inversion of the plate. Dry the inverted plates on a paper towel.

124. Resuspend each cell pellet in roughly 300 μL of Complete RPMI medium, and consolidate all the stained TCR-transduced effectors in each pool into one tube. Count the number of T cells per pool and dilute to 2.5 million cells/mL in Complete RPMI medium.

?TROUBLESHOOTING

125. T cell-target co-incubation. For each pool of TCR-transduced effectors, plate desired targets in 100 μL of Complete RPMI medium in 96-well plates, using a flat-bottom plate for adherent cell targets, and a round-bottom plate for non-adherent targets (discussed in detail in Preparation of target cells).

126. Include control conditions in any TCR specificity test. We recommend testing the reactivity of each pool of TCR-transduced cell lines using triplicate wells of unstimulated T cells (100 μL of Complete RPMI medium) as a negative control, and of positive control conditions, such as PMA (100 ng/μL in 100 μL) and Ionomycin (final conc.: 20 μg/mL 100 μL), or PHA-L (8 μg/mL in 100 μL).

127. Plate effector pools for the screening assay by dispensing 100 μL (0.25×10^6 cells) effector pool cell suspension (diluted to 2.5 M cells/mL in **Step 124**) to each well with targets, for a total volume per well of 200 μL of Complete RPMI medium. Centrifuge all 96-well plates at 500*g* rpm for 2 min at RT to bring the effector and target cells into contact at the bottom of the wells, and incubate overnight (14–20 h) at 37°C, 5% CO_2.

128. Flow cytometry assessment of CD137 upregulation. After overnight incubation, transfer effector T cell pools cultured against any condition with adherent cell targets into wells of a 96-well round-bottom plate for flow cytometry, pipetting vigorously to ensure complete resuspension and transfer of T cells.

129. Centrifuge plates for 3 min at 500*g* at RT. Pour out the media through inversion of the 96-well round bottom plate.

130. Wash the pellets with 200 μL FACS buffer, and centrifuge again for 3 min at 500*g* at RT. Discard the supernatant by plate inversion.

131. Prepare a staining master mix as follows, using the recommended or analogous markers and fluorophores for testing activation of T cells:

CD137 assay master mix

Antibody/Reagent	Volume per sample
FACS Buffer	50 μL
Anti-Human CD137:PE Antibody (*For Jurkat, substitute Anti-Human CD69:PE Antibody)*	2 μL (1:25)
Anti-Human CD8:BV785 Antibody	1 μL (1:50)
Anti-Murine TCR Beta: PE-Cyanine7 Antibody *(For Jurkat, it is also possible to substitute Anti-Human CD3:PE-Cyanine7 Antibody as a marker of transduction)*	2 μL (1:25)

132. With a multichannel pipette, dispense 50 μL of the CD137 assay master mix per well. Mix by pipetting and incubate in the dark for 18 minutes at RT.

133. Wash each well with 150 μL of FACS buffer, centrifuge at 500*g* for 3 min at RT and discard supernatant by plate inversion. Repeat this step two additional times, washing with 200 μL of FACS buffer.

134. Resuspend cells in roughly 170–200 μL of FACS buffer, depending on the desired flow rate of cells through the flow cytometer.

135. Acquire cells using a flow cytometer equipped with high-throughput sampler (HST). Recall previously established settings with compensation for the analyzed parameters (Equipment Setup, Flow cytometer compensation) and calibrate the instrument using Calibration Rainbow Fluorospheres. Acquire an aliquot of cells and establish physical parameters (Forward and side scatter) to properly analyze the effector cells. Confirm 1) that staining of effectors with CellTrace dyes has produced events with a matrix of dye combinations allowing discrimination of distinct populations with different intensities of CellTrace CFSE, Far-Red and Violet within each pool; and 2) that effectors in wells without stimulation (negative controls) have low background of CD137 expression, while positive controls (PHA and PMA/Ionomycin) induce strong upregulation of CD137 on all effector cells (Fig. 3).

!CRITICAL STEP: As collection of flow cytometry data from dozens or hundreds of wells is a time-consuming process, set up a high-quality fluorescent compensation prior to running your samples (Equipment Setup, Flow cytometer compensation). Ensure that the highest dye concentrations (High CFSE, High Far-Red, High-Violet) emit signals that are not out of the range of detectable light intensities; Make sure that cells stained with intermediate dilutions (Middle and Low) give rise to distinct events that are not overlapping with other stained populations.

?TROUBLESHOOTING

136. Acquire events from all the wells using the HTS setting on the flow cytometer, recording at least 1000 events per subpopulation, as identified though fluorescence of CellTrace dyes (Fig. 3). This number of events can most often be achieved by collecting roughly 100–120 μL from each well.

!CRITICAL STEP: Both staining strategy and transduction efficiency affect the acquisition of data through flow cytometry, as the number of TCR-transduced (mTRBC+) cells recorded per subpopulation is inversely correlated with the number of TCR-transduced cell lines per pool and is directly correlated with transduction efficiency. When analyzing pools with a high number of TCR-transduced cell lines (e.g. 63) and/or with low levels of TCR transduction (<20% average mTRBC/cells), we recommend increasing the total number of acquired events.

137. Analysis of CD137 upregulation. Analyze flow cytometry data using FlowJo software and gate TCR-transduced effector cell lines as described in Fig. 3. Briefly, this consists of the following steps: 1) gate on singlets, with similar FSC-A and FSC-H parameters; 2) identify lymphocytes, based on FSC-A and SSC-A; 3) gate on CD8+ or CD8- (as a proxy for CD4+) effectors, depending on the expected restriction of a given TCR (as established from single-cell or functional data); 4) gate on each subpopulation, defined based on fluorescence of the three CellTrace dyes, according to the scheme of staining (Supplementary Table 2); 5) identify TCR-expressing cells by positivity for murine TCRβ-chain (transduction marker); 6) analyze CD137 surface expression on such transduced cells.

!CRITICAL STEP: We suggest grouping samples for analysis based on their most direct control. If conditions involve peptide-pulsed APCs, these should be grouped and analyzed together with their corresponding background control (DMSO-pulsed APCs). TCR reactivity against tumor cells should make use of background CD137 expression of effectors in the absence of stimulation and/or cultured with non-tumor controls.

138. In each TCR-transduced cell line, assess the level of activation induced through TCR stimulation. For the CD137 upregulation measured upon stimulation with each target, subtract the background level of CD137 expression detected in the same wells on internal controls (TCR control 1 or TCR control 2, Supplementary Table 2). We recommend using background effectors transduced with TCRs with the same CD4/CD8 restriction as that of tested TCRs. For example, to evaluate the reactivity of a TCR identified from a CD8+ T cell clonotype, calculate the percentage of CD137+ T cells among CD8+mTRBC+ effectors for both the tested TCR and a non-reactive CD8-restricted control TCR, whose reactivities are measured simultaneously against each target. Then, subtract these values measured in the same stimulation condition (%CD137 of tested TCR - %CD137 of control TCR): the presence of an internal control enables the reliable comparison of CD137 upregulation across multiple wells.

!CRITICAL STEP: We recommend assessing reactivity against positive controls (PMA-Ionomycin and PHA) at this step. Rarely, CD4 TCRs may have a lower level of CD137 upregulation in response to stimulation with PMA-Ionomycin. Although we expect CD137 upregulation close to 100%, in our experience for CD4 TCRs, analysis is possible with a CD137 upregulation of at least 20%. This lower rate of positivity is usually due to the high level of apoptosis induced by supraphysiological stimulus (PMA-Ionomycin), which causes lack of detection of CD137 among CD8- (CD4+) cells.

139. Assess the reactivity of each TCR-transduced cell line against APCs stimulated with peptides. For each TCR in a given pool of TCR-transduced effectors, determine the percentage of CD137+ cells among mTRBC+ (CD8+ or CD8-, depending on TCR restriction) when cultured in the presence of APC pulsed with peptides. In this case, we recommend subtracting the background level of CD137 measured on each TCR-transduced cell line when cultured with DMSO-pulsed APCs.

!CRITICAL STEP: This approach provides the relative CD137 upregulation induced by TCR recognition of each peptide, compared to its own background reactivity against the same APCs in the absence of a pulsed peptide. This is recommended when using APCs that might increase the level of basal activation, due to high expression of HLAs or costimulatory molecules (e.g. when using dendritic cells), or due to the presence of viral antigens (e.g. when using EBV-transformed LCLs). However, even in these cases, we recommend monitoring the level of activation of internal controls to assess whether CD137 upregulation measured on effectors in each condition is specific.

140. For any TCR displaying substantial upregulation of CD137 to a given condition, confirm reactivity by monitoring the level of expression of mTRBC+ cells in the tested population when stimulated with specific targets, non-specific targets or controls. In the case of a strong recognition mediated by cognate antigen-HLA complexes, TCR molecules should be sequestered from the surface of reactive effectors, thus causing decreased detection of mTRBC (Fig. 3). Low/Absent TCR downregulation measured on effector cells in the presence of APCs pulsed with high doses of peptide might be indicative of non-specific recognition and would necessitate the repetition of testing with targets pulsed with lower peptide doses for confirmation. On the contrary, TCR downregulation can be modest or low when measuring reactivity of TCR-transduced effectors against cellular targets expressing physiologic levels of antigens, as can be the case with endogenous processing and presentations of proteins.

Troubleshooting:

Troubleshooting advice can be found in Table 1.

Timing

Part 1. Cloning of the TCR fragment into the plasmid backbone

Steps 1–10, TCR selection and fragment design: 4 h

Step 11, Order TCR fragments: 3 weeks

Steps 12–14, Plasmid linearization: overnight, together with step 11

Steps 15–16, Gel purification of linearized plasmid: 3 h, together with step 11

Steps 17–20, Test of linearization efficiency, overnight, together with step 11

Steps 21–23, Gibson assembly of TCR fragments into linearized plasmid: 45 min

Steps 24–33, Bacterial transformation and growth: overnight

Steps 34–36, Plasmid preparation: 3 h

Steps 37–39, Plasmid sequencing and dilution: overnight

Part 2. Parallel production of lentiviruses and T cell transduction

Steps 40–46, Expansion and plating of 293T cells: 6 d

Steps 47–52, Preparation of transfection reagents: 1 h

Step 53–54, Transfect plasmids into 293-T cells: overnight

Preparation and lentiviral transduction of primary T cells—Steps 55–62, Preparation of primary T cells for lentiviral transduction: 2 d, together with steps 40–54

Steps 63–71, Lentiviral transduction of T cells and T cell expansion: 6 d

Steps 72–77, Dynabead removal: 6 h

Step 78–79, Plating and further primary T cell expansion: 8–14 days

Preparation and lentiviral transduction of Jurkat cells—Steps 80–82, Expansion and plating of Jurkat cells: 2 d

Steps 83–94, Jurkat cell transduction: 4 d

Steps 95–96, Expansion of TCR-transduced Jurkat cell lines: >4 d

Steps 97–107, Flow cytometric assessment of transduction efficiency: 3 h, together with steps 78–79 or 95–96

Part 3: Flow cytometric assessment of T cell reactivity

Steps 108–111, Organize TCR-transduced effector pools, 0.5 h

Steps 112–124, Staining of T cells with vitality dyes: 4 h

Steps 125–127, T cell-target co-incubation: overnight

Steps 128–136, Flow cytometric assessment of CD137 upregulation: 14 h

Steps 137–140, Analysis of flow cytometric results: 4 h

Box 1 Preparation of target cells

Steps 1, Selection and preparation APCs: variable

Steps 2–6, Peptide pulsing and preparation for T cell co-incubation: 3 h, together with steps 120–135

Steps 7–8, Preparation of cellular targets, overnight or 0.5 h

Anticipated results

Selection of TCRs from human specimens

The strength of this protocol is that it optimizes and simplifies the parallel reconstruction of hundreds of individual TCRs and it provides tools for their simultaneous screening against a customizable panel of targets, thus minimizing the use of cellular material. To demonstrate the efficiency of TCR reconstruction and expression in primary T cells and to test the ability to deconvolute TCR antigen specificity, we originally applied this methodology to T cell clonotypes detected in a glioblastoma multiforme (GBM) lesion. This set of experiments provided insights on the neoantigen specificity of TCR clonotypes in GBM patients treated with immunotherapies[23] and established the technological platform that was utilized for our subsequent studies in melanoma[4,21,45]. As previously reported, single-cell RNA and TCR profiling of CD45+ CD3+ T cells infiltrating a GBM biopsy revealed the presence of 266 TILs that could be grouped in 223 TCR clonotypes based on the identity of TCR α- and TCR β-chains (Supplementary Table 4). These clonotypes could be classified as CD8+, CD4+ or double negative (DN, CD4-CD8-) based on transcriptional expression of *CD4, CD8A* and *CD8B* genes. We also scored the activation and inhibition profile of each clone, by assessing the average expression of a panel of gene transcripts associated with activated or exhausted T cell states (Fig. 6A, Supplementary Table 4). We selected 98 TCR clonotypes based on: i) expansion within the tumor microenvironment (TCRs expressed by >1 cell, 24 TCR clonotypes); ii) high (> median) activation and/or inhibitory scores; iii) detection of TCR chains in peripheral blood, as available from previous studies[23]. For five of the 24 expanded clones, 2 TCR α-chains were consistently detected in >1 cells, and therefore we reconstructed each potential TCR α/β-chain pairing. For singleton clones that expressed 2 TCR α-chains, we selected the one with the highest level of expression, as determined by UMI count[4]. Using the methods described in **Steps 1–39**, we generated 103 TCR plasmids, which encoded for TCRs expressed by 52 CD4+, 41 CD8+ and 5 DN clonotypes (Fig. 6B-**left**). Sequencing of plasmids confirmed the presence of correct TCR inserts in all 103 TCR plasmids.

TCR transduction

Lentiviral transduction of TCR constructs in CD3+ T cells isolated from the peripheral blood of a healthy donor (**Steps 40–79**) resulted in the successful expression of the exogenous murinized TCR chains for 102 of 103 TCR constructs (99%), with an average transduction rate of 35% (range: 9.55–84.42% mTRBC+/CD3+ T cells, Fig. 6C). The intensity of mTRBC detection through flow cytometry was variable, even among TCR-transduced cell lines with highly similar transduction rates (Fig. 6D). This variability might reflect different conformation or stability of each TCR α/β-chain pair folded on the surface of T cells; in all cases, the intensity of mTRBC expression was sufficient to distinguish transduced cells from their untransduced counterparts. The expression levels of each TCR on the surface of T cells were maintained across multiple independent rounds of transduction performed using T cells from different donors (Supplementary Fig. 3A), even as the percentage of transduced cells differed substantially, thus suggesting that mTRBC detection and TCR stability are intrinsic features of each TCR α/β-chain pair construct. In line with our previous findings (Fig. 5E), the level of surface expression of murinized TCRs correlated with their ability to displace endogenous human TCRs (Supplementary Fig. 3B). Overall, this protocol allowed us to express 99% of the tested TCRs in primary T cells; a similar success rate was observed in our previous studies reporting reconstruction of >700 TCRs[4,21], thus demonstrating the robustness of this approach. In our experience, failed TCR expression in primary T cells could be due to: i) preferential expansion of plasmids with incorrect TCR inserts from bacterial growth; ii) lack of stability of folded TCR α/β-chains on the surface of T cells; or iii) reactivity of TCRs against HLA alleles expressed by the selected healthy donors, causing fratricide and depletion of transduced T cells. Of note, the last issue can be solved by expressing a TCR in T cells from donors with a different set of HLA alleles (see Troubleshooting, **Step 135**).

TCR reactivity screening

Once expressed in T cell lines, the reactivity of TCRs was assessed by measuring CD137 upregulation of transduced T cells (mTRBC+) in response to stimulation with the patient's APC pulsed with a series of antigens, as previously described (**Steps 108–140**, Box 1, **Steps 1–6**, Fig. 6B). As an example, we report here the antigen specificity screening of 102 GBM TIL-TCRs successfully cloned and expressed in primary T cells (Fig. 6C). Effectors were divided in 3 pools, each of which included 34 TCR-transduced T cell lines and 1 internal control (untransduced T cells or T cells transduced with an irrelevant TCR). The individual effectors in each pool (n=35) were labeled with combinations of CellTrace proliferation dyes (CT-CFSE: 3 dilutions, CT-Far Red: 3 dilutions, CT-Violet: 4 dilutions, Supplementary Table 2). After staining, TCR-transduced T cell lines were pooled, and each pool was cultured with HLA-matched patient-derived EBV-LCLs pulsed with a series of peptide antigens. Reactivity was analyzed on transduced CD8+ T cells for CD8-restricted TCRs and on CD4+ T cells for CD4 or DN-TCRs.

Selection of Antigens—In this example, the selection of antigens for TCR reactivity screening was guided by the availability of sequencing data (e.g. whole exome sequencing (WES) or bulk RNA-sequencing (RNA-seq)), proteomic data (e.g. HLA immunopeptidome) or immune data (e.g. *in vivo* immunization with known antigen, detection of antigen-specific

T cells), technologies which open the possibility of inferring or predicting the antigens expressed by the target cells of interest. Here, we evaluated WES and RNA-seq of the primary GBM specimen from the same patient[23] to generate a list of target antigens, which included (Fig. 6B-**right**): i) long peptides (25mers) covering each tumor mutation, identified by comparing the genetic profiles of GBM tumor and normal tissue; ii) 12 peptide pools comprising a total of 96 short peptides (9–11mers, 4 peptides per pool) predicted to bind patient's HLA alleles and derived from tumor-associated antigens (TAAs, n=32) overexpressed within the analyzed GBM specimen, as established by comparison of tumor RNA-seq versus controls (GTEx[56]); iii) 12 peptide pools comprising a total of 96 short viral peptides reported as binders of the patient's HLA alleles in IEDB (Immune Epitope Database)[57]; iv) lysates of cell lines infected with CMV of Flu viruses (Zeptometrix).

Results—As a first step, to assess the reliability of the reactivity assay, we evaluated TCR-reactivity in response to negative or positive controls. Of note, when EBV-LCLs are used as APCs, it is crucial to estimate the potential recognition of viral antigens, including those arising from endogenous presentation of EBV-related proteins. Nearly all the tested TCRs displayed low reactivity to negative controls (no target), while they were strongly activated in response to PMA/Ionomycin (>75% CD137+), and moderately (<25%) in response to PHA (Fig. 7A). For one TCR (TCR#08), a substantial (>5%) basal reactivity was observed in all conditions, even in negative controls. With this activation profile, high and non-specific TCR activation precludes the possibility of reliably investigating the specificity of this TCR, and thus it was thereafter excluded from subsequent analysis. Six TCRs conferred T cell activation when T cells were cultured with autologous EBV-LCLs pulsed pool of viral peptides[57]. For 4 of these TCRs (TCR#24, TCR#53, TCR#55, TCR#75, Fig. 7A), CD137 was upregulated with similar intensities in response to *in vitro* stimulation with autologous EBV-LCLs, regardless of the presence or absence of viral or control peptides. Such a pattern of reactivity suggests reactivity against EBV peptides endogenously presented by EBV-LCLs. Two TCRs (TCR#47, TCR#51) exhibited high reactivity to multiple viral pools pulsed on EBV-LCLs. To investigate whether these TCRs were specific for EBV antigens, we compared activation measured in presence of autologous EBV LCLs and with autologous B cells isolated from PBMCs (Fig. 7B). The signal transduced by 5 TCRs (TCR#24, TCR#47, TCR#51, TCR#55, TCR#75) was almost completely abrogated when T cells were cultured with primary autologous B cells, which lack EBV virus in an active replicative stage, demonstrating specificity for EBV-related antigens. Conversely, TCR#53 conferred high CD137 upregulation in all conditions, suggesting potential recognition of self-HLA antigen complexes on autologous APCs. Importantly, when we looked at scTCR-seq of primary TILs, we noted that TCR#53 was sequenced as a secondary TCR in TILs with expression of non-self reactive TCR#35 (Supplementary Table 4, Fig 7A). If compared to TCR#53, TCR#35 had a high expression and stability (Fig 6D), thus suggesting that in vivo primary TILs with dual expression of TCR#35–53, the expression of the primary TCR#35 might prevail at the expense of the reactivity of the secondary TCR#53.

In screenings against viral pools, 3 TCR-transduced effectors (TCR#24, TCR#47, TCR#51) exhibited a level of activation which was higher when tested with APCs pulsed with specific peptide pools compared to DMSO-pulsed EBV-LCLs (Fig. 7A), indicating potential

recognition of selected peptides. To assess this, we deconvoluted the reactivity of the 3 TCRs by re-screening their reactivity against autologous EBV-LCLs pulsed with lower doses (10^6 pg/mL) of viral peptides from positive pools (Fig. 7C, Supplementary Table 4). For each of these 3 TCRs, only one peptide was able to trigger high TCR reactivities at low peptide doses (Fig. 7C, red arrows), in each case consistent with those initially measured with high pooled peptide doses (10^7 pg/mL, Fig. 7A). These peptides mapped to EBV-specific proteins (Fig. 7D), and further assessment of TCR reactivity against autologous EBV-LCLs pulsed with increasing doses of cognate peptides defined the high affinity of these TCRs against EBV epitopes (Fig. 7E). Therefore, in these examples we document how it is possible to detect multiple EBV-specific TCRs using autologous EBV-LCLs, B cells and viral peptides. Ideally, the same strategy can be adopted to investigate TCR-specificity for other viruses, by testing T cell reactivity against virus-infected or non-infected target cells. Notably, this is feasible only when there is availability of autologous target cells that can be infected by specific viral strains. To overcome this limitation, we tested lysates of virally infected cell lines (Zeptometric) as a source of antigens with which to pulse autologous EBV-LCLs. This screening revealed that 4 CD4 TCRs exhibited specific recognition of human cytomegalovirus (hCMV) proteins, while 1 CD4 TCR reacted against Influenza A (FluA) antigens pulsed on autologous EBV-LCLs (Fig. 7F). This test was particularly effective for deconvoluting the specificity of CD4-restricted TCRs, since complex antigen mixtures (such as those generated by cell lysates) taken up by endocytosis are preferentially presented by APCs through HLA class II molecules[58]. Indeed, for 4 of 5 of these TCRs, CD137 upregulation was higher if measured on TCR-transduced CD4+ T cells rather than among CD8+ effectors (Fig. 7G), suggesting an activation mediated by antigen-HLA class II complexes through CD4 costimulation.

We also extended the screening of the specificity of GBM-TCRs to TAA peptides or tumor mutations, to identify TCRs with potential anti-tumor reactivity. None of the TCR-transduced cell lines showed specific recognition of TAA-peptide pools (Fig. 7H). As previously reported[23], 2 CD4-restricted TCRs (TCR#60, TCR#103) exhibited recognition of one neoantigen (NeoAg#07) generated from a single nucleotide variant (SNV), while none of the TCRs recognized peptides derived from novel open reading frame (NeoORFs) generated by insertion or deletion mutations (Fig. 7I). Detection of TCR reactivity against increasing doses of cognate antigens demonstrated that the NeoAg#07-reactive TCRs displayed intermediate avidity and high specificity to mutated peptide, as TCR-transduced cell lines did not exhibit major activation in the presence of the wildtype peptide (Fig. 7J).

Overall, these data demonstrate how this protocol can be applied to study the specificity of TCRs identified from single-cell sequencing data. In this example, we were able to deorphanize 13 TCRs among those identified in 97 TIL clonotypes. Detection of antigen-reactivity in primary T cells was not affected by the level of expression of murinized TCRs or by their competition with the endogenous human TCRs, since we were able to deorphanize TCRs with different expression patterns (Fig. 6D); these included lowly expressed TCRs with high competition with human TCRs (virus-specific TCR#51 and TCR#47, Supplementary Fig. 3B). As expected, the viral-specific TCRs constitute a major portion of the repertoire; still, the screening identified rare neoantigen-specific TCRs with potential for antitumor activity (Fig. 7K). The rate and type of TCR deorphanization of

our approach strongly depended on the knowledge of immunogenic antigens in a specific context: in GBM-TILs, where tumor-specific TCRs are known to be rare and immunogenic tumor antigens are poorly characterized, the rate of TCR-deorphanization was 14% using a curated library of viral, neoantigen and TAA epitopes. Conversely, when we applied this protocol to investigate the specificity of TILs in melanoma - a context in which antitumor specificities are abundant and immunogenic antigens have been well-described - the rate of TCR deorphanization ranged from 25 to 75%[4]. Once TCR cognate antigens are detected, it is possible to link T cell specificity with gene signatures expressed by T cells harboring the deorphanized TCRs, to gain insight into the T cell programs driving the function of viral and tumor antigen-specific TCRs (Fig. 7L). Although only a fraction of TCRs were deorphanized in this case, this system enables repetition of TCR expression and screening to test more targets, including peptides derived from detection of HLA-binding immunopeptidomes or antigenic libraries designed on the basis of sequencing data and expressed in HLA-matched APCs. Importantly, each reiteration of the screening can produce results in just 2–3 weeks and only requires expertise in cloning, T cell culture and flow cytometry. In conclusion, this protocol provides a useful tool designed to test the specificity of roughly 100 TCRs at a time in parallel against a diverse set of antigenic targets, with the potential to provide insights into biologic mechanisms underlying T cell function in infection, cancer and autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests

G.O is consultant for Bicycle Therapeutics. C.J.W. is an equity holder of BioNtech, Inc.; is a member of the SAB of Adventris, Aethon Therapeutics, and Repertoire; and receives research funding from Pharmacyclics. A.A. declares no competing interests.

Data availability

All the raw data and derived data of this manuscript are available from the corresponding authors upon reasonable request. The single-cell transcriptomic and TCR datasets referred in section “Anticipated results” have been previously reported[23] and are available through dbGaP portal with accession number phs001519.v1.p1.

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Key Points:

- This protocol combines Gibson assembly, a miniaturized lentiviral transduction system and combinatorial dye staining of pool-organized effector T cells to rapidly screen and simultaneously deconvolute the reactivity of up to hundreds of TCRs selected from single-cell TCR sequencing data
- This strategy is suitable for uncovering TCR reactivity against patient-derived samples with limited cell availability or curated panels of antigens to answer precise biological questions

Box 1:

Preparation of target cells

!CRITICAL: These steps provide different options for the preparation of target conditions, depending on the type of target cells and the format of antigens.

Preparation of peptide-pulsed APCs

1. Select an appropriate APC system for pulsing of peptide antigens. Each type of APC requires optimization of culture conditions for isolation, expansion, and manipulation with peptide antigens. Importantly, the generation of APCs from patient material guarantees the expression of the proper HLA alleles. This protocol is compatible with the use of different types of APCs, including:
   - Primary DCs or monocyte-derived DCs, which can be used to present pulsed or mini-gene driven antigens,[5,7,59] if available in sufficient numbers.
   - Primary B cells, which can be isolated through the use of CD19 Microbeads (Miltenyi Biotec, cat. no. 130-050-301).[7,59]
   - B cells immortalized through infection with the Epstein-Barr Virus, defined as lymphoblastoid cell lines (EBV-LCLs). These can be generated upon isolation of B cells from PBMCs and infection with EBV.[4,21,50,60,61]
   - Monoallelic cell lines, derived from HLA-negative cells (B721.221 cells,, K562, etc.) and transduced to express one or more patient-specific HLA alleles[44,62], which can be used to investigate the HLA-context of a given antigen's presentation.

   !CRITICAL STEP: For each type of APCs, it is important to experimentally define a proper ratio of effector and target cells, minimizing the level of background activation of TCR-transduced cell lines as much as possible.
2. Pulsing APCs for peptide antigen presentation (suggested amounts have been optimized for EBV-LCLs and B721.221 cells). On the day of effector-target co-incubation (starting at **Step 120**), resuspend APCs at a density of 5 million cells/mL in RPMI with 1% P/S (to maximize uptake of administered peptides). Plate at least 0.5×10^6 cells (100 μL) per pool of TCRs in a 96-well round-bottom plate, to ensure generation of enough peptide-pulsed APCs for reactivity testing.
3. Add 1 μL of the antigen per 100 μL of cell suspension. Antigens can include peptides reconstituted at an appropriate concentration in Hybri-Max DMSO, or cell lysates purchased or generated by thermal lysis[21,63,64].

   !CRITICAL STEP: Different peptide formulations and concentrations can be used to stimulate the reactivity of TCR-transduced cell lines. Generally,

crude peptides are sufficient for initial TCR specificity screening, but using pure peptide is advised to minimize the risk of false-positive results. For large-scale peptide screening of TCR-transduced T cells or Jurkats, a peptide concentration of 10^7 pg/mL can be used to pulse APCs (Fig. 5B). CD137 upregulation on effectors cultured with APCs pulsed with serial dilutions of peptides (>90% purity) can be used to assess TCR functional avidity (Fig. 5B).

!CRITICAL STEP: Antigen presenting cells pulsed with peptide pools or gene-modified with tandem mini-gene libraries (Fig. 5D, Anticipated Results) can also be used to test multiple antigens at once. In such cases, it is important to design pooling strategies to minimize the number of epitopes competing for the same HLA alleles. When possible, we recommend pooling peptides or minigenes predicted to bind to different HLA alleles. Additionally, when using peptides, we recommend pulsing APCs with high doses of each peptide (10^7 pg/mL) to overcome differences in HLA-binding between weak or strong epitopes and to maximize the chance of presentation on HLA molecules.

!CRITICAL STEP: This step is labor-intensive when using a large number of conditions. We recommend properly organizing target peptides in stock plates several days before the immune assays, and dispensing peptides using a multichannel pipette to facilitate their efficient pulsing onto APCs.

4. Incubate APCs with pulsed antigens for 2–3 h at 37°C, 5% CO_2. Pulsing can be started prior to labelling effectors with CellTrace dyes.

5. After pulsing, centrifuge the APCs for 3 min at 500*g* at RT. Carefully aspirate at least 50% of the media in each well and replace it with Complete RPMI medium, to dilute excess peptide and DMSO solvent in the medium, and to reintroduce FBS to cultures.

6. Plate 0.25×10^6 APCs per target plate in 100 μL of Complete RPMI medium (effector-target ratio 1:1). Proceed to **127**.

!CRITICAL STEP: When using EBV-LCLs as APCs, it is recommended to test non-immortalized patient-derived B cells isolated from PBMCs in parallel. This negative control allows for discrimination of EBV antigen-induced reactivity. Further, we recommend testing negative controls including APCs (autologous EBV-LCLs or HLA-modified B721.221 cells) pulsed with peptide solvent (DMSO) or irrelevant peptides, such as Ovalbumin Class I epitope (SIINFEKL) and Ovalbumin Class II epitope (ISQAVHAAHAEINEAGR).

Preparation of other cellular targets

7. Plate target cells at the desired Effector:Target ratio.

- For adherent cells: Plate cells on the day prior to target-T cell incubation (**Step 127**) in a 96-well flat-bottom plate in 100 μL of Complete RPMI medium.
- For non-adherent cells: Plate cells on the same day as target-T cell plating (**Step 127**) directly into the 96-well round-bottom plate used for the assay, in 100 μL of Complete RPMI medium.

!CRITICAL STEP: When testing TCR reactivity against viable cells harvested from precious samples with limited availability (primary tumor, patient tissues, etc.), we recommend testing the reactivity of pools in triplicate, to ensure unequivocal results and to minimize the chance that repeated testing is needed.

8. Proceed to **Step 127**, adding 0.25×10^6 T cells in 100 μL of Complete RPMI medium and incubating overnight.

!CRITICAL STEP: Depending on the type of cellular target, the number of plated cells may be optimized and adjusted. We recommend a ratio of 10:1 effector to target cells for large adherent cells, as 2.5×10^4 cells are sufficient to cover the area of the well and to elicit strong TCR recognition[4,21].

Box 2:

Recommendations for selecting TCRs

Since identification of correct pairing of TCR α and β-chains is pivotal for the reconstruction and activity of TCRs, we recommend selection of TCRα/β-pairs from clones detected within single-cell TCR-seq (scTCR-seq) data in at least 2 cells, possibly with high quality transcriptome, as established from scRNA-seq (between 250 and 10,000 reads, and mitochondrial gene content from scRNA-seq of <20%). Alternatively, other strategies might be designed to ensure identification of correct TCR α and β-chain pairing (e.g. isolation and growth of T cell clones *in vitro* followed by bulk TCR sequencing from RNA transcripts[65–67]). We recommend the selection of TCR clonotypes with RNA expression of 1 TCRα-chain and 1 TCRβ-chain. However, ~10% of T cell clones express 2 TCR α-chains[68]; to investigate the specificities of these clones, 2 different TCRs (one for each α/β TCR pairing) must be cloned and screened. This also applies to rare T cell clones showing expression of 2 distinct TCRβ-chains. In most cases, only one chain pairing displays stable surface TCR expression and/or target reactivity.

Box 3:

Culture and expansion of Lenti-X 293T cells

1. Thaw a new vial of Lenti-X 293T cells (passage 1) in Complete DMEM medium in a 175 cm^2 flask; grow cells in an incubator with 5% CO_2 at 37°C.
2. Upon reaching 70–80% confluency, split the cells as follows using warm (37°C) trypsin and Complete DMEM medium. Prepare a 50 mL conical tube with 10 mL of warm Complete DMEM medium. Aspirate the medium from the cells in flask, add 10 mL of PBS without disturbing the cell layer and gently wash to remove residual serum.
3. Aspirate PBS and add 5 mL of warm 0.05% Trypsin-EDTA; incubate for 30 s at 37°C to detach cells.
4. Once cell detachment has been visually confirmed, dissociate the Lenti-X 293T cells into a single cell suspension using a 5 mL serological pipette. Quickly transfer the cell suspension to the 50 mL tube with warm media to block the trypsin reaction. Wash residual cells in the flask with ~10–20 mL of Complete DMEM medium and transfer it into the collection tube.
5. Centrifuge at 500*g* for 5 min at RT, aspirate the supernatant, and count the number of viable cells in the resultant pellet using a hematocytometer or other cell counting methodology.
6. Plate roughly 1×10^6 Lenti-X 293T cells per 175 cm^2 flask, in as many flasks as cell count permits (passage 2), and grow in an incubator 37°C, 5% CO_2.
7. At 70–80% confluency, repeat Box 3, **Steps 4–7**. Resuspend the Lenti-X 293T cells at a concentration of 5×10^6 cells/mL in Freezing medium, and freeze 1 mL aliquots of the expanded Lenti-X 293T cells (after passage 2) for long-term storage in liquid nitrogen. Aliquots should be slow-frozen to −80°C followed by transfer to liquid nitrogen.

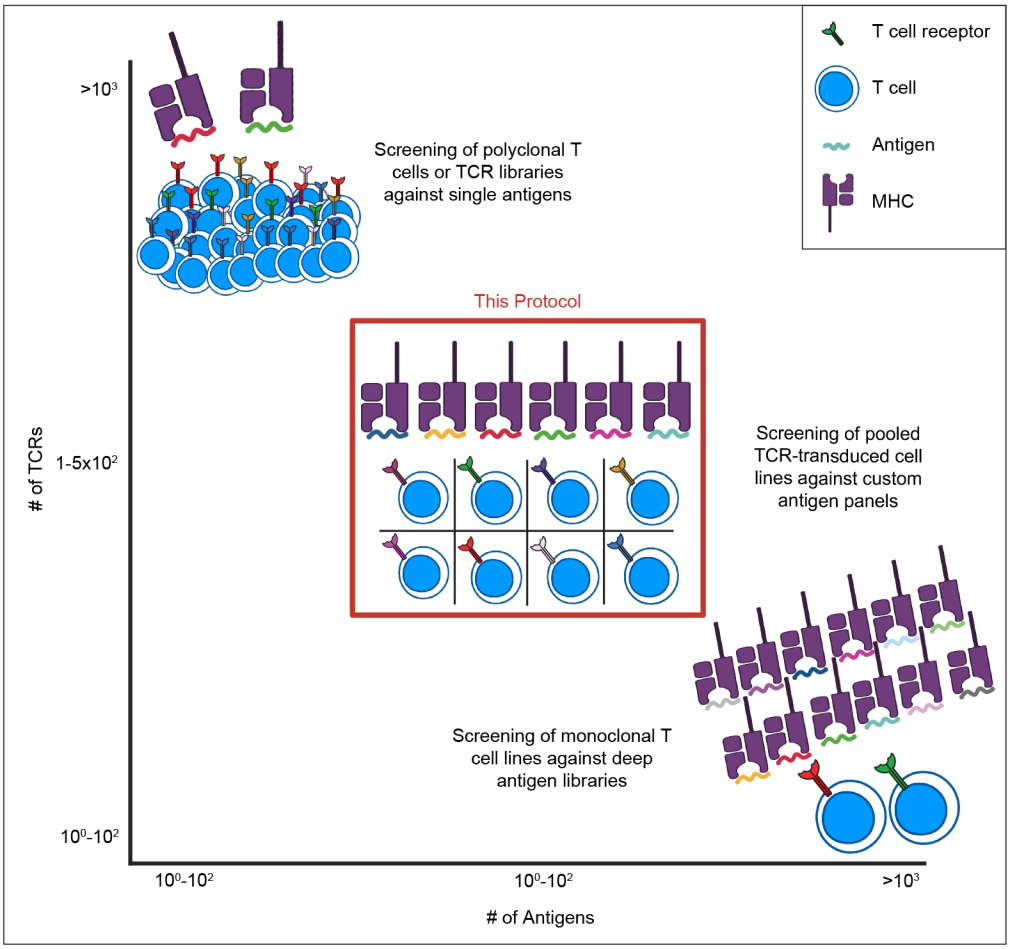

Fig. 1 |. A medium throughput approach to screen TCRs specificity.

Common approaches for screening TCR specificity generally rely on: 1) testing of polyclonal T cells (either primary cells or TCR libraries) with high TCR diversity (>10^5) against a small number of antigens (top-left); or 2) testing T cell clones against a very large peptide library covering thousands of peptides (bottom-right). This protocol outlines an approach to screen dozens to hundreds of TCRs per assay, tested in parallel against a supervised panel of antigens. We envisage the utility of such a strategy to test the reactivity of ~100 TCRs per assay against 100–1000 targets. Created with Biorender.com.

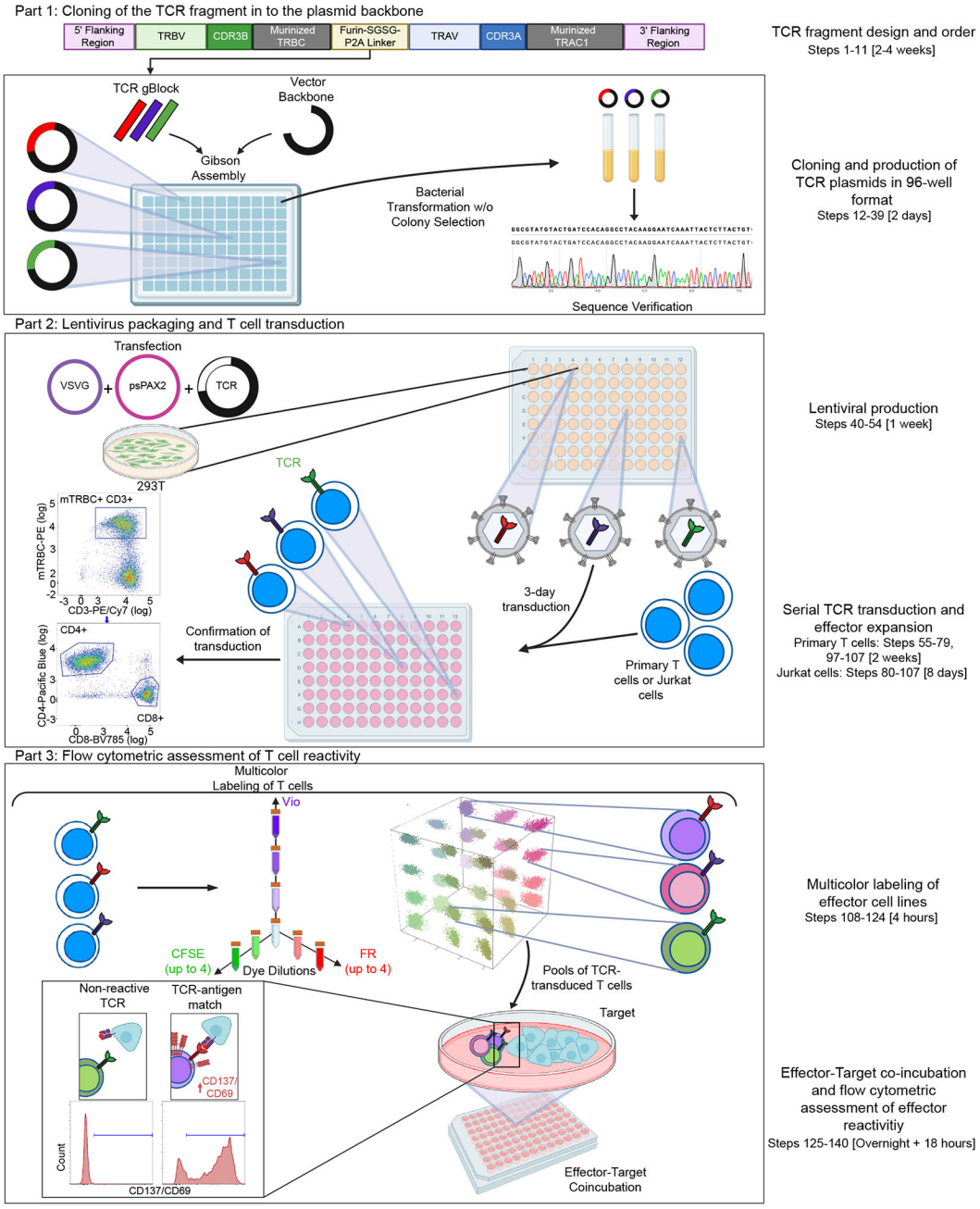

Fig. 2 |. Overview of an optimized workflow for the rapid parallel reconstruction and specificity screening of multiple TCRs.

Scheme of the 3 major phases of this protocol. **Part 1:** User-defined TCR fragments are cloned into a lentiviral backbone plasmid, expanded in bacteria without colony selection, and sequenced to confirm proper integration of the TCR fragment. **Part 2:** TCR plasmids are then transfected into Lenti-X 293T cells in 96-well plates for miniaturized production of lentiviral particles and transduction of primary T cells harvested from healthy donors or TCR-KO Jurkat cells. TCR expression is assessed by flow cytometry through the detection of surface expression of the murinized TCR constant chain (mTRBC) included in TCR constructs. TCR-transduced cell lines are expanded over 2 weeks. **Part 3:** TCRs are stained

with a combination of dilutions of 3 dyes (CellTrace Far Red (FR), CellTrace Violet (Vio) and CellTrace Carboxyfluorescein succinimidyl ester (CFSE)), resulting in an array of up to 64 (36 in the example) TCR-transduced cell lines identifiable by flow cytometry. Upon labelling, effectors can be grouped in distinct pools (e.g. 3 pools 32–36 effectors and internal controls, for a total of 96 TCR-cell lines); such labelling enables investigation of TCR transduction signal (measured as surface upregulation of CD137 on primary T cells or CD69 on Jurkat cells) in pools of effector cell lines upon *in vitro* stimulation with different antigens. Created with Biorender.com

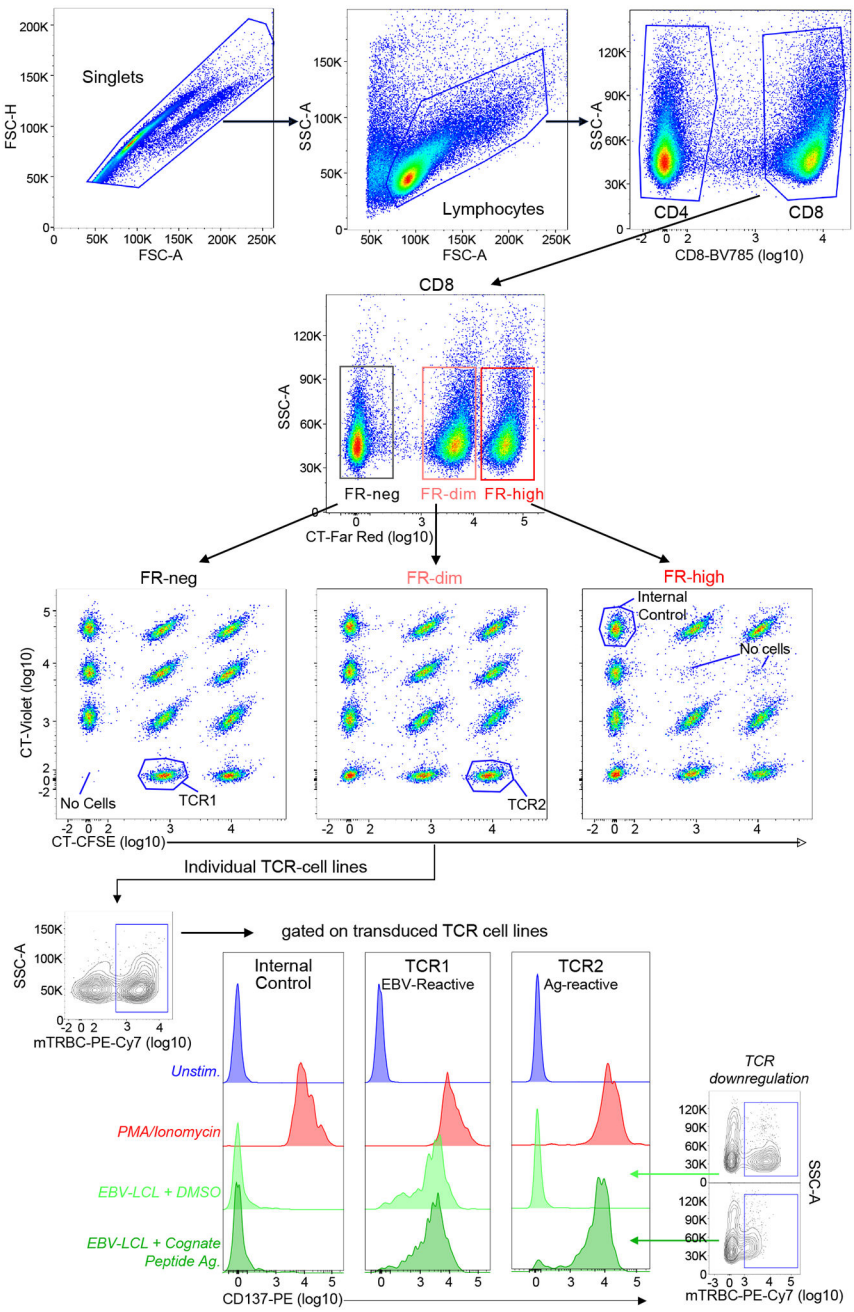

Fig. 3 |. Flow cytometric strategy for the analysis of the reactivity of multiple TCR-cell lines.

Representative flow cytometry plots depicting the gating strategy for determining TCR specificity of pools of TCR-transduced effectors. In a representative experiment, 33 different TCR-cell lines were labeled, pooled and tested. Only events in gate “Singlets” were recorded. Based on the TCR costimulatory restriction, the analysis can be performed on CD8+ or CD8-(CD4+) T cells. TCR-transduced cell lines are distinguished by the fluorescence levels of three CellTrace dyes, Far Red (FR), CFSE, and Violet dyes (Materials). Labelling with combination of 3 dilutions of FR (FR-high, FR-dim (1:10 dilution), and FR-negative (FR-neg)) and CFSE, and 4 dilutions of Violet allowed

flow-cytometric identification of each population within the pool of effectors. CD137 upregulation was monitored on TCR-transduced T cells identified by positivity for murine constant TCR-beta chain (mTRBC). The reactivity profiles of representative CD8+ T cells transduced with 3 different TCRs is depicted (bottom): left – T cells transduced with an Irrelevant TCR (Internal Control), which was included among the effectors to assess the level of background reactivity; center – T cells transduced with an EBV-specific TCR, displaying CD137 upregulation in any culture condition that included autologous (HLA-matched with the subject from whom TCRs were identified) EBV-immortalized lymphoblastoid cell lines (EBV-LCLs) pulsed with DMSO or with tested peptides; right – T cells transduced with a TCR reactive towards a specific antigen, as assessed through upregulation of CD137 upon culture with the cognate peptide. Recognition of the cognate antigen is confirmed by profound TCR downregulation in association with antigen reactivity. For each TCR-transduced cell line, CD137 expression in absence of target cells or in presence of PMA + Ionomycin is assessed as a negative and positive control, respectively.

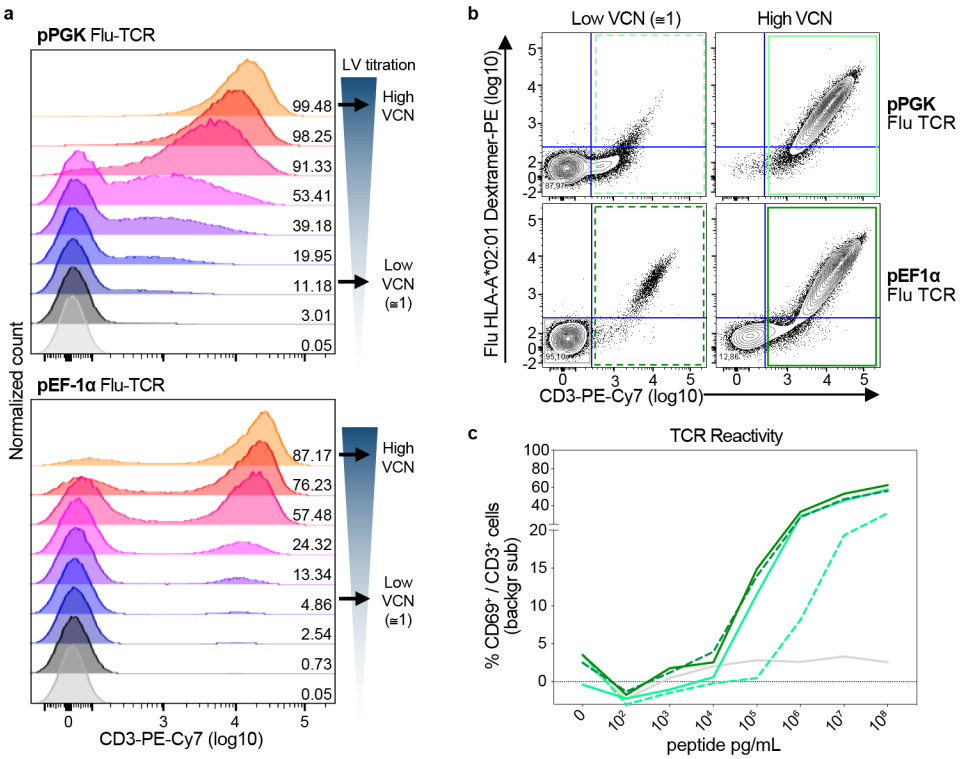

Fig 4. |. Expression and reactivity of TCRs under the control of different promoters.

a. A flu-specific TCR (Supplementary Table 2) was cloned in a backbone plasmid downstream of a PGK promoter (top) or a EF-1α promoter (bottom). The resulting two plasmids were used to produce lentiviral (LV) particles coding for a pPGK Flu-TCR (top) or pEF-1α Flu TCRs (bottom), which were then added at increasing concentration (bottom to top) to infect TCR-deficient CD8+CD4+ Jurkats. The surface level of expression of the TCR complex was monitored through flow cytometric detection of the CD3 marker. While the intensity of expression of the pPGK Flu-TCR increased with the proportion of transduced cells, pEF-1α Flu TCR was consistently detected at high levels on transduced Jurkats, independently of the transduction efficiency. For each construct, Jurkats with 5–15% TCR expression were considered as transduced by single lentiviral particles (vector copy number [VCN] ≅ 1): their reactivity was compared to that of cells transduced at high levels with multiple integrations of the TCR-transgene (high VCN). **b.** Flow cytometric bi-dimensional plots depicting the ability of candidate TCRs to bind cognate peptide-HLA complexes (y axis) among Jurkats transduced with low (left) or high (right) VCN of pPGK Flu-TCR (top) or pEF-1α Flu TCRs (bottom). Colored gates - TCR-transduced cells, as measured through CD3 expression (x axis). **c.** Reactivity of TCR-transduced Jurkat cells is detected as upregulation of the CD69 activation marker, measured through flow cytometry after an overnight stimulation with HLA-A*0201 targets pulsed with increasing doses of cognate antigen. Colored full or dashed lines - TCR-expressing CD3+ Jurkats transduced under different conditions (VCN ≅ 1 or high, pPGK or pEF-1α Flu TCRs) or untransduced CD3- cells (grey), as indicated by gates in panel b. CD69 expression measured on Jurkat cells transduced with an irrelevant TCR was subtracted. Overall, the ability of the tested TCR to bind and react to the cognate antigen was independent of transduction efficiency when the

TCR was expressed under the control of the EF-1α promoter, which granted its high and stable expression even when the transduction efficiency was low.

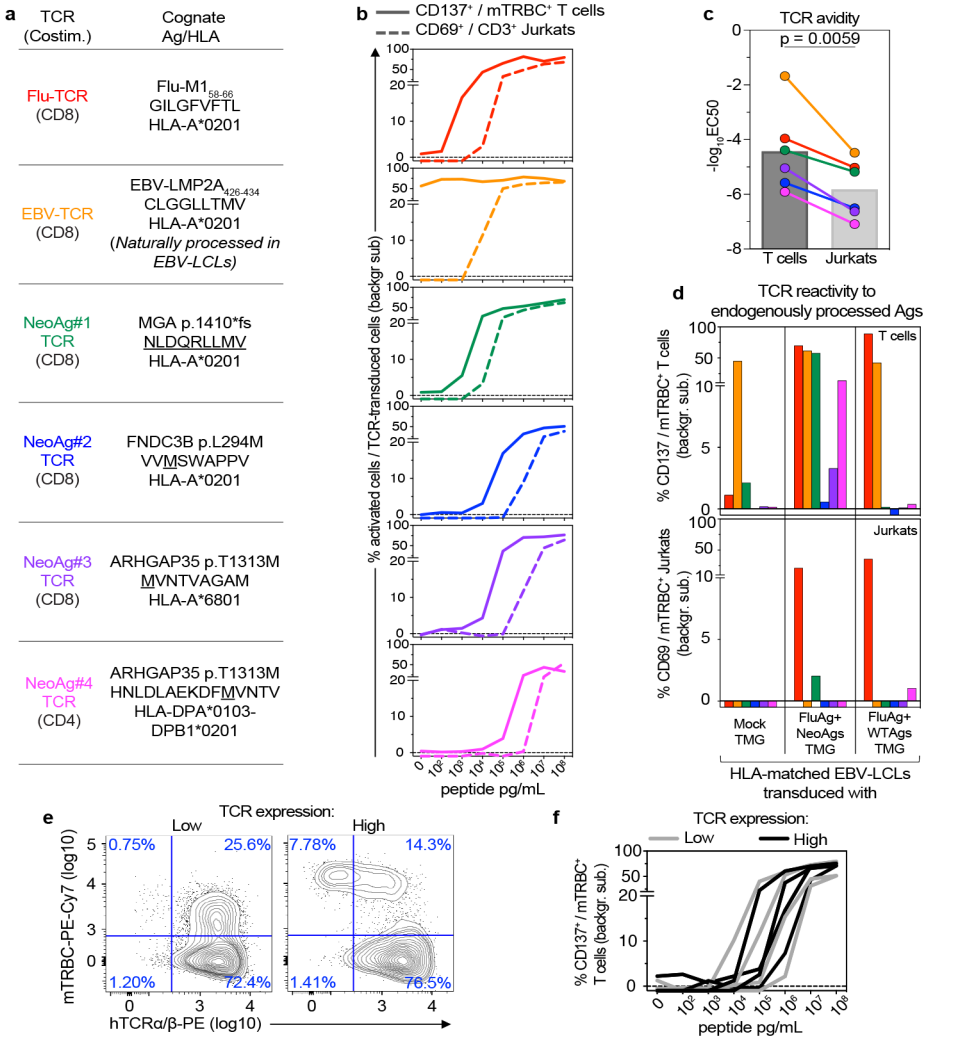

Fig. 5 |. Comparison between Jurkats and primary T cells as cellular systems for the assessment of TCR reactivity.

a. Characteristics of 6 TCRs with known specificity, used for a validation experiment (Supplementary Table 2). These TCRs were murinized, cloned and expressed through lentiviral transduction in TCR-deficient CD4+ CD8+ Jurkats or in human T cells isolated from the peripheral blood of healthy donors. Mutated amino acid residues are underlined. Different colors are used to distinguish TCRs in subsequent panels. **b.** After overnight incubation with HLA-matched EBV-LCLs pulsed with increasing doses of cognate peptides, TCR reactivity was assessed, based on upregulation of CD69 or CD137 surface expression on CD3+ TCR-transduced Jurkat cells (dashed lines) or on CD8+ or CD4+ transduced (mTRBC+) primary T cells (solid lines), respectively. **c.** Avidity of TCRs, as measured based on CD69 or CD137 signals detected respectively in TCR-transduced Jurkats or T cells. EC50: half-maximal effective concentration of peptide associated with TCR-transduction signals, as calculated from curves in panel b. Significance of comparison was calculated using a two-tailed paired *t-test*. **d.** TCR reactivity against endogenously processed cognate antigens, as measured in TCR-transduced Jurkat cells (bottom) or CD4+ or CD8+ primary T cells (top). TCR reactivity (CD69 or CD137 upregulation) was

assessed on TCR-transduced cells after overnight incubation with HLA-matched EBV-LCLs transduced with tandem mini-genes (TMGs) encoding the immunogenic epitope Flu-M1 and the neoantigens (NeoAgs) targeted by the tested TCRs (panel a) or their wildtype counterparts (WT). Background TCR reactivity was assessed against HLA-matched EBV-LCLs transduced with a mock TMG (with a GFP replacing the cognate antigens). EBV antigens were endogenously expressed in all the targets. **e.** Bidimensional FACS-plots showing co-expression of exogenous murinized TCRs (mTRBC, y-axis) and endogenous human TCRs (hTCRα/β, x-axis) among CD3+CD8+ primary T cells transduced with two representative murinized MLANA-specific TCRs with different patterns of expression (left: low; right: high). **f.** Reactivity of 8 representative MLANA-specific TCRs exhibiting different patterns of surface expression (high: black; gray: low; Supplementary Table 1) in T cells. TCR transduction signal (CD137 upregulation on mTRBC+ primary T cells) was assessed after overnight incubation with HLA-matched EBV-LCLs pulsed with increasing concentrations of $MLANA_{27–35}$-peptide. For all the panels: analysis was conducted on CD8+ or CD4+ mTRBC+ T cells based on the CD4/CD8 TCR restriction (panel a, Supplementary Table 1); for analyses on both TCR-transduced Jurkats and T cells, the background reactivity measured on cells transduced with an irrelevant TCR was subtracted. Overall, the detection of CD137 upregulation in primary T cells allowed for a more sensitive assessment of TCR reactivity compared to the detection of CD69 upregulation in Jurkats.

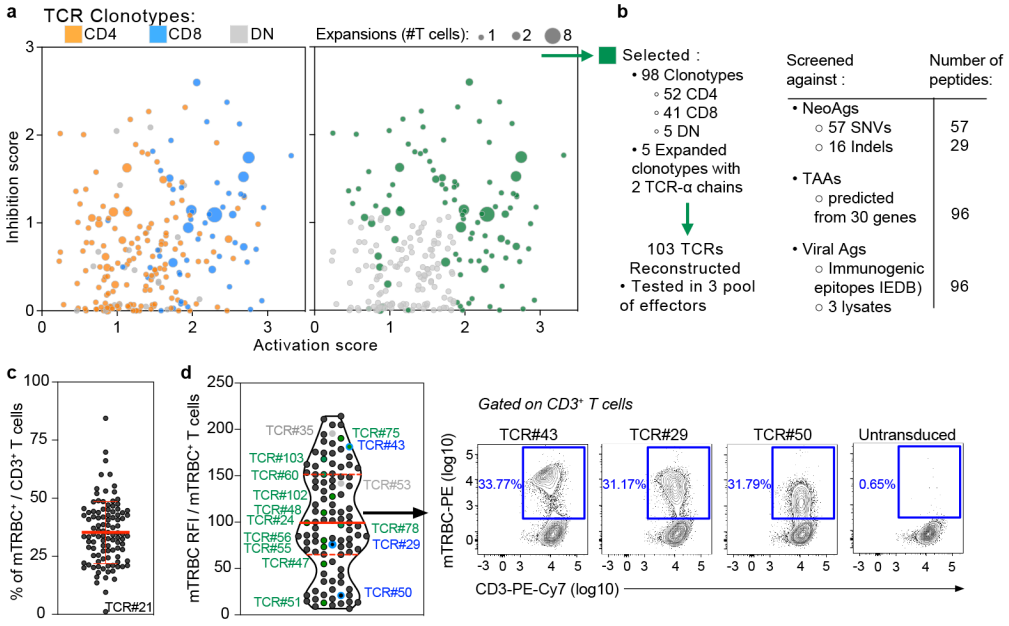

Fig. 6 |. Selection and reconstruction of TCRs from T cell clonotypes infiltrating a glioblastoma lesion.

a. Selection of TCRs from T cell clonotypes detected in a glioblastoma (GBM) lesion. After single-cell RNA and TCR-sequencing of tumor infiltrating lymphocytes, 223 unique clonotypes were identified and scored for the mean expression of activation and inhibitory genes (respectively x and y axis, see Supplementary Table 3). Dots represent distinct TCR clonotypes, classified as CD4, CD8 or CD4/8 double negative (DN, left plot, color legend) based on the expression of CD4/CD8 lineage markers (Supplementary Table 3). Size of dots is proportional to clonotype expansion, measured as number of TCR-expressing T cells sequenced within the tumor microenvironment. TCR clonotypes were selected (right plot, green dots) based on: i) expansion (# T cells ≥2); ii) highest activation and/or inhibitory scores or iii) detection in peripheral blood (see Supplementary Table 3). **b.** Features of 98 TCRs (left) and antigens (right) selected for reconstruction and specificity screening. Five expanded clonotypes were characterized by recurrent detection of 2 TCR-α chains (Supplementary Table 3) and were reconstructed separately, for a total of 103 tested TCRs. The resulting TCR-cell lines were divided in 3 pools of effectors, based on the dye labeling strategy described in the Protocol. TCR-transduced T cells were screened against the patient's EBV-LCLs pulsed with different peptides (right) encoding for i) personal NeoAgs, including short nucleotide variant (SNVs) or novel open-reading frame generated insertion and deletions (Indels); ii) epitopes predicted as immunogenic in 30 tumor associated antigens (TAAs) overexpressed in GBM; and iii) immunogenic viral epitopes reported in IEDB as binders of patients' class I HLAs or viral lysates (Zeptometric). **c.** Efficiency of TCR expression among TCR-transduced T cells. The selected 103 TCRs were murinized, cloned in plasmids and lentivirally transduced in CD3+ T cells from a healthy subject. TCR expression on the surface of T cells was monitored by flow cytometric detection of murine TCR-beta constant chain (mTRBC). 102 out of 103 TCRs were successfully expressed. Red horizontal lines and error bars depict mean value with SD. **d.** Violin plot depicting the expression levels of 102 TCRs (dots) on the surface of transduced cells,

measured as fluorescence intensity of mTRBC expression relative to the one detected on untransduced T cells (RFI, relative fluorescence intensity). TCRs referenced throughout the text are labeled (blue circles: representative TCRs; green dots: de-orphanized TCRs; grey dots: TCRs from a single clone with 2 TCR-α chains). Horizontal lines - median expressions and quantiles. Bidimensional flow cytometric plots depicting TCR expression (mTRBC+) on CD3+ T cells transduced with 3 distinct TCRs are shown on the left, in comparison with untransduced cells (UT). Distinct TCRs showed different patterns of surface expression (high, intermediate and low), despite similar transduction efficiencies.

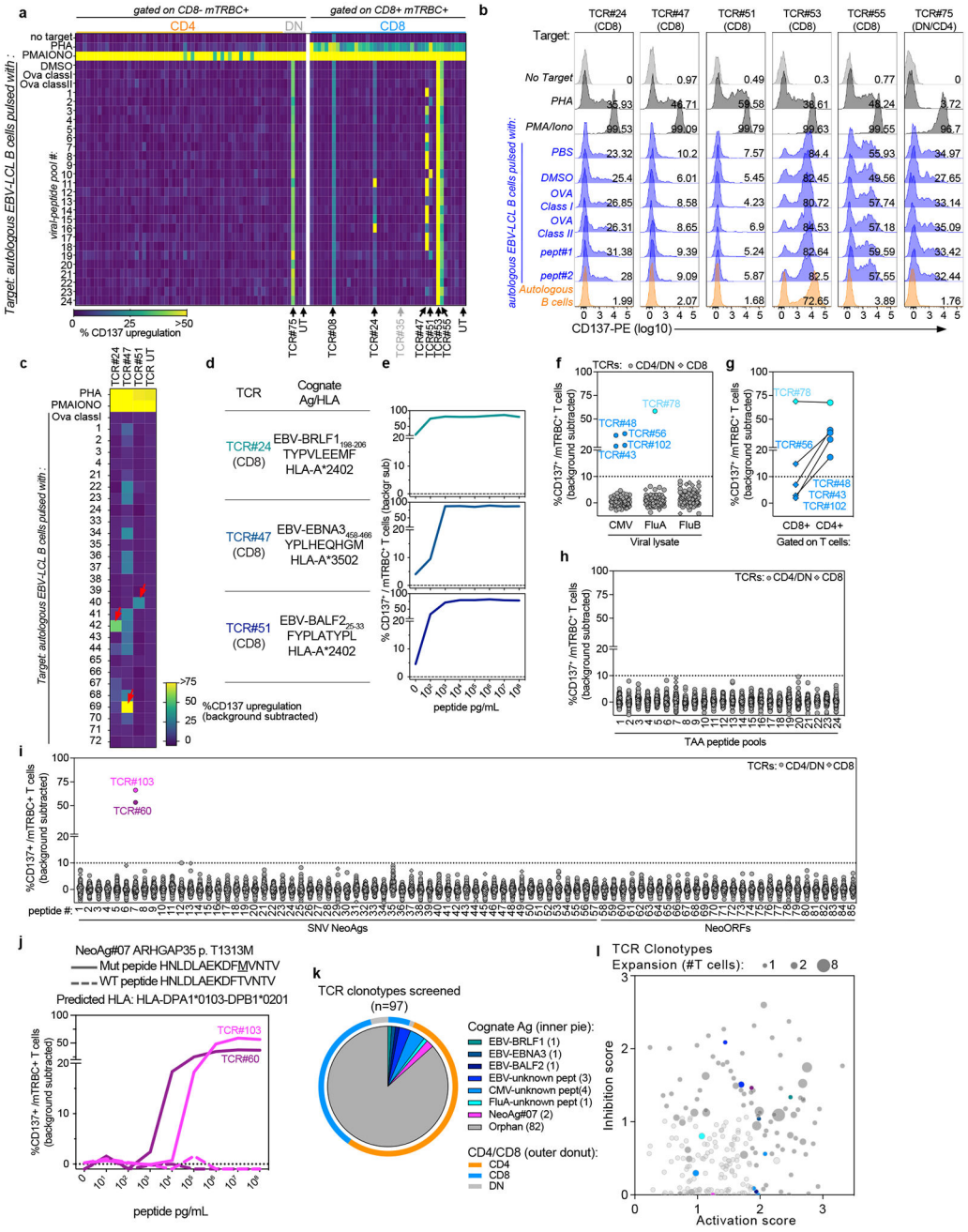

Fig. 7 |. Specificity screening of TCRs from dozens of clonotypes infiltrating a GBM lesion.

a. Heatmap depicting CD137 upregulation detected on TCR-transduced mTRBC+ T cell lines (columns) after overnight stimulation with a single GBM patient's EBV-LCLs pulsed with different pools of viral peptides (rows, 10^8 pg/mL). CD137 upregulation was analyzed on CD8+mTRBC+ T cells for CD8 clonotypes or on CD8-mTRBC+ T cells for CD4/DN clonotypes (Supplementary Table 3). Reactivity in the absence of target exposure or against cells pulsed with DMSO or with irrelevant peptides (OVA class I and II) were tested as negative controls; T cell lines were stimulated with PHA or PMA/Ionomycin as positive controls. Reactivity of untransduced (UT) T cells is reported for both CD4+ and CD8+

T cells. TCRs reactive against EBV-LCLs or distinct viral peptide-pools are highlighted with black arrows. **b.** Flow cytometry plots documenting CD137 upregulation measured on TCR-cell lines with reactivity against EBV-LCLs and/or viral peptides (highlighted in a). Reactivity is shown in the presence of positive and negative controls (dark and light gray, respectively) and against the patient's EBV-LCLs pulsed with representative peptides (blue); CD137 upregulation is further measured against primary autologous B cells (orange), which do not carry actively replicating EBV virus. The absence of TCR reactivity against B cells provided direct inference of EBV-specificity for TCR#24, TCR#55 and TCR#75. **c.** Heatmap depicting deconvolution of specificity of 3 TCRs reactive against viral peptide pools. TCR reactivity was tested against targets pulsed with lower doses of individual viral peptides (10^6 pg/mL). The background CD137 signal measured in the presence of DMSO-pulsed EBV-LCLs is subtracted. The activity profile of untransduced T cells is shown in parallel. Red arrows - the highest reactivity detected per TCR. **d.** Characteristic of the cognate antigens of 3 TCRs with de-orphanized viral specificity. **e.** Avidity of 3 antiviral TCRs with de-orphanized specificity. T cell reactivity of TCR-transduced cell lines is measured against autologous EBV-LCLs pulsed with increasing doses of cognate antigens. Signals from T cells transduced with an irrelevant TCR was measured in parallel and subtracted as background. **f-g.** Reactivity of CD4/DN TCRs (circles) or CD8-TCRs (diamonds) measured against autologous EBV-LCLs pulsed with lysates of cell lines infected with CMV, FluA or FluB viruses. Background reactivity measured against PBS-pulsed target was subtracted. Reactivity was considered positive when >10% (dotted line). Five TCRs with high reactivity were identified, including 4 CMV-specific (blue) and 1 FluA-specific (cyan) TCRs. Their CD4-CD8 restriction was further documented in **g,** by analyzing CD137 upregulation in response to the cognate viral lysates on CD4+ or CD8+ TCR-transduced T cells. **h-i,** Screening of TCR reactivity against the patient's EBV-LCLs pulsed with peptides encoding tumor associated antigens (h) or neoantigens (i). CD137 upregulation is measured on TCR transduced (mTRBC+) CD4+ (circles) or CD8+ (diamonds) T cells based on known co-receptor restriction. Basal reactivity measured in the presence of DMSO-pulsed targets was used as background. Two CD4 TCRs specific for the same NeoAg#07 were identified as reactive (>10% CD137 upregulation, dotted line), as previously reported[23]. **j.** Avidity of 2 NeoAg-specific CD4-TCRs. The TCR-transduction signal (CD137 upregulation) was measured against the patient's EBV-LCLs pulsed with increasing doses of mutated (solid lines) or control wildtype (WT, dashed lines) antigens. Background CD137 expression of T cells transduced with an irrelevant TCR was measured in parallel and subtracted. The sequences of mutated and WT cognate peptides and the predicted HLA restriction are annotated. **k.** Summary of TCR specificity, as determined after TCR reconstruction and specificity screening. Pie colors indicate the cognate antigens of the TCR; the outer ring colors show the lineage of TCR clonotype, as determined by single cell RNA expression (Supplementary Table 3). Most of the de-orphanized TCRs demonstrated viral specificity. **l.** Knowledge of TCR reactivity allows investigation into the phenotype of antigen-specific tumor-infiltrating T cells. The plot reports the activation and exhaustion status of TCR clonotypes infiltrating a glioblastoma biopsy, as measured through scores obtained from RNA expression of gene signatures (see Fig. 5a, Supplementary Table 3). Colors of the T cell clonotypes (dots) refers to TCR-specificity, as reported in panels d-k. Dark gray clonotypes represent orphan TCR clonotypes; light gray dots represent clones not

screened for TCR reactivity. Size of dots is proportional to clonotype expansion within the tumor microenvironment.

Table 1:

Troubleshooting table

Troubleshooting			
Step	**Problem**	**Possible reason**	**Solution**
15	≥3 lines appear in gel	Incomplete digestion	Repeat digestion and/or run the gel for longer to achieve better separation of correct-length product
20	Bacteria transformed with the water control grew	The backbone plasmid was incompletely digested or the cut ends annealed	Repeat backbone digestion, confirming a predominance of the linearized plasmid
31	Aerated cover shows evidence of drops/spilling of bacterial media	Deep-well plates were insufficiently secured and contamination between samples may have occurred	Secure the plates more tightly to minimize spilling. Include additional plates with an unseeded test well located close to bacteria- seeded wells to assess the degree of spillage during shaking
37	Contaminant peaks are present at the start of the gBlock upon sequencing	Closed plasmid may be present in addition to the proper insertion product	Repeat the Gibson Assembly (**Steps 21–23**) using a higher (150 – 200ng) proportion of insert compared to linearized backbone (10 – 50 ng)
54	No evidence of GFP+ in 293-T cells	Preparation of transfection reagents was unsuccessful	Repeat the transfection of 293-Ts (**Step 48–54**), checking plasmid concentration and *Trans*IT-LT1 incubations
57	Not enough T cells for transduction after Pan T selection	Insufficient number of PBMCs thawed	Thaw a higher number of PBMCs. The yield of T cell selection should be roughly 20–40% of the PBMCs thawed the day before
62	T cells are not clumped around the Dynabeads, or are elongated	The quality and viability of the thawed T cells is poor	Thaw PBMCs from a different healthy donor. Thaw the cells gently to ensure high cell viability
65, 68, 70, 85, 89	Media in 293-T plates is yellow, rather than pink or orange	293-T cells were too densely plated	Replate 293Ts, confirming correct cell count to achieve 50–80% confluency
78	T cells do not significantly expand after removal of activation beads	T cells are of poor health and suffered during transduction	Use a different donor's PBMCs for transduction and add complete T cell medium with cytokines to the viral supernatant in the last round of transduction (**Step 90**)
		T cells were seeded at too low concentration for expansion	Increase the density of cells per mL of media to roughly 1×10^6 T cells/mL, and do not replenish the medium until it becomes orange/yellow
104	Untransduced cells show TCR expression	Media was contaminated with viral supernatant or transduced cells from adjacent wells	Use special care to transfer reagents precisely during transfection and transduction
107	The ratio of CD4/CD8 is highly skewed	Patient PBMC sample is enriched with CD4 cells	Confirm that both CD4+ and CD8+ T cells are similarly transduced with TCR
		CD8 T cells take longer to expand	Wait longer before performing the TCR specificity testing
	Transduction rate is low	Preparation of reagents, production of lentivirus, or T cell activation were suboptimal	If it is observed only on a few TCR-transduced cell lines: Confirm TCR plasmid concentration. TCRs isolated from clones with 2 TCR-alpha chains might not assemble or be unstable.
			If it is observed on all TCRs: Repeat transfection and transduction using new packaging plasmids and ensuring strict adherence to protocol
121	Proportional dye dilutions are not visible in dried T cell pellets	The ratio of dye:T cells was insufficient	Repeat staining with a 0.3 μL more of each dye used for the highest dilution
			Count T cells again to ensure an accurate number of stained cells

Troubleshooting			
Step	**Problem**	**Possible reason**	**Solution**
124	Not enough T cells for the assay after staining with CellTrace dyes	Too many cells were lost during washing steps	Repeat the staining using a greater excess of T cells
		Viability of cells was low and DMSO in each dye led to cell death	
		Initial cell count was an underestimate	
135	Background in unstimulated T cell wells is high, and/or the transduction rate is unexpectedly low for certain TCRs	TCR is activated by HLA expressed in donor T cells causing fratricide	Transduce donor T cells from a different healthy donor with a different set of HLA alleles