

Phage display-derived TCR-T cells targeting KRASG12V and CTAG1B suppress colorectal and esophageal tumors

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Abstract

Background

T cell receptor-engineered T cell (TCR-T) therapy enables recognition of intracellular tumor antigens presented by peptide-human leukocyte antigen (pHLA) complexes, but identification of antigen-specific TCRs with suitable functional properties remains challenging. We developed a phage display-based workflow to discover and functionally evaluate TCRs targeting cancer-testis antigen CTAG1B (NY-ESO-1) and the oncogenic neoantigen KRASG12V in esophageal and colorectal cancer, respectively.

Methods

A naïve $\alpha\beta$ TCR phage-displayed library was generated from T cells obtained from healthy donors heterozygous for HLA-A*02:01 and HLA-A*11:01. Following three rounds of biopanning against CTAG1B_(157–165)/HLA-A*02:01 and KRASG12V_(8–16)/HLA-A*11:01 complexes, antigen-reactive candidates were identified by monoclonal phage ELISA and screened for pHLA selectivity. Selected TCRs were expressed in primary human T cells and evaluated for antigen-dependent cytotoxicity, cytokine secretion, tumor-cell recognition, normal-cell reactivity, and antitumor activity in xenograft models.

Results

Selected TCRs achieved transgenic TCR expression exceeding 70% and mediated peptide dose-dependent cytotoxicity and antigen-dependent cytokine secretion. CTAG1B-specific TCR-T cells recognized esophageal tumor cells, whereas KRASG12V-specific TCR-T cells recognized colorectal tumor cells in an antigen-dependent and HLA-restricted manner, with limited reactivity against the normal and control cell types evaluated in vitro. In vivo, both TCR-T cell groups significantly suppressed tumor progression in their respective xenograft models. In vivo, CTAG1B- and KRASG12V-specific TCR-T cells significantly suppressed tumor progression in their respective xenograft models, with the strongest-performing receptors reducing mean endpoint tumor weight by approximately 80% and 60%, respectively, relative to control T cells. Transferred human CD3⁺ T cells and cells expressing the introduced TCR remained detectable approximately 30 days after treatment, while increased CD8⁺ T cell density within treated tumors supported localization of the adoptively transferred T cell population. LDH-based analyses showed no marked increase in treatment-associated tissue injury.

Conclusions

These findings demonstrate a phage display-based workflow for identifying and functionally evaluating antigen-specific TCRs against both cancer-testis antigens and tumor-specific neoantigens and support further development of this approach for solid tumor TCR-T therapy.

Background

Colorectal cancer (CRC) and esophageal cancer (EC) remain major contributors to global cancer mortality despite advances in systemic therapy [1–3]. CRC is the third most commonly diagnosed malignancy worldwide and a leading cause of cancer-related death, with metastatic disease associated with poor long-term survival despite the use of combination chemotherapy, targeted agents, and immune checkpoint inhibitors [4]. Most CRC cases are microsatellite stable (MSS), a subtype that shows limited responsiveness to current immunotherapies because of low tumor mutational burden and an immunologically “cold” tumor microenvironment [5]. Similarly, EC, encompassing both esophageal adenocarcinoma and squamous cell carcinoma, is associated with aggressive disease progression and poor long-term survival, with reported 5-year survival rates of approximately 15–25% [6, 7]. Although immune checkpoint blockade has shown benefit in subsets of patients with CRC and EC, responses remain limited and are often associated with specific biomarkers, including PD-L1 expression or high tumor mutational burden [8]. The scarcity of highly specific, immunogenic tumor antigens that can be effectively targeted while limiting off-tumor toxicity remains an important challenge. In addition, the immunosuppressive tumor microenvironment (TME) can limit T cell persistence, tumor localization, and cytotoxic function [9, 10]. These limitations support the development of alternative immunotherapeutic strategies capable of eliciting tumor antigen-specific T cell responses.

T cell receptor-engineered T cell (TCR-T) therapy has emerged as an immunotherapeutic strategy capable of targeting intracellular tumor antigens presented as peptide-human leukocyte antigen (pHLA) complexes. Unlike chimeric antigen receptor (CAR)-T cells, which recognize cell-surface antigens in an MHC-independent manner [11, 12], TCR-T cells can recognize peptides derived from intracellular proteins, including tumor-associated antigens and tumor-specific neoantigens. However, the clinical development of T cell therapies, including CAR-T and TCR-T cells, remains constrained by several challenges, including limited receptor affinity and specificity, restricted target and receptor repertoires, cross-reactivity and safety concerns, and tumor-mediated immune evasion [13–15]. Identifying tumor antigens with restricted normal-tissue expression, together with TCRs that selectively recognize the intended pHLA complex, is therefore important for improving therapeutic specificity and safety.

Two classes of antigens have attracted particular interest for TCR-based immunotherapy: cancer-testis antigens and tumor-specific neoantigens. CTAG1B (also known as NY-ESO-1), a well-characterized cancer-testis antigen, is expressed in multiple malignancies, including esophageal cancer, while showing restricted expression in normal tissues [16–19]. Its immunogenicity and restricted normal-tissue expression make it an attractive target for TCR-based therapies, particularly in HLA-A*02:01-restricted tumors [16, 18]. Conversely, oncogenic driver mutations such as KRAS represent ideal neoantigen targets due to their tumor specificity and essential role in cancer progression. KRAS mutations occur in approximately 50% of colorectal cancers, with codon 12 substitutions among the most prevalent [20]. KRASG12V is a recurrent oncogenic variant that drives constitutive activation of downstream signaling pathways, including MAPK and PI3K [21]. KRAS mutations can be maintained across primary and metastatic tumor sites [22], providing a potentially stable therapeutic target. From an immunological

perspective, KRASG12V generates a tumor-specific neoantigen that is absent from normal tissues, making it an attractive target for mutation-specific TCR recognition. Previous studies have demonstrated that mutant KRAS peptides can be naturally processed, presented by MHC class I molecules, and recognized by cytotoxic T cells [23–25], supporting their potential for TCR-based immunotherapy.

Despite these advantages, conventional TCR discovery frequently relies on patient-derived T cells, including tumor-infiltrating lymphocytes, which can constrain the diversity and availability of receptors for screening. Display-based approaches provide an alternative means of interrogating large TCR repertoires independently of tumor-derived patient samples. However, identification of pHLA-binding receptors alone does not establish their functional activity when expressed in primary T cells or their ability to recognize naturally processed antigen on tumor cells. The use of phage-displayed TCR libraries to identify receptors against distinct classes of tumor antigens, followed by systematic specificity screening and functional validation in primary human T cells, remains underexplored.

In this study, we constructed a naïve phage-displayed TCR library using T cells from healthy donors heterozygous for HLA-A*02:01 and HLA-A*11:01. These HLA alleles were selected based on their population prevalence and their ability to present the tumor epitopes investigated in this study. HLA-A*02:01 is widely distributed and has been extensively studied in TCR-based approaches targeting cancer-testis antigens such as CTAG1B, including the well-characterized CTAG1B_(157–165) epitope [26, 27]. HLA-A*11:01 is also prevalent in multiple populations and can present KRAS-derived neoantigens, including KRASG12V [28, 29]. These defined pHLA targets therefore provided a basis for selecting and evaluating antigen-specific TCR candidates against two distinct classes of tumor antigens.

Through iterative biopanning and secondary specificity screening, we identified TCR candidates recognizing KRASG12V_(8–16)/HLA-A*11:01 and CTAG1B_(157–165)/HLA-A*02:01 complexes. Selected receptors were subsequently expressed in primary human T cells and evaluated for antigen-dependent effector function, tumor-cell recognition, normal-cell reactivity, and antitumor activity in vivo. By integrating phage display-based TCR discovery with functional evaluation, this study evaluated a workflow for identifying and functionally prioritizing TCR candidates against both a cancer-testis antigen and a tumor-specific neoantigen.

Methods

Study design and reproducibility

This study was designed to identify and functionally evaluate phage-derived TCRs targeting KRASG12V and CTAG1B epitopes. In vitro experiments were performed with at least three biological replicates unless otherwise stated, with technical replicates included where applicable. In vivo experiments were conducted using predefined group sizes based on prior experience with the xenograft models and in accordance with the approved Institutional Animal Care and Use Committee (IACUC) protocol. No statistical method was used to predetermine sample size. Mice were randomized to treatment groups

after tumors reached approximately 300–400 mm³. In vitro experiments were not performed under blinded conditions, whereas investigators performing tumor measurements were blinded to treatment allocation.

Cell lines and culture conditions

All cell lines were authenticated and routinely tested for mycoplasma contamination. Cell line identities and Research Resource Identifiers (RRIDs) are provided in Supplementary Table 1. T2/HLA-A*11:01 cells, an HLA-A*02:01-knockout T2 derivative engineered to express HLA-A*11:01, were provided by Wenfang Chen from the Li laboratory and used for HLA-A*11:01-restricted peptide-presentation assays. Cells were maintained under standard culture conditions (37°C, 5% CO₂) in the appropriate growth media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Growth media for the primary cells and cell lines used in this study are provided in Supplementary Table 1. Cell lines were generally used within five passages after thawing and were not used beyond passage 10. HLA allotype and antigen expression in tumor cells or antigen-presenting cells were evaluated by flow cytometry or RT-PCR, as described in our previous studies [16, 30]. Antibody information, including clone or catalog numbers and vendors, is provided in Supplementary Table 4. Antigen and HLA characterization of the cell lines is summarized in Supplementary Table 2.

Generation of stable cell lines

Stable cell lines expressing the indicated transgenes were generated in-house by lentiviral transduction of the parental cell lines at a multiplicity of infection (MOI) of 5. Transduced cells were selected with 3 µg/mL puromycin using the resistance marker encoded by the transgene vector. The puromycin concentration was determined using a dose-response assay that produced > 90% cell death within 3 days. Cell viability was assessed using the CCK-8 assay (Biosharp) according to the manufacturer's instructions.

Human T cell isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors following vendor screening procedures (Miaoshun Biotechnology, China). Donors were screened by the vendor for infectious agents, including HIV, hepatitis B and C viruses, cytomegalovirus, Epstein-Barr virus, and human T-lymphotropic virus. CD3⁺ T cells were isolated from PBMCs by negative selection (STEMCELL Technologies) according to the manufacturer's protocol. HLA genotyping was performed by the vendor before sample distribution. Three healthy donors, each heterozygous for HLA-A*02:01 and HLA-A*11:01, were used for TCR library construction. No additional inclusion or exclusion criteria were applied beyond the vendor screening procedures.

RNA extraction and cDNA synthesis

Total RNA was extracted from 5×10^6 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions under RNase-free conditions. RNA was isolated by chloroform phase separation, isopropanol precipitation, and ethanol washing, followed by resuspension in RNase-free water. RNA concentration and purity were assessed by spectrophotometry (A260/A280), and RNA integrity was evaluated by 2% agarose gel electrophoresis. First-strand cDNA was synthesized using PrimeScript IV reverse transcriptase (TaKaRa) according to the manufacturer's protocol. cDNA quality was assessed by PCR amplification of the housekeeping genes ACTB, GAPDH, and B2M, as previously described [16].

Construction of TCR phage display library

TCR α - and β -chain variable regions were amplified using degenerate primers designed from sequences curated in the International Immunogenetics Information System (IMGT) database and adapted from previously described protocols [16]. Amplified products were cloned into the pSCTR-amp phagemid vector using NcoI and NotI restriction sites. Recombinant phagemids were transformed into TG1 electrocompetent *Escherichia coli* cells by electroporation. Library diversity and quality were assessed by next-generation sequencing (NGS), including analysis of sequence diversity and productive rearrangement frequency. Sequencing data were processed using a standardized Python (v3.8) pipeline to assess clonotype distribution, diversity metrics, and sequence enrichment across panning rounds. Uniform read-depth and quality thresholds were applied across samples. The TRAV and TRBV gene identities of the selected TCR candidates advanced for functional evaluation are provided in Supplementary Table 6.

Peptide selection and pHLA preparation

Relevant target and control peptides were purchased from GenScript (China). Peptides were selected according to the target epitopes and their corresponding HLA restrictions. Soluble peptide-HLA (pHLA) complexes were prepared in-house using previously described protocols [26, 31]. Briefly, HLA proteins were expressed as inclusion bodies and refolded in the presence of the corresponding peptides. Refolded pHLA complexes were purified using an AKTA Pure chromatography system (Cytiva/GE Healthcare) by sequential ion-exchange and size-exclusion chromatography. Eluted fractions were analyzed by SDS-PAGE, and fractions showing the expected purity profile were pooled, concentrated, and biotinylated for subsequent phage-display selection and specificity assays. Peptide sequences, HLA restrictions, and experimental applications are provided in Supplementary Table 3.

Phage display and biopanning

Three rounds of phage-display biopanning were performed as previously described [16], using approximately 1×10^{11} plaque-forming units (PFU) as input for each round. Briefly, biotinylated pHLA complexes were immobilized on streptavidin-coated magnetic beads and incubated with the phage-displayed TCR library. Unbound phages were removed by washing, whereas bound phages were eluted by trypsin digestion and amplified in TG1 cells for the subsequent round of selection. Enrichment was assessed by calculating output-to-input phage ratios across successive rounds. Negative selection against control pHLA complexes was incorporated to reduce enrichment of nonspecific or cross-reactive binders. The peptides and pHLA complexes used for selection and specificity assessment are listed in Supplementary Table 3. Following the third round of biopanning, individual clones were screened by monoclonal phage ELISA, with each clone tested in triplicate wells to identify candidate pHLA-reactive binders.

TCR specificity assay

TCR specificity was evaluated by phage ELISA as previously described [16, 30]. Briefly, streptavidin-coated plates were incubated with biotinylated pHLA complexes, followed by incubation with phage-displayed TCRs. After washing, bound phages were detected using horseradish peroxidase (HRP)-conjugated anti-M13 antibody followed by TMB substrate development. Absorbance was measured at 450 nm. Clones with $OD_{450} > 0.5$ were designated as putative binders and advanced to secondary specificity screening. Putative binders were subsequently evaluated for preferential binding to the cognate pHLA relative to control pHLA complexes. The cognate and control pHLA complexes used for specificity assessment are detailed in Supplementary Table 3.

Lentiviral production and T cell engineering

Selected TCR sequences were cloned into the third-generation lentiviral transfer vector pLTV-mC under the control of the EF-1 α promoter. The transfer, envelope, and packaging plasmids used for lentiviral production are listed in Supplementary Table 5. Lentiviral particles were produced by co-transfecting 293T cells with the packaging plasmids pMDLg/pRRE and pRSV-Rev and the envelope plasmid pMD2.G using polyethyleneimine (PEI). Viral supernatants were collected at 48 and 72 h after transfection, clarified by filtration through a 0.45- μ m filter, and concentrated by ultracentrifugation. Viral titers were determined by transducing J.RT3-T3.5 cells followed by flow cytometric analysis after 3 days of incubation.

Primary human T cells were activated using anti-CD3/CD28 stimulation (Thermo Fisher Scientific) in the presence of recombinant human IL-2 (100 IU/mL). After 24 h of activation, T cells were transduced with lentiviral particles at a multiplicity of infection (MOI) of 10 and expanded for 9–12 days before functional analysis. Surface expression of the introduced TCR was quantified by flow cytometry using a murine TCR β -specific antibody, allowing discrimination of the introduced TCR from endogenous human TCR. Only cultures with > 50% transgenic TCR expression were used for downstream functional assays.

In vitro functional assays

In vitro functional assays were performed using at least three biological replicates unless otherwise stated. Appropriate controls, including effector-only and target-only conditions, were included for background correction in cytotoxicity and cytokine assays. TCR-T cell-mediated cytotoxicity was assessed using the CytoTox 96® LDH release assay (Promega) according to the manufacturer's instructions. TCR-T cells were co-cultured with tumor target cells or T2 cells pulsed with the cognate or control peptides listed in Supplementary Table 3, with HLA matching as appropriate. For peptide dose-response experiments, T2 target cells were pulsed with serial concentrations of the cognate peptide before co-culture with the corresponding TCR-T cells, as previously described [16, 30]. Effector-to-target (E:T) ratios were varied as indicated for the individual experiments. Following incubation, supernatants were collected and LDH activity was measured by absorbance at 490 nm. Percent cytotoxicity was calculated relative to spontaneous and maximum lysis controls. IFN γ and TNF α secretion was measured in co-culture supernatants using ELISA kits (Sino Biological) according to the manufacturer's instructions, with cytokine concentrations determined from standard curves generated for each assay.

In vivo xenograft studies

All animal experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC; approval no. 2023078) at the Guangzhou Institutes of Biomedicine and Health. Female NOD/SCID/IL2 γ ^{-/-} mice (6–8 weeks old) were subcutaneously inoculated with 4×10^6 SW480 colorectal cancer cells or ECA109 esophageal cancer cells per mouse. Once tumors reached approximately 300–400 mm³, mice were randomized to treatment groups, including untransduced T cell controls and the corresponding TCR-T cell treatment groups. For the in vivo studies, TCR-T products and their corresponding untransduced controls were generated from purified human CD8⁺ T cells and expanded before adoptive transfer. TCR-T cells (2×10^6 cells per mouse) were administered intravenously. Recombinant human IL-2 (25,000 IU per mouse) was administered intraperitoneally once daily for 5 consecutive days, beginning on the day of TCR-T cell infusion. Tumor growth was monitored every 3 days using digital calipers, and tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. At the study endpoint, tumors were excised, weighed, and processed for downstream analyses. Investigators performing tumor measurements were blinded to treatment allocation. No animals were excluded from analysis unless predefined humane endpoints were reached.

Post-mortem tissue analyses: treatment-associated tissue injury, tumor histopathology, and T cell localization

At the experimental endpoint, mice were euthanized and tissues were collected for LDH-based assessment of treatment-associated tissue injury, tumor histopathology, and evaluation of T cell localization. Post-mortem analyses were performed as described below.

A) Serum collection and LDH analysis

Whole blood (approximately 500–1000 μ L) was collected by cardiac puncture and allowed to clot at room temperature for 45 min before centrifugation at $1500 \times g$ for 10 min. Serum was collected while

avoiding hemolyzed samples and was either analyzed immediately or stored in aliquots at -80°C. Serum LDH levels were measured using the CytoTox 96® LDH assay (Promega) according to the manufacturer's instructions as an indicator of treatment-associated tissue injury.

B) Ex vivo assessment of tissue injury by LDH release

Tumors and major organs, including the liver, lung, kidney, heart, and spleen, were harvested at the experimental endpoint, rinsed in PBS, and processed for ex vivo LDH analysis. Resected tumors were divided into separate portions immediately after excision to permit parallel downstream analyses. Fresh tumor tissue was used for LDH analysis, while separate portions were reserved for histological and immunofluorescence analyses. Fresh tissues were mechanically dissociated into single-cell suspensions and passed through 70 µm cell strainers. Cells were resuspended in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin and seeded in 96-well plates at equal cell densities. Samples were incubated at 37°C with 5% CO₂ for 4 h to allow spontaneous LDH release. Following incubation, supernatants were collected after centrifugation and analyzed using the CytoTox 96® LDH assay (Promega).

C) Tumor histology and immunofluorescence analysis of T cell localization

Tumor tissues collected at the study endpoint were fixed in 10% neutral-buffered formalin for 24 h, paraffin-embedded, and sectioned at 4–5 µm. Hematoxylin and eosin (H&E) staining was performed to evaluate tumor morphology, cellularity, and necrosis, with histological assessment performed in a blinded manner. Adjacent sections were subjected to immunofluorescence staining following antigen retrieval and blocking using Alexa Fluor 594-conjugated anti-human CD8α antibody. Nuclei were counterstained with DAPI. Fluorescent images were acquired by confocal microscopy, and intratumoral human CD8⁺ T cell density was quantified to assess T cell localization within tumor tissues. Antibody information is provided in Supplementary Table 4.

D) Flow cytometric analysis of splenic T cell distribution

To evaluate the persistence and phenotype of adoptively transferred T cells at the study endpoint, spleens were mechanically dissociated into single-cell suspensions and passed through 70-µm cell strainers. Red blood cells were lysed using ammonium-chloride-potassium (ACK) lysis buffer, followed by washing in PBS supplemented with 2% FBS. Cells were stained with antibodies against human CD3, murine TCRβ, CD69, CD25, and PD-1 and analyzed by flow cytometry. Human CD3⁺ T cells were quantified to assess persistence of the adoptively transferred T cell population. Expression of the introduced TCR was assessed by murine TCRβ-specific staining, while CD69, CD25, and PD-1 expression was evaluated to characterize the phenotype of persisting T cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism versions 9 and 10. Data are presented as mean $\pm$ SD unless otherwise indicated. Comparisons between two groups were performed using unpaired two-tailed t tests, with Welch's correction. Multiple-group comparisons were performed using one-way or two-way analysis of variance (ANOVA), as appropriate. One-way ANOVA was followed by Tukey's multiple-comparisons test, whereas two-way ANOVA was followed by Šídák's multiple-comparisons test. $P < 0.05$ was considered statistically significant. Statistical significance was denoted as follows: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$. Next-generation sequencing (NGS) data were analyzed using Python (v3.8) with custom scripts and publicly available computational tools.

Results

Construction and quality assessment of the naïve phage-displayed TCR library

To generate a source of TCRs for antigen-specific selection, we constructed a naïve phage-displayed TCR library using CD3⁺ T cells from healthy PBMC donors heterozygous for HLA-A*11:01 and HLA-A*02:01. Following the library construction, next-generation sequencing (NGS) was used to assess sequencing quality, sequence diversity, construct length, V-J gene usage, and sequence productivity. Per-base quality scores remained high across both α - and β -chain reads, with no evident decline in sequencing quality across the variable regions (Fig. 1A). Nucleotide diversity was low within the molecular identifier regions but increased markedly across the variable regions of both chains (Fig. 1B(i-ii)). A high proportion of productive TCR α and TCR β rearrangements was also observed.

Length-distribution analysis showed that most α - and β -chain sequences clustered around the expected construct lengths, with relatively few shorter or aberrant sequences (Fig. 1C(i-ii)). V and J gene segment analysis showed broad representation across the α - and β -chain repertoires without marked dominance of individual gene segments (Fig. 1D). Sequence classification further identified a substantial proportion of productive in-frame sequences, together with smaller fractions of partial and frameshifted sequences (Fig. 1E).

Following three rounds of biopanning against the respective pHLA complexes, phage output increased progressively while library diversity decreased (Fig. 1F). Despite this reduction, multiple sequences remained represented after selection. These results demonstrated sufficient library quality and diversity for subsequent isolation of antigen-reactive TCR candidates.

Isolation and validation of antigen-specific TCRs

To identify antigen-reactive TCRs, the naïve phage-displayed TCR library, with an initial library size of approximately 6×10^9 CFU, was subjected to three iterative rounds of biopanning against KRASG12V₍₈₋₁₆₎/HLA-A*11:01 and CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01 pHLA complexes. Following selection,

individual clones were screened by monoclonal phage ELISA. A subset of clones showed binding signals above the KM13 helper phage control (Fig. 2A(i-ii), C(i-ii)). An OD₄₅₀ threshold of > 0.5 was used to identify putative binders for subsequent specificity screening.

Secondary screening against control pHLA complexes showed that only a subset of the initially identified binders retained preferential recognition of the target pHLA. For KRASG12V₍₈₋₁₆₎/HLA-A*11:01, clones A5, A24, A25, and B14 showed selective binding with minimal or no detectable reactivity against the control pHLA complexes tested (Fig. 2B(i-ii)). Similarly, screening of CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01 binders identified clones C2, C13, C14, C18, C19, D11, D15, D22, and D28 with preferential binding to the target pHLA, although the degree of reactivity against control pHLA complexes varied among individual clones (Fig. 2D(i-ii)). These analyses identified a panel of KRASG12V- and CTAG1B-reactive TCR candidates. Selected receptors from this panel were subsequently advanced for engineering and functional evaluation in primary human T cells. The TRAV and TRBV gene identities of the selected TCR candidates are provided in Supplementary Table 6.

Antigen and HLA characterization of target tumor models

Antigen and HLA characteristics relevant to selection of the tumor models were determined in the present study or established in our previous studies [16, 30, 32], as summarized in Supplementary Table 2. RT-PCR and flow cytometric analyses demonstrated CTAG1B expression in esophageal cancer samples relative to matched normal controls, while RT-PCR analysis confirmed the presence of KRASG12V in colorectal cancer samples. HLA expression was also assessed previously [16, 30]. The antigen and HLA profiles of the tumor models used for subsequent TCR-T cell functional studies are summarized in Supplementary Table 2.

Expansion and functional characterization of engineered TCR-T cells

Selected TCR candidates were expressed in primary human T cells and expanded in vitro. Transduced T cells continued to proliferate over the 11-day culture period, maintained viability, and showed substantial fold expansion by day 11 (Fig. 3A(i-iii)). Flow cytometric analysis confirmed surface expression of the introduced TCRs, with transgenic TCR expression exceeding 70%, as determined by murine TCR β -specific staining within the human CD3⁺ population (Fig. 3B; Supplementary Fig. S1). Murine TCR β -specific staining was used to distinguish the introduced TCRs from endogenous human TCRs.

The engineered TCR-T cells were subsequently evaluated for antigen-dependent effector function using peptide-pulsed target cells. CTAG1B-specific TCR-T cells showed peptide dose-dependent cytotoxicity against cognate peptide-pulsed target cells (Fig. 3C(i)) and secreted IFN γ following stimulation with the cognate peptide, while responses to irrelevant peptide controls remained low (Fig. 3C(ii)). Similarly, KRASG12V-specific TCR-T cells showed peptide dose-dependent cytotoxicity (Fig. 3D(i)) and preferential IFN γ secretion in response to the cognate mutant peptide compared with control peptides (Fig. 3D(ii)).

Consistent with these findings, selected TCR-T cells showed antigen-specific cytotoxicity against HLA-appropriate T2 cells pulsed with the corresponding CTAG1B₍₁₅₇₋₁₆₅₎ or KRASG12V₍₈₋₁₆₎ peptide (Supplementary Fig. S2). Responses observed across decreasing peptide concentrations indicated functional activity under conditions of decreasing peptide availability, although the density of presented pHLA complexes was not directly quantified.

Tumor-cell recognition, HLA restriction, and normal-cell reactivity of TCR-T cells

Following validation of antigen-dependent activity against peptide-pulsed targets, we evaluated the ability of the engineered TCR-T cells to recognize tumor cells with defined antigen and HLA profiles. TCR-T cells secreted IFN γ in response to tumor cells co-expressing the cognate antigen and the appropriate HLA molecule, whereas responses to cells lacking either the cognate antigen or the appropriate HLA molecule were low (Fig. 4A(i-ii)).

TCR-T cells also showed effector-to-target (E:T) ratio-dependent cytotoxicity against tumor cells. CTAG1B-specific TCR-T cells lysed esophageal tumor cells (Fig. 4B(i)), while KRASG12V-specific TCR-T cells lysed colorectal tumor cells (Fig. 4C(i)). Cytotoxicity was accompanied by increased TNF α secretion (Fig. 4B(ii), C(ii)). Antigen-dependent cytotoxicity was further assessed using peptide-pulsed T2 cells, with TCR-T cells preferentially lysing targets loaded with the cognate peptide compared with irrelevant peptide controls (Fig. 4D).

Potential off-target reactivity was assessed against normal and control cell targets, with the corresponding antigen/HLA-matched tumor cell included as a positive control in each assay. CTAG1B-specific TCR-T cells retained cytotoxic activity against ECA109 cells while showing limited cytotoxicity against the normal esophageal epithelial Het-1A and colon-derived CCD-841CoN cells, HLA-engineered 293T control cells, and PBMCs from two independent healthy donors not used for TCR-T cell generation (Fig. 4E(i)). Similarly, KRASG12V-specific TCR-T cells retained cytotoxic activity against SW480 cells while showing limited cytotoxicity against the corresponding normal and control cell targets, including Het-1A, CCD-841CoN, HLA-engineered 293T cells, and PBMCs from the same two independent healthy donors (Fig. 4E(ii)).

CTAG1B- and KRASG12V-specific TCR-T cells suppress tumor growth in vivo

The antitumor activity of the engineered TCR-T cells was evaluated in esophageal and colorectal cancer xenograft models. In the esophageal cancer model, CTAG1B-specific TCR-T cells significantly suppressed tumor progression relative to control T cells, as shown by reduced longitudinal tumor growth and lower endpoint tumor weights (Fig. 5A(i-ii)). The magnitude of tumor control differed among the tested TCRs. TCRT-C18 showed the strongest activity, reducing mean endpoint tumor weight from approximately 1.0 g in the control group to 0.2 g (~ 80% reduction). TCRT-D15 reduced mean tumor

weight to approximately 0.35 g (~ 65% reduction), while TCRT-C19 produced a more moderate reduction to approximately 0.45 g (~ 55% reduction). Gross images of the resected tumors are shown in Supplementary Fig. S3A.

KRASG12V-specific TCR-T cells also suppressed tumor progression in the colorectal cancer model (Fig. 6A(i-ii)). TCRT-A5 showed the strongest activity, reducing mean endpoint tumor weight from approximately 1.0 g in the control group to 0.4 g (~ 60% reduction). TCRT-A24 reduced mean tumor weight to approximately 0.4–0.45 g (~ 55–60% reduction), whereas TCRT-B14 showed a more modest reduction to approximately 0.7–0.8 g (~ 25–30% reduction). Gross images of the resected tumors are shown in Supplementary Fig. S3B.

LDH levels measured in serum and harvested organs at the study endpoint remained low across treatment groups in both tumor models, with no marked increase associated with TCR-T cell treatment (Fig. 5B(i-ii), Fig. 6B(i-ii)).

Approximately 30 days after treatment, human CD3⁺ T cells remained detectable in the spleens of treated mice in both tumor models, and murine TCR β -specific staining identified persisting T cells expressing the introduced TCRs (Fig. 5C(i-ii), Fig. 6C(i-ii)). The frequency of murine TCR β ⁺ cells at endpoint was lower than the pre-injection transgenic TCR expression level (> 70%). Phenotypic analysis showed generally low CD69 expression and modest CD25 expression at endpoint, with differences among individual TCR constructs (Fig. 5C(iii-iv), Fig. 6C(iii-iv)). PD-1 expression was also variably detected among the persisting T cells (Fig. 5C(v), Fig. 6C(v)).

H&E staining showed treatment-associated histopathological differences in tumors from TCR-T cell-treated mice compared with controls, including reduced tumor cellularity and disrupted tissue organization (Fig. 5D, Fig. 6D). Immunofluorescence analysis demonstrated CD8⁺ T cells within tumors from treated mice in both models (Fig. 5E(i), Fig. 6E(i)). Quantitative analysis showed increased intratumoral CD8⁺ T cell density relative to control tumors (Fig. 5E(ii), Fig. 6E(ii)). Because the TCR-T cell products used in these experiments were generated from purified human CD8⁺ T cells, the increased intratumoral CD8⁺ T cell density supports tumor localization of the adoptively transferred T cell population.

Discussion

In this study, we developed a phage display-based workflow for identifying antigen-specific TCRs and evaluated selected receptors targeting CTAG1B_(157–165)/HLA-A*02:01 and KRASG12V_(8–16)/HLA-A*11:01. Following pHLA-directed selection and specificity screening, selected TCRs were expressed in primary human T cells and showed antigen-dependent effector activity against peptide-pulsed targets and tumor cells. The engineered TCR-T cells also suppressed tumor growth in CTAG1B-positive esophageal and KRASG12V-positive colorectal cancer xenograft models. Together, these findings show that the phage

display workflow can be used to identify functionally active TCRs against both a cancer-testis antigen and a tumor-specific neoantigen.

Library quality, phage selection, and specificity screening

The diversity and quality of the starting TCR repertoire are important considerations in receptor discovery using display-based approaches. NGS analysis showed broad sequence diversity across the variable regions of both TCR chains, representation of multiple V and J gene segments, and a high proportion of productive sequences. These characteristics provided a suitable starting repertoire for pHLA-directed selection [26, 33–36]. Iterative biopanning increased the recovery of antigen-reactive phage while reducing overall library diversity. Importantly, enrichment alone did not establish pHLA selectivity. Several clones identified during the initial ELISA screen showed reactivity against control pHLA complexes, whereas a smaller subset retained preferential recognition of the intended target. This finding supports the use of secondary specificity screening when selecting TCR candidates from phage-displayed libraries. Differences observed between the KRASG12V- and CTAG1B-directed selections may reflect differences in pHLA presentation and structural constraints governing TCR recognition [9, 37, 38], although these factors were not directly examined in the present study.

Functional activity after expression in primary T cells

A central consideration in display-based TCR discovery is whether receptors selected on the basis of pHLA binding retain functional activity when expressed in primary T cells. Primary T cells expressing the selected TCR constructs maintained viability and expanded under the culture conditions used, with transgenic TCR expression exceeding 70%. Previous studies have demonstrated that genetically modified T cells can maintain viability following transduction and that culture conditions influence T cell expansion and functional properties [39, 40]. The engineered cells mediated peptide dose-dependent cytotoxicity and cytokine secretion, demonstrating functional responses across decreasing peptide concentrations. These cellular assays provide evidence of functional sensitivity but do not directly measure TCR-pHLA binding properties, which could be further investigated through quantitative affinity measurements and pHLA multimer staining.

The selected receptors also recognized tumor cells in an antigen-dependent and HLA-restricted manner. Responses were strongest when the target cells expressed both the cognate antigen and the appropriate HLA molecule, while responses were low when either component was absent, consistent with the requirements for pHLA-specific TCR recognition [41–43]. This distinction is particularly important for TCR-based therapy because antigen expression alone is insufficient; the relevant peptide must be processed and presented by the restricting HLA molecule.

Normal-cell reactivity and preliminary safety assessment

The normal-cell reactivity experiments provided an initial assessment of specificity beyond the tumor models. Both CTAG1B- and KRASG12V-specific TCR-T cells retained activity against their antigen/HLA-matched tumor targets while showing limited cytotoxicity against the normal epithelial cells, HLA-engineered 293T controls, and independent healthy-donor PBMCs evaluated in this study. These findings provide preliminary evidence of limited reactivity against the tested cell types and support further evaluation of the safety profiles of these receptors [43–45]. Broader testing against normal tissues and alternative pHLA complexes will be necessary to more fully define potential on-target/off-tumor and cross-reactive recognition.

In vivo efficacy, construct differences, persistence, and tumor localization

The in vivo studies extended these observations by showing antitumor activity in two xenograft models. CTAG1B-specific TCR-T cells suppressed esophageal tumor growth, whereas KRASG12V-specific TCR-T cells suppressed colorectal tumor growth. However, the magnitude of tumor control differed among receptors directed against the same antigen. This construct-dependent activity indicates that antigen binding and receptor expression alone may not fully predict therapeutic performance [46–49]. Differences in receptor-intrinsic properties, productive pHLA engagement, and downstream T cell activation may contribute to these differences, although the mechanisms were not directly examined in the present study [14, 50, 51]. These observations reinforce the need for functional evaluation after receptor discovery rather than prioritizing candidates solely on the basis of binding during the screening process.

Human CD3⁺ T cells remained detectable in the spleen approximately 30 days after treatment, and murine TCR β -specific staining identified persisting cells expressing the introduced TCRs. The frequency of murine TCR β ⁺ cells was lower than the pre-injection transgenic TCR expression level. This reduction could reflect contraction of the transferred population, changes in receptor expression, or other mechanisms that cannot be distinguished from the endpoint analysis alone [52, 53]. CD69 and CD25 expression was generally low or modest at this late time point, with differences among individual TCR constructs [54–59]. PD-1 expression was also variable. Although PD-1 can be induced following antigen encounter and sustained activation, its expression alone is insufficient to establish functional exhaustion [60–62]. Longitudinal phenotypic and functional analyses will therefore be needed to define how early effector activity relates to subsequent persistence and functional state.

The histopathological and immunofluorescence findings were consistent with the observed antitumor activity. H&E staining showed reduced tumor cellularity and disrupted tissue organization in treated tumors, although these changes alone cannot establish specific remodeling of the tumor microenvironment. CD8⁺ T cells were detected within tumors from treated mice, and quantitative analysis showed increased intratumoral CD8⁺ T cell density. Because the infused TCR-T products were generated from purified human CD8⁺ T cells, these findings support tumor localization of the adoptively

transferred T cell population. Effective localization of transferred T cells to tumor tissue is an important requirement for adoptive T cell therapy in solid tumors [63].

Treatment-associated tissue injury, limitations, and future perspectives

LDH measurements in serum and harvested organs showed no marked increase associated with TCR-T cell treatment in either xenograft model. Together with the limited reactivity observed against the normal cell types evaluated in vitro, these findings provide an initial assessment of treatment-associated tissue injury, consistent with the importance of evaluating cytotoxicity and potential off-tumor effects during preclinical TCR-T cell development [41, 64].

Although the present findings provide encouraging evidence of the antitumor activity and specificity of the selected TCRs, certain considerations remain relevant to their further evaluation. The normal-cell and peptide cross-reactivity assessments provide an initial characterization of receptor specificity, which could be extended through broader screening in future studies. Additionally, while the xenograft models enabled evaluation of antitumor activity in vivo, their immunodeficient nature limits representation of the broader immune interactions within the tumor microenvironment. Finally, immune phenotyping was primarily conducted at the study endpoint, providing insight into the functional state of the engineered T cells at that time, with longitudinal characterization remaining an area for further investigation.

Despite these limitations, the present study demonstrates the potential of phage-displayed TCR libraries for identifying therapeutically relevant receptors against intracellular tumor antigens. The identification and functional validation of TCRs targeting CTAG1B, a cancer-testis antigen, and KRASG12V, a tumor-specific neoantigen, demonstrate the applicability of this approach across distinct classes of pHLA targets. By linking receptor discovery to functional validation in primary human T cells, tumor-cell recognition, and antitumor activity in vivo, this study provides a framework for advancing candidate TCRs toward further preclinical evaluation.

Conclusions

In this study, we developed and functionally evaluated a phage display-based platform for identifying TCRs targeting the KRASG12V neoantigen and the cancer-testis antigen CTAG1B. Using a naïve $\alpha\beta$ TCR library generated from healthy donors heterozygous for HLA-A*11:01 and HLA-A*02:01, we identified pHLA-reactive TCR candidates recognizing KRASG12V₍₈₋₁₆₎/HLA-A*11:01 and CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01. Selected receptors were expressed in primary human T cells and mediated antigen-dependent cytotoxicity and cytokine secretion in vitro. CTAG1B-specific TCR-T cells recognized esophageal cancer cells, whereas KRASG12V-specific TCR-T cells recognized colorectal cancer cells in an antigen-dependent and HLA-restricted manner. In vivo, the engineered TCR-T cells suppressed tumor growth in esophageal and colorectal cancer xenografts. Human CD3⁺ T cells and cells expressing the introduced

TCR remained detectable at the study endpoint, while increased intratumoral CD8⁺ T cell density supported localization of the adoptively transferred T cell population.

These findings support the utility of phage-displayed TCR libraries for identifying functionally active TCR candidates against distinct classes of tumor antigens. The differences in activity among TCR candidates recognizing the same target also emphasize the importance of secondary specificity screening and functional validation following receptor discovery. Further studies incorporating quantitative TCR-pHLA binding measurements, pHLA tetramer or multimer characterization, broader peptide and normal-tissue cross-reactivity assessment, and validation in more physiologically representative models will be required before clinical translation. Overall, this study provides a framework for the discovery and preclinical evaluation of antigen-specific TCRs for adoptive T cell therapy in solid tumors.

Abbreviations

ANOVA

Analysis of variance

CAR

Chimeric antigen receptor

CFU

Colony-forming units

CRC

Colorectal cancer

EC

Esophageal cancer

ELISA

Enzyme-linked immunosorbent assay

FBS

Fetal bovine serum

H&E

Hematoxylin and eosin

HLA

Human leukocyte antigen

HRP

Horseradish peroxidase

IACUC

Institutional Animal Care and Use Committee

IFN γ

Interferon gamma

LDH

Lactate dehydrogenase

MHC
Major histocompatibility complex
MOI
Multiplicity of infection
MSS
Microsatellite stable
NGS
Next-generation sequencing
PBMC
Peripheral blood mononuclear cell
PD-1
Programmed cell death protein 1
PD-L1
Programmed death-ligand 1
PFU
Plaque-forming units
pHLA
Peptide-human leukocyte antigen
RT-PCR
Reverse transcription polymerase chain reaction
SDS-PAGE
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCR
T cell receptor
TCR-T
T cell receptor-engineered T cell
TME
Tumor microenvironment
TNF α
Tumor necrosis factor alpha
TRAV
T cell receptor alpha variable
TRBV
T cell receptor beta variable

Declarations

Ethics approval and consent to participate

All animal experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Guangzhou Institutes of Biomedicine

and Health (approval no. 2023078). Human peripheral blood mononuclear cells (PBMCs) used in this study were commercially sourced as de-identified samples from Miaoshun Biotechnology. No identifiable private information was obtained or used in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

CRedit authorship

Obed B. Amissah: Writing - original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Wenfang Chen: Methodology, Investigation, Formal analysis. Belinda E. Baiden: Writing - review & editing, Visualization. Rajesh Basnet: Data curation. Zhiyuan Li: Validation, Supervision, Project administration, Funding acquisition. Gloria B. Kim: Writing - review & editing, Validation, Supervision, Resources, Funding acquisition.

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Author Contribution

O.B.A.: Writing - original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. W.C.: Methodology, Investigation, Formal analysis. B.E.B.: Writing - review & editing, Visualization. R.B.: Data curation. Z.L.: Validation, Supervision, Project administration, Funding acquisition. G.B.K.: Writing - review & editing, Validation, Supervision, Resources, Funding acquisition. All authors reviewed the manuscript.

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Data Availability

The data supporting the findings of this study are available within the article and its supplementary materials. TCR α - and β -chain variable gene identities (TRAV and TRBV) for the selected TCR candidates are provided in Supplementary Table 6. The Python scripts used in this study are available from the corresponding authors upon reasonable request.

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Figures

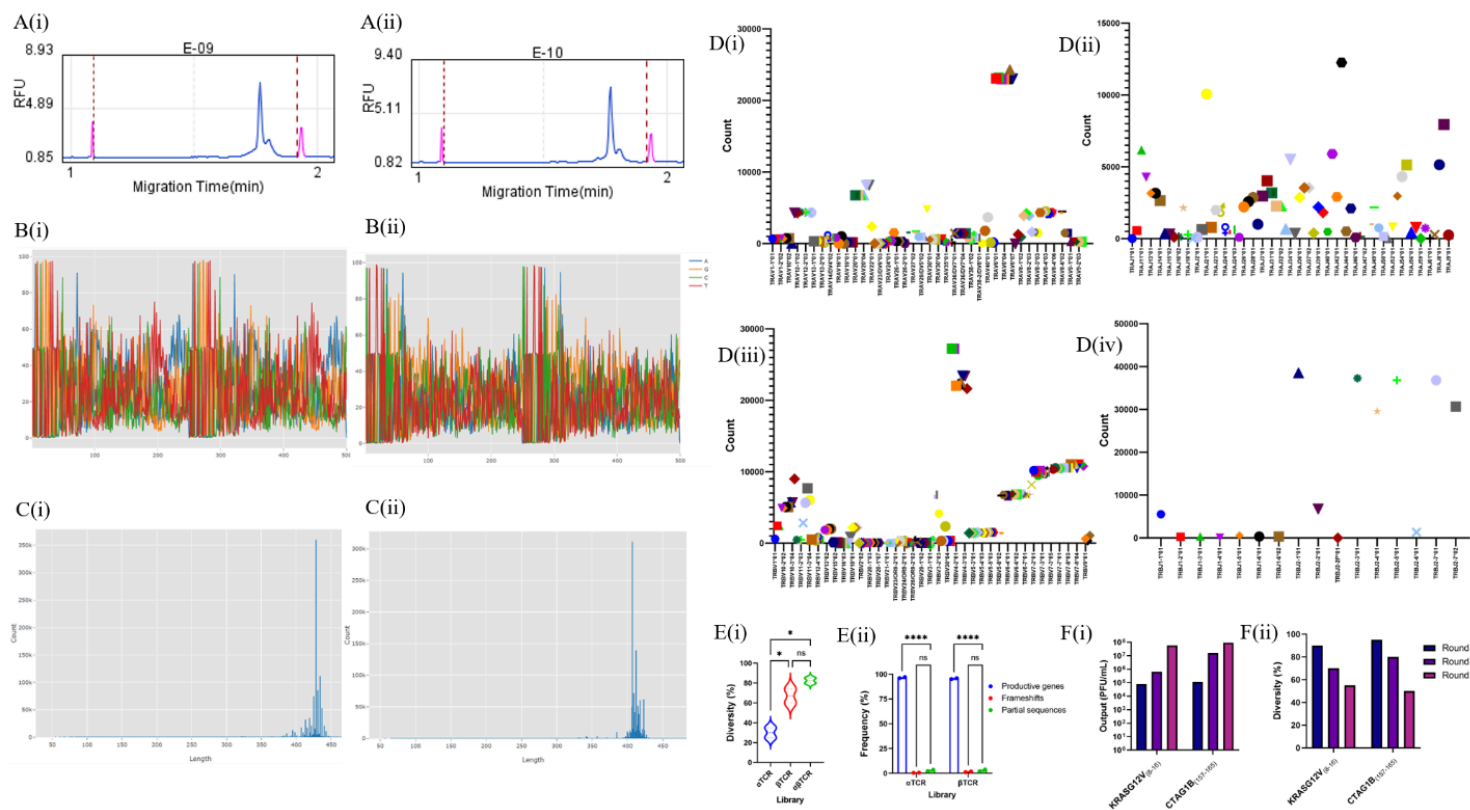


Figure 1

Next-generation sequencing quality assessment and diversity profiling of the naïve $\alpha\beta$ TCR library. A, Per-base sequencing quality profiles for the α -chain (E-09) and β -chain (E-10) libraries, showing high Phred quality scores across the read length with defined peaks corresponding to variable regions. Dashed lines indicate the boundaries of molecular identifier (MID) and coding regions. B, Nucleotide diversity across sequencing reads for the α -chain (B(i)) and β -chain (B(ii)) libraries. Positions 0-55 and 251-300 correspond to molecular identifier regions, while positions 56-250 and 301-500 represent the variable regions of the TCR α and TCR β chains, respectively. C, Length distribution of assembled α -chain (C(i)) and β -chain (C(ii)) sequences, showing a dominant peak consistent with the expected full-length variable-region constructs and relatively few shorter sequences. D, Frequency distribution of V and J gene segment usage across the α - and β -chain repertoires, showing broad representation of gene segments without marked dominance of individual segments. E, Classification of sequence productivity, showing the proportions of productive (in-frame), partial, and frameshifted sequences within the library. F, Phage output and library diversity following iterative rounds of biopanning, starting from an initial *E. coli* library size of 6×10^9 CFU, illustrating enrichment dynamics and changes in sequence diversity.

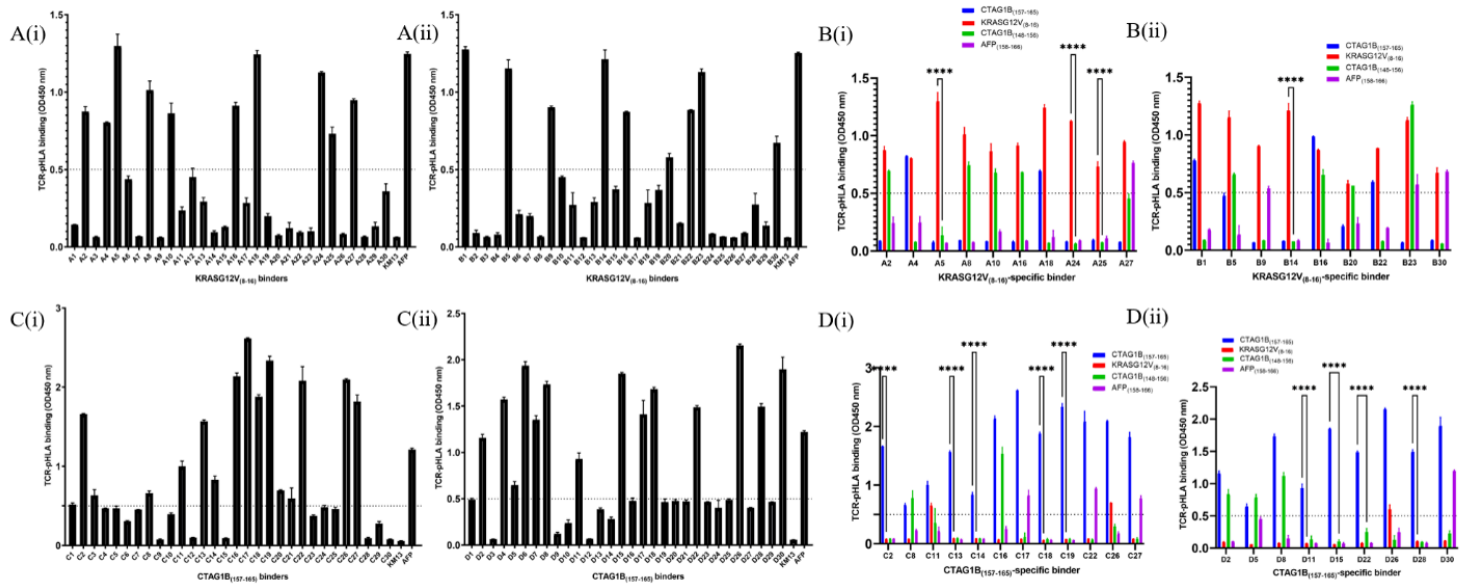


Figure 2

Identification and specificity profiling of antigen-binding TCRs following phage display selection. A-B, Selection against KRASG12V₍₈₋₁₆₎/HLA-A*11:01. Following three rounds of biopanning of the naïve phage-displayed TCR library, with approximately 1×10^{11} PFU used as input for each round, 30 individual clones were isolated and screened by monoclonal phage ELISA. Binding signals were evaluated relative to the KM13 helper phage control, and clones with OD450 >0.5 were designated as putative binders (A, i-ii). Candidate binders were subsequently evaluated by secondary specificity screening against a panel of control pHLA complexes. Clones showing selective binding to KRASG12V₍₈₋₁₆₎/HLA-A*11:01 with minimal or no detectable reactivity against the control pHLA complexes tested were prioritized as pHLA-specific TCR candidates (B, i-ii). C-D, Selection against CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01. The same screening workflow was applied following three rounds of biopanning, with approximately 1×10^{11} PFU used as input for each round. Thirty individual clones were screened by monoclonal phage ELISA, and clones with OD450 >0.5 were designated as putative binders (C, i-ii). Candidate binders were subsequently evaluated by secondary specificity screening against a panel of control pHLA complexes. Clones showing preferential recognition of CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01 with minimal or no detectable reactivity against the control pHLA complexes tested were prioritized as antigen-specific TCR candidates (D, i-ii).

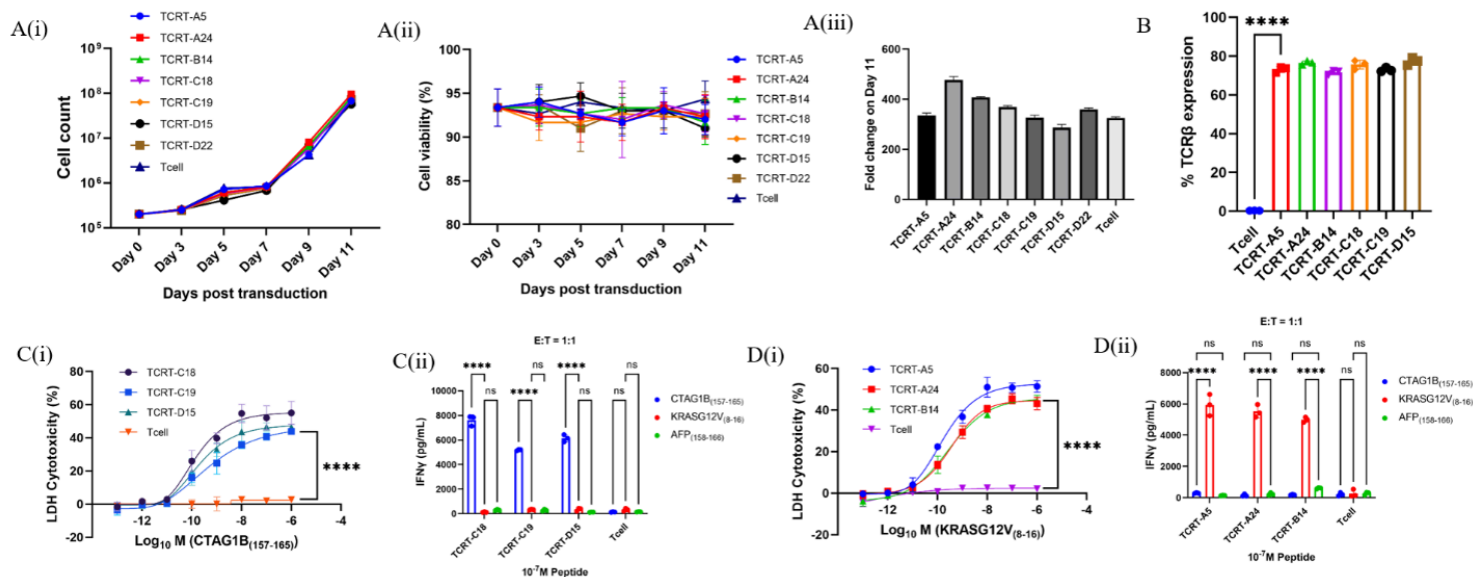


Figure 3

Expansion, surface TCR expression, and functional characterization of engineered TCR-T cells. A, Primary human T cells were activated, lentivirally transduced with selected TCR constructs, and expanded in vitro over an 11-day period. Cell number and viability were monitored at defined intervals during expansion (A, i-ii), and fold expansion was calculated at day 11 (A, iii). B, Surface expression of the introduced TCRs was assessed by flow cytometry using human CD3 and murine TCRβ-specific staining. The frequency of transgenic TCR-expressing cells was determined based on murine TCRβ positivity within the human CD3⁺ population, with representative flow-cytometric identification of human CD3⁺/murine TCRβ⁺ cells provided in Supplementary Fig. S1. C, Functional activity of CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01-specific TCR-T cells was evaluated using peptide-pulsed T2 target cells. Antigen-dependent cytotoxicity was quantified by LDH release across a range of peptide concentrations (C, i), and IFNγ secretion was measured following stimulation with cognate or irrelevant peptide controls at 10^{-7} M (C, ii). D, KRASG12V₍₈₋₁₆₎/HLA-A*11:01-specific TCR-T cells were evaluated using peptide-pulsed T2/HLA-A*11:01 target cells. Cytotoxicity was measured across a range of peptide concentrations (D, i), and IFNγ secretion was assessed following stimulation with cognate or control peptides (D, ii).

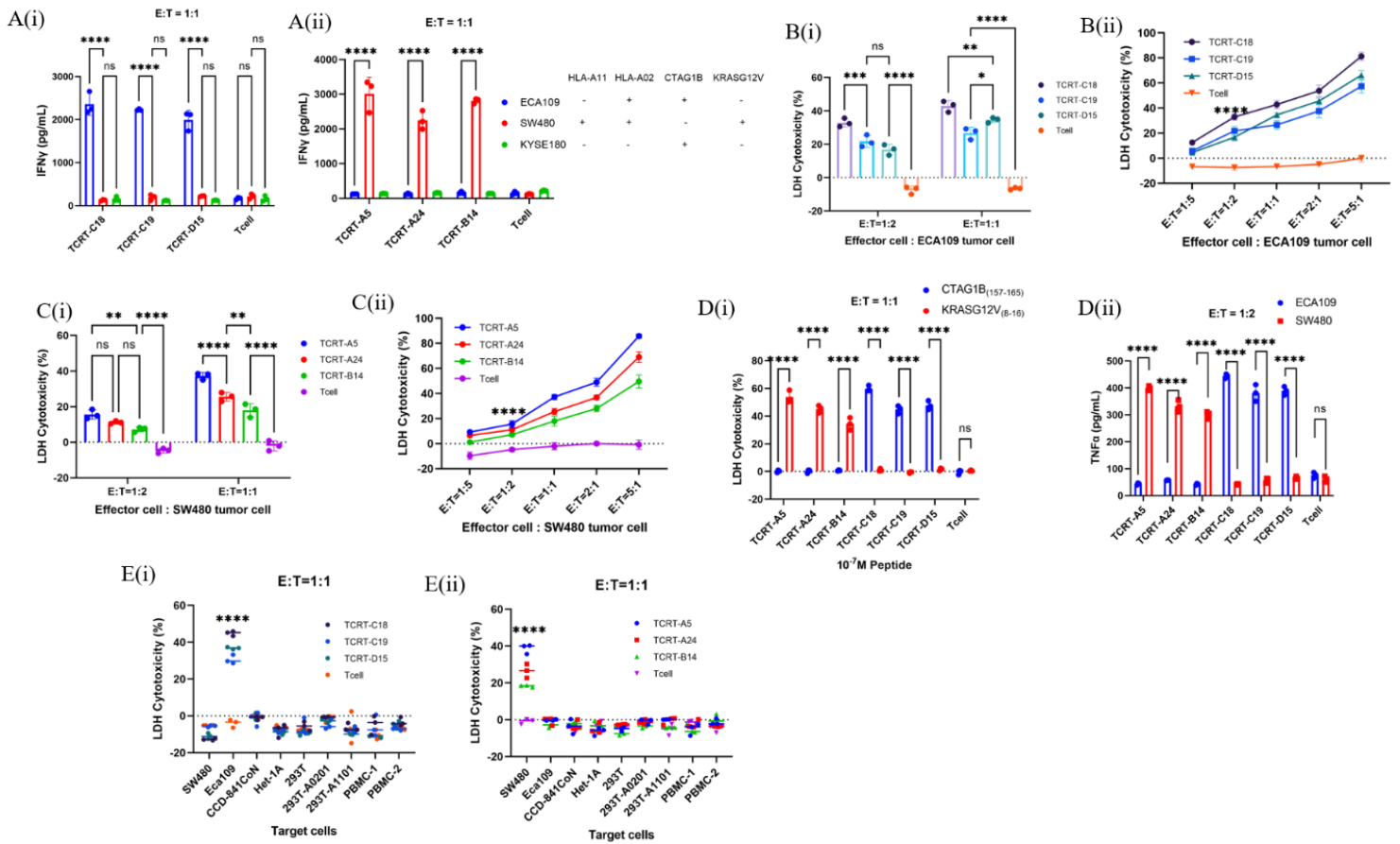


Figure 4

Tumor-cell recognition, HLA restriction, and normal-cell reactivity of engineered TCR-T cells. A, IFN γ secretion by TCR-T cells was assessed following co-culture with esophageal carcinoma cell lines ECA109 and KYSE180 and the colorectal carcinoma cell line SW480. Responses were evaluated in cells co-expressing the cognate antigen and the appropriate HLA molecule and in cells lacking either or both components (A, i-ii). B-C, Cytotoxic activity was evaluated across increasing effector-to-target (E:T) ratios. CTAG1B₍₁₅₇₋₁₆₅₎/HLA-A*02:01-specific TCR-T cells were tested against esophageal tumor cells (B), whereas KRASG12V₍₈₋₁₆₎/HLA-A*11:01-specific TCR-T cells were tested against colorectal tumor cells (C). Cytotoxicity was quantified by LDH release (B, i; C, i), and TNF α secretion was measured as an additional functional readout (B, ii; C, ii). D, Antigen-dependent recognition was further evaluated using peptide-pulsed T2 cells. TCR-T cells were tested against targets loaded with cognate or irrelevant control peptides, and cytotoxicity was assessed by LDH release. E, Potential off-target reactivity was assessed by co-culturing TCR-T cells with tumor-positive controls, normal epithelial cells, including colon-derived CCD-841CoN and esophageal-derived Het-1A cells, HLA-engineered 293T control cells, and independent healthy-donor PBMCs not used for TCR-T cell generation. (i) CTAG1B-specific TCR-T cells were evaluated against ECA109 cells as the antigen/HLA-matched positive tumor control together with the indicated normal and control cell targets, including 293T/HLA-A*02:01 cells and PBMCs from two independent healthy donors (PBMC-1 and PBMC-2). (ii) KRASG12V-specific TCR-T cells were evaluated against SW480 cells as the antigen/HLA-matched positive tumor control together with the indicated normal and

control cell targets, including 293T/HLA-A*11:01 cells and PBMCs from the same two independent healthy donors. Cytotoxicity was quantified by LDH release.

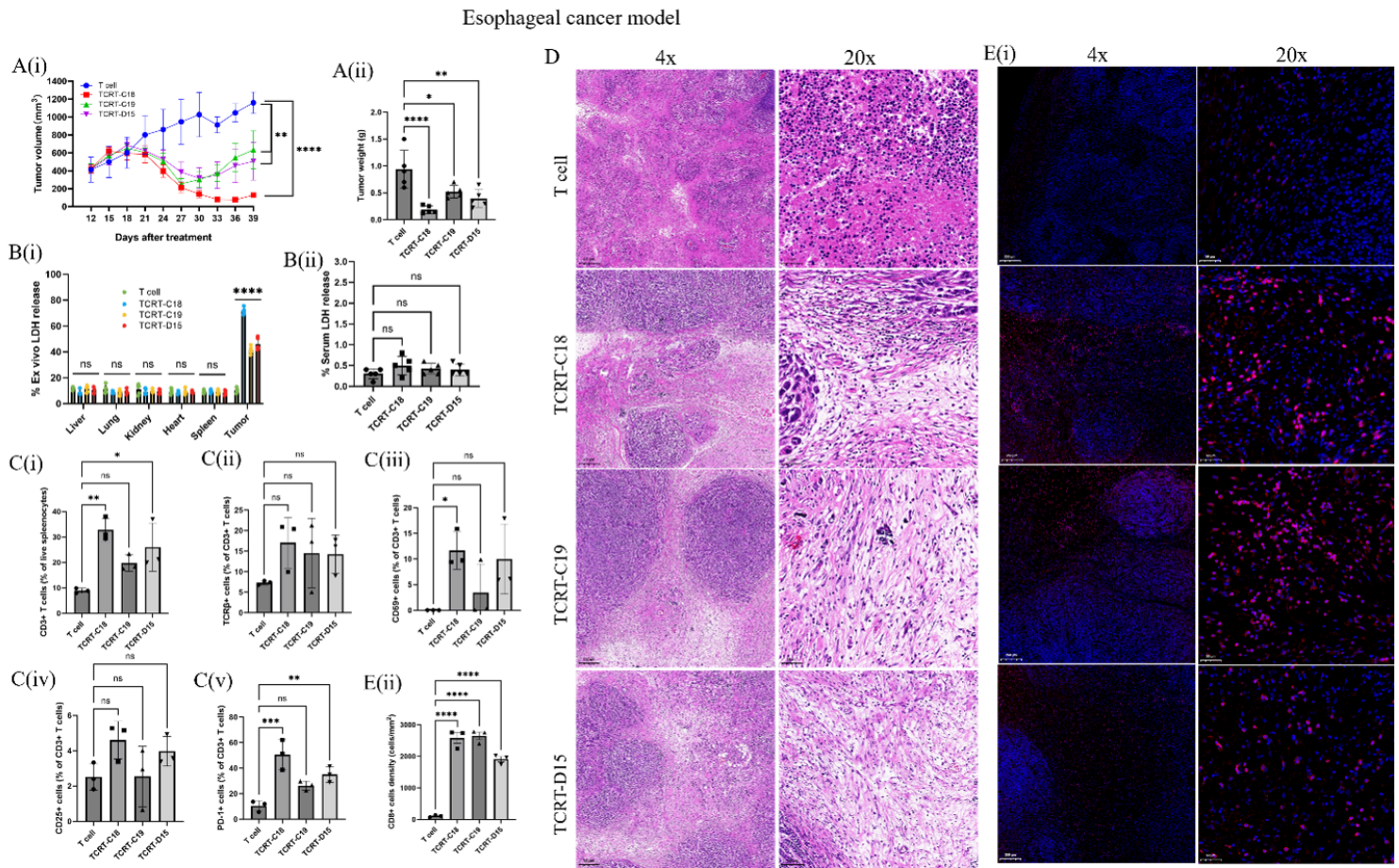
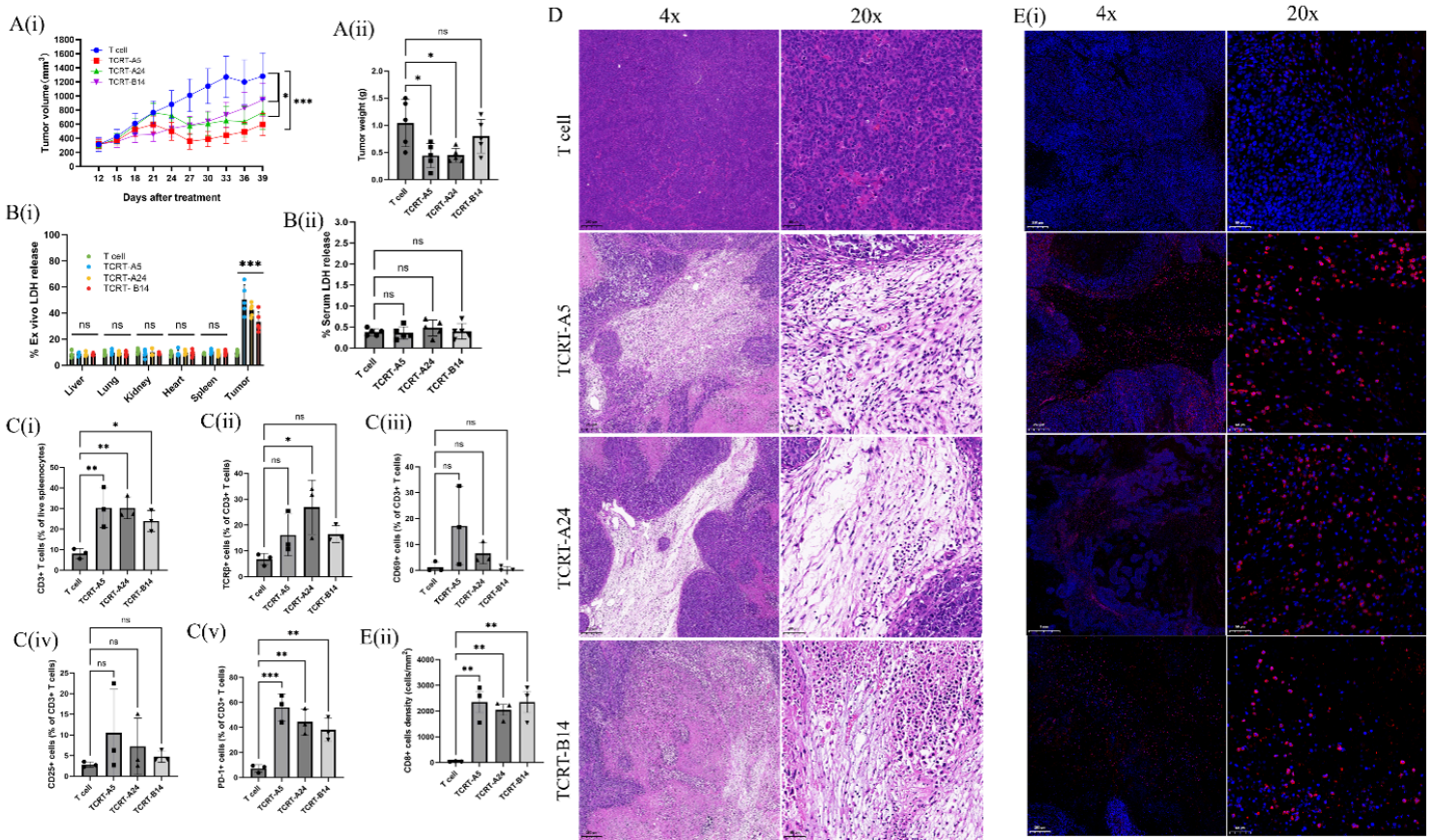


Figure 5

In vivo antitumor activity, persistence, and tissue-associated effects of CTAG1B-specific TCR-T cells in esophageal cancer xenografts. A, Antitumor activity following adoptive transfer of CTAG1B-specific TCR-T cells. Tumor progression was monitored longitudinally by caliper measurement (A, i), and excised tumor weights were determined at study endpoint (A, ii). B, LDH-based assessment of treatment-associated tissue injury at study endpoint. LDH release was measured in harvested organs (B, i) and serum (B, ii). C, Persistence and phenotypic characterization of adoptively transferred TCR-T cells. Splenocytes harvested approximately 30 days after treatment were analyzed by flow cytometry. Live cells were gated on human CD3⁺ T cells, followed by assessment of transgenic TCR expression using murine TCRβ-specific staining and phenotypic markers including CD69, CD25, and PD-1 (C, i-v). D, Representative hematoxylin and eosin (H&E)-stained tumor sections showing treatment-associated histopathological differences between TCR-T cell-treated and control groups. E, Immunofluorescence analysis of tumor sections for CD8⁺ T cell localization. Representative images show CD8⁺ T cells within tumor tissues (E, i), with quantitative analysis of CD8⁺ cell density shown in E, ii. Nuclei are counterstained in blue.

**Figure 6**

In vivo antitumor activity, persistence, and tissue-associated effects of KRASG12V-specific TCR-T cells in colorectal cancer xenografts. A, Antitumor activity of KRASG12V-specific TCR-T cells in colorectal tumor-bearing mice. Tumor progression was monitored longitudinally by caliper measurement (A, i), and excised tumor weights were determined at study endpoint (A, ii). B, LDH-based assessment of treatment-associated tissue injury at study endpoint. LDH release was measured in harvested organs (B, i) and serum (B, ii). C, Persistence and phenotypic characterization of adoptively transferred TCR-T cells. Splenocytes collected approximately 30 days after treatment were analyzed by flow cytometry. Live cells were gated on human CD3⁺ T cells, followed by assessment of transgenic TCR expression using murine TCRβ-specific staining and phenotypic markers including CD69, CD25, and PD-1 (C, i-v). D, Representative H&E-stained tumor sections showing treatment-associated histopathological differences between TCR-T cell-treated and control groups. E, Immunofluorescence analysis of tumor sections for CD8⁺ T cell localization (E, i), with quantitative analysis of CD8⁺ cell density shown in E, ii.

Supplementary Files

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