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ESPEC-SUIT: a versatile and robust platform to identify and track antigen-specific T cell receptors in patients with cancer

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ABSTRACT

Background Methods to identify and characterize antigen-reactive T cell receptors (TCRs) represent important tools to understand and exploit T cell responses in patients with cancer and beyond. Current methods are hampered by the rarity of individual T cell clones and limited applicability to monitor both major histocompatibility complex (MHC) class I-restricted and MHC class II-restricted responses, hence insufficiently reflecting the entire antigen-reactive repertoire of a patient. To obtain broad and deep insight into polyclonal, antigen-specific TCR repertoires, we developed the 'epitope-specific expansion culture with subsequent identification of TCRs' (ESPEC-SUIT) assay to identify and track antigen-specific TCRs.

Methods *In vitro* stimulation of peripheral blood mononuclear cells with (vaccine-targeted) neoantigens was verified in cytokine secretion assays and read-out by TCRβ repertoire sequencing (TCRseq). Candidate antigen-reactive clonotypes were defined by specific expansion in cultures stimulated with relevant antigen, followed by TCR cloning and validation in co-cultures of TCR transgenic effector cells and peptide-presenting targets. Using TCRseq information, candidate and validated clonotypes were traced and characterized in bulk and single-cell repertoire sequencing data of longitudinally collected blood samples and tumor tissue.

Results In a cohort of 32 patients with cancer, we demonstrate that ESPEC-SUIT supports strong, robust and reproducible expansion of $CD4^+$ and $CD8^+$ T cells in response to various antigens. TCRseq revealed highly polyclonal neoepitope-specific T cell responses, which can be further characterized with respect to cross-reactivity, affinity or human leukocyte antigen (HLA) restriction. In a subcohort of 10 patients, we selected 341 ESPEC-SUIT-derived TCRs for cloning and *in vitro* functional validation from >2000 candidates and confirmed antigen-reactivity for >75%. We exemplify the usefulness of this TCR discovery method for downstream analysis in neoepitope vaccinated patients with glioma, where we found longitudinal changes in candidate TCR frequencies in blood mirroring antigen-specific *ex vivo* Enzyme-Linked ImmunoSpot (ELISpot) responses. Furthermore, up to 67% of candidates could be detected in on-treatment brain tumor tissue and exhibited gene expression signatures overlapping with clonotypes of confirmed specificity to the vaccine antigen.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Understanding tumor-specific T cell responses is crucial for the conception of novel treatment options and for deciphering mechanisms of response and treatment resistance, in particular in patients receiving immunotherapy.

⇒ Many studies have interrogated the overall T cell response, disregarding antigen specificity (limited mechanistic insight) or used sophisticated technology to identify few antigen-specific receptors (limited breadth and sensitivity).

WHAT THIS STUDY ADDS

⇒ *Method*: by supplying an easy-to-use assay with high sensitivity, specificity and true-positive rate, we enable identification of a large number of antigen-reactive T cell receptors (TCRs) per antigen, which can be further characterized for therapeutic exploitation or *in vivo* behavior.

⇒ *Insight*: we show that T cell responses to tumor antigens can be highly polyclonal. For a recurrent brain tumor antigen, we demonstrate that vaccination with a long, neoepitope-encoding peptide induced peripheral expansion of tumor antigen-reactive T cells, many of which were also found infiltrating brain tumor tissue.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ As antigen specificity is encoded in the TCR sequence, epitope specific expansion culture with subsequent identification of TCRs (ESPEC-SUIT) enables interrogation of antigen-specific T cell responses in patient cohorts with cancer and other diseases.

⇒ Such next-generation immune monitoring allows T cell characterization and tracing at unprecedented depth, both over time in blood and spatially within tumor tissue.

⇒ Identification of (tumor) antigen-reactive TCRs using ESPEC-SUIT can improve our understanding of failed versus successful T cell-tumor interactions and provides a resource for selecting tumor-specific TCR candidates with therapeutic potential.

Conclusion ESPEC-SUIT provides unprecedented insight into highly polyclonal, antigen-specific T cell responses and enables discovery of large numbers of TCRs for a given antigen. It represents an efficient, cost-effective and scalable framework for the interrogation of tumor-reactive T cell responses in patients with cancer.

BACKGROUND

A large body of evidence shows that endogenous antitumor T cell responses occur in many patients with cancer.[1–4] While these might not always effectively control tumors as a result of tumor-mediated immune suppression, relevant immune responses can be unleashed by immune checkpoint blockade,[5] induced and/or boosted by vaccination,[6 7] or harnessed in passive immunotherapy approaches.[8–10] Central to an effective antitumor immune response are T cell receptors (TCR), whose specificities are encoded at the sequence level by V(D)J recombination. Based on their interaction with antigens presented on major histocompatibility complex class I (MHC I) or class II (MHC II) molecules, they can mediate effector responses of cytotoxic and helper T cells, both of which can exert antitumor activity.[11]

Methods to identify and characterize (tumor) antigen-reactive TCRs therefore represent important tools to understand and exploit relevant T cell responses. Recently, impressive efforts have been made to identify tumor-reactive TCRs within tumor-infiltrating lymphocytes (TILs) by probing the TCR repertoire[12 13] or interrogating transcriptional profiles,[3 4 14] although many of these methods are antigen-agnostic and thus cannot differentiate T cell populations with different specificities. Parallel efforts have focused on the identification of tumor-reactive TCRs from the peripheral blood repertoire based on phenotypic or transcriptional features.[15 16] Due to the low frequency of individual antigen-reactive clonotypes in the circulation, blood-based assays often have limited sensitivity, require enrichment of antigen-specific T cells and/or reagents such as MHC multimers, mainly applicable to MHC I-restricted antigens.[17 18] Approaches using antigen-specific *in vitro* expansion make relevant T cells more accessible.[2 19–21] Resulting data are encouraging, but caution against expanding unwanted responses due to repetitive stimulation.[20]

We have developed an assay to reliably expand and identify antigen-reactive TCRs from patients with cancer for further characterization, validation and mapping of tumor-specific T cells, and termed it '*epitope-specific expansion culture with subsequent identification of TCRs*' (ESPEC-SUIT). Our protocol efficiently identifies antigen-reactive, MHC I-restricted as well as MHC II-restricted TCRs that have been primed *in vivo*. ESPEC-SUIT provides both deep and broad insight into antigen-specific TCR repertoires without any specialized reagents, such as custom MHC multimers, and thus allows sensitive characterization of patient-individual responses based on standard blood sampling. Here, we demonstrate the performance of ESPEC-SUIT across dozens of patients, validating several hundred TCRs selected from >2000 identified candidates, and exemplify the usefulness of this approach in patients vaccinated with peptides encoding neoepitopes. We uncovered a surprising breadth of antigen-specific T cell responses to neoepitopes both in the native repertoire and in response to neoepitope-specific vaccines. ESPEC-SUIT provides access to a vast number of candidate TCRs for a given antigen, far exceeding other methods.[20 22–24] The resulting libraries of antigen-reactive receptors can be screened for desired features such as human leukocyte antigen (HLA) restriction, affinity and avidity, to select for the most suitable candidates for future clinical application.

ESPEC-SUIT thus represents an efficient, cost-effective and scalable framework for the interrogation of antigen-specific responses in patient cohorts with cancer and other diseases. It allows insight into spontaneous T cell responses and the effects of treatments inducing or expanding antigen-specific T cells, such as vaccines or immune checkpoint inhibitors.

METHODS

Sampling and patient consent

Blood and tissue samples were collected from 32 patients with various tumor types (see online supplemental table 1). Some experiments used blood donations (buffy coats) of healthy volunteers, obtained via the German Red Cross blood donation service.

Twenty-nine of the analyzed patients, all diagnosed with glioma (including astrocytomas, oligodendrogliomas, glioblastomas and diffuse midline gliomas), were undergoing peptide-based vaccination at the time of sampling. Vaccine targets included recurrent glioma-associated antigens (neo-1, n=21 patients; neo-2, n=6 patients) as well as patient-individual neoepitopes. Of three additional patients (diagnosed with liposarcoma, melanoma, colorectal cancer), two (i4, i6) had in the past received peptide-based vaccinations covering patient-individual mutations or fusions. With the exception of patient i3, who received a vaccine combining a

short (9-mer) and a long (13-mer) peptide spanning a patient individual fusion event, all administered vaccines consisted of long peptides (16–27-mers). Basic patient characteristics, including diagnosis, age, gender as well as—where applicable—details on vaccines received and time since last vaccination, are summarized in online supplemental table 1. Up to 150 mL of heparinized blood was obtained from patients by venipuncture. Blood was diluted in phosphate-buffered saline (PBS) and Biocoll Separation Solution (BS.L6115, Biochrom) and transferred to Leucosep tubes (227290, Greiner Bio-One) for density-gradient centrifugation according to the manufacturer's instructions. Recovered peripheral blood mononuclear cells (PBMCs) were washed and frozen in a two-component freezing medium: resuspension of cells in medium A (60% X-Vivo20 (04-448Q, Lonza), 40% fetal calf serum (FCS, S0615, Sigma-Aldrich)), followed by addition of an equal volume of medium B (80% FCS, 20% dimethyl sulfoxide (DMSO), D2650-100ML, Sigma-Aldrich) and immediate transfer to −80°C in a controlled rate freezing device. Cells were subsequently transferred to liquid nitrogen for storage until analysis.

Where available, tumor samples were snap-frozen and stored at −196°C until DNA/RNA extraction or transported on ice in X-Vivo15 (BE02-060Q, Lonza) and processed to single-cell suspensions as previously described[25] within 4 hours of resection.

Epitope-specific expansion cultures

PBMCs were thawed, washed and rested overnight in X-Vivo20 (04-448Q, Lonza) supplemented with 2% human AB serum (H4522-100ML, Sigma-Aldrich). On day 1, cell concentration was adjusted to 2×10^6 cells/mL and cells were plated at 500 µL per well into a 24-well plate. To serve as antigen-presenting cells (APC), at least one well per peptide was pulsed with 4 µg/mL peptide of interest and incubated at 37°C, 5% CO_2. Wells without peptide addition served as controls ('no peptide'). In parallel, an equal number of wells was similarly plated and incubated, then added to the APC plate after 4 hours of incubation, achieving a final concentration of 1×10^6 cells per mL and well. On days 4, 7, 9 and 11, half the medium per well was replaced with fresh cytokine-containing medium at final cytokine concentrations of 50 IU/mL hIL-2 (Proleukin, Ch-B. 5 02 519J, Novartis), 25 ng/mL hIL-7 (130-095-362, Miltenyi), 25 ng/mL hIL-15 (130-095-764, Miltenyi). If vigorous proliferation was observed, cells of the respective well were resuspended, split into two wells and each well replenished with 500 µL cytokine-containing medium (with final concentrations as above). On days 13–15, cells were harvested, counted and used for (i) TCR repertoire analysis (1×10^6 cells per culture pelleted and cryopreserved after optional enrichment of $CD4^+$ or $CD8^+$ T cells using magnetic bead-based positive selection (human CD4/CD8 microbeads, 130-45-101/201, Miltenyi)), (ii) scV(D)J sequencing (up to 1×10^6 cells cryopreserved in DMSO-containing freezing medium) or (iii) functional assessment of epitope-based expansion of relevant T cell populations after overnight resting in cytokine-free media. Expansion cultures were performed using a variety of antigens including shared neoepitopes (neo-1, neo-2), patient individual neoeptiopes as well as recall and control antigens (Epstein-Barr-virus ('EBV') $BMLF\text{-}1_{280\text{-}288}$ (GLCTLVAML), 'TpD' (ILMQYIKANSKFIGIPMGLPQ-SIALSSLMVAQ),[26] 'Influenza I' $M1_{58\text{-}66}$ (GILGFVFTL), 'Influenza II' $M1_{306\text{-}318}$ (PKYVKQNTLKLAT), Cytomegalovirus ('CMV') $pp65_{495\text{-}503}$ (NLVPMVATV), CEFX recall antigen pool (PM-CEFX-1, JPT), Myelin-Oligodendrocyre-Glycoprotein $(MOG)_{35\text{-}55}$ (MEVGWYRSPFSRVVHL YRNGK)). In some cases, peptides were combined into pools containing 2 µg/mL per peptide.

A detailed step-by-step description of the ESPEC method is available at protocols.io (dx.doi.org/10.17504/protocols.io.rm7vzkq42vx1/v1). See online supplemental table 2 for an overview of which samples were included in each assay.

Interferon γ Enzyme-Linked ImmunoSpot of *ex vivo* PBMC or ESPEC cultures

T cell responses before and after epitope-specific expansion were monitored using Enzyme-Linked ImmunoSpot (ELISpot) assays. White-bottom multiscreen high-throughput screening plates (MSIPS4W10, Merck Millipore) were hydrophilized with 35% EtOH, washed with sterile water and coated overnight at 4°C with anti-human interferon (IFN)γ (15 µg/mL 1-D1K, Mabtech, in PBS). Plates were then washed with PBS, blocked for 1 hour with X-Vivo20 (04-448Q, Lonza) containing 1% bovine serum albumin (BSA, A3294-100G, Sigma-Aldrich), followed by washing with X-Vivo20. For *ex vivo* assays, thawed PBMCs after overnight resting were seeded at $3\text{–}4\times10^5$ cells/well, while post-ESPEC 0.25×10^4 (vaccinated patients) to 1.25×10^5 (patients not currently undergoing vaccination) cells/well were plated. Assays were performed in 100–200 µL volume and included unstimulated and peptide-stimulated (10–20 µg/mL) conditions, as well as positive controls (10 µg/mL staphylococcal enterotoxin B (S4881-1mg, Sigma-Aldrich), 0.5 µg/mL CMV-pp65 (130-093-435, Miltenyi) plus 0.5 µg/mL AdV5 Peptivator (130-093-496, Miltenyi) or 0.02 µg/mL phorbol 12-myristate 13-acetate (PMA, P8139-1, Merck) plus 1 µg/mL ionomycin (19657, Merck). Cells expanded without peptide were assayed against all peptides assessed in the respective patient. After 44 hours, cells were removed by washing, and plates were developed according to the instructions of the IFNγ-ALP-Flex-Kit (3420-2A, Mabtech). Spot-forming units (SFU) were determined using an ImmunoSpot Analyzer (Cellular Technology Ltd.). A positive response was defined as >30 SFU above background (unstimulated). Frequencies of reactive cells were calculated as SFU×100/number of cells plated per well.

TCRβ deep-sequencing

For TCR deep-sequencing (TCRseq) of patient PBMC, tissue or post-ESPEC cells, genomic DNA was isolated using the DNeasy Blood and Tissue Kit (69504, Qiagen);

RNA was isolated using QIAGEN AllPrep DNA/RNA Mini-Kit (69504, Qiagen). RNA integrity was determined using Agilent 4150 TapeStation, with High-Sensitivity RNA Screen Tapes (5067-5579, Agilent).

In most cases, TCRβ-chain (TRB) deep-sequencing was performed using hsTCRBv4b Kit (Adaptive Biotechnologies) according to the manufacturer's protocol. Post-ESPEC and tumor samples were sequenced at survey-level using an input of 252 ng and 1.6–3.5 µg genomic DNA/well, respectively; PBMC samples were sequenced at deep or survey level, using 1.3 µg/well genomic DNA as input. Libraries were sequenced on Illumina MiSeq V.3 or NextSeq550 V.2.5 mid-output (both 150 cycles) by the DKFZ Genomics & Proteomics Core Facility. Data processing (demultiplexing, trimming, gene mapping) was done using Adaptive Biotechnologies proprietary platform. On average, ~73 000 TCR templates per sample and more than seven reads/TCR were observed.

For some samples, RNA-based TRB sequencing was performed using DriverMap-AIR TCR-BCR Profiling-Kit (DMAIR-HTBR-24, Celllecta) according to the manufacturers' protocol, using 100 ng (post-ESPEC) or 600–1000 ng (tumor) RNA as input. RNA-based TRB libraries were sequenced on Illumina NextSeq550 or NovaSeq6k (both 300 cycles) by the DKFZ Genomics & Proteomics Core Facility. Alignment of raw sequencing output was performed using MIXCR[27] with *cellecta-human-rna-xcr-umi-drivermap-air* preset. On average, ~3 million TCR templates per sample and 46 reads/TCR were observed.

Data analysis and visualization was performed using Immunoseq Analyzer (Adaptive Biotechnologies), MIXCR (V.4.7.0),[27] VDJtools (V.1.2.1)[28] and R-based tools (immunarch V.0.6.6[29]; ggplot2, VennDiagram). Diversity is the total number of unique clonotypes in a sample; clonality is defined as '1–Shannon's entropy/$\log_2$(diversity)',[30] also known as Pilou evenness. Tracing of clonotypes was based on CDR3 amino acid sequences. To allow comparison between patients and experiments, VDJtools was used to down-sample clonotype counts to 10 000, a number detected in the majority of post-ESPEC samples. Due to the low T cell infiltration of tumor samples, data were down-sampled to the size of the tumor repertoire for comparisons between TIL and post-ESPEC samples. Clonotype frequencies are reported as the relative frequencies within a given sample.

Single cell sequencing

Single cell suspensions of freshly resected brain tumor tissue were enriched for tumor-infiltrating T cells using FACS as described previously.[25] Enriched T cells were resuspended in 0.04% BSA in PBS, counted and 12 000 cells were loaded for combined single-cell RNA/V(D)J sequencing. Cryopreserved post-ESPEC samples were thawed in the presence of 50 IU/mL benzonase (YCP1200-500KU, speed BioSystems), washed once in PBS and up to 1×10^6 cells were stained for 20 min on ice with hashing antibodies (clones LNH-94/2M2, TotalSeq-C0251-C0258, Biolegend) diluted 1:2500 in 100 µL FACS buffer. Cells were washed twice (centrifugation at 430× g) in 2.5 mL FACS buffer, taken up in PBS and counted. Three to four hashed samples were pooled at equal ratio and up to 40 000 cells loaded for one sequencing reaction. Gene expression libraries, TCR-V(D)J libraries and feature barcode libraries were prepared according to the manufacturer's protocols (10x Genomics 5′ RNA and V(D)J kits, versions 1.0–1.1 10x Genomics, 5′ RNA and V(D)J kits, V.1.0–1.1), sequenced on the NovaSeq6000 platform (Illumina) and aligned using Cellranger V.6.1.2. Single-cell RNA sequencing gene count matrices were imported into R (V.4.3.3) and analyzed using Seurat (V.5.1.0). Cells with >15% mitochondrial gene expression and/or non-T cell ($CD3E^-$) were excluded from the analysis. Raw count matrices were log normalized and integration of datasets was performed using harmony (V.1.2.1). The top 30 principal components were used to perform uniform manifold approximation and projection dimensionality reduction.

TCR selection and cloning

Candidate antigen-reactive TCRs were selected for testing based on their enrichment in post-ESPEC expansion cultures as compared with control cultures (no peptide). A cut-off of $>10^{-3}$ in the antigen-stimulated culture and $<4\times10^{-4}$ in the control culture was applied to repertoire data down-sampled to 10 000 TCR counts. TCRselect, an R-based online tool for selection of relevant clonotypes, can be reached at https://tcrselect.dkfz.de.

TCRαβ pairs of candidate clonotypes were extracted from single-cell V(D)J-sequencing results of post-ESPEC T cells or TIL using the makeTCR online tool (https://edgreen21.shinyapps.io/makeTCR/).[31] Details regarding the cloning procedure and constructs are described by Hamberger *et al*[31] and at Addgene: makeTCR collection. If multiple plausible TCRα chains were detected for a candidate TCRβ sequence, both pairs were cloned and tested. VDJ regions of TCRs were ordered as synthetic DNA fragments (Twist Biosciences) and cloned (NEBridge Golden Gate Assembly Kit, BsmBI-v2 E1602S) in 96-well format as chimeric TCRs, using murine TRA or TRB constant region sequences.[32] Murine TRA/TRB sequences were modified to include an additional disulfide bond to improve stability and avoid mismatches with the endogenous human TCR after transduction into human T cells.[33 34] To enable *in vitro* transcription, TCR constructs were PCR-amplified using primers (TAATACGACTCACTATAGGGACAGAACACAGGCCACCATG and TCAGCTGGACCACAGTCTCAGG) to add a T7 promoter, and the resulting PCR product was used as a template for the T7-mScript Standard mRNA-Production-System (C-MSC100625, CELLSCRIPT). mRNA was m^7G capped and enzymatically polyadenylated following the manufacturer's instructions.

TCR expression and *in vitro* functional validation

For recombinant TCR expression, expanded healthy donor T cells (see online supplemental methods) were thawed and rested overnight in X-Vivo15 with 2% human AB serum. On the next day, cells were washed once with PBS, and 2×10^6 T cells in 20 µL P3 solution (Lonza, supplemented according to the manufacturer's recommendations) were electroporated with 750 ng TCR-encoding RNA using the Lonza 4D-Nucleofector (program EO-115). After electroporation, cells were plated into 48-well plates containing TexMACS media (130-097-196, Miltenyi) with 2% human AB serum (H4522-100ML, Sigma-Aldrich). 18–24 hours after electroporation, cells were collected. 50 IU/mL benzonase (YCP1200-50KU, Speed BioSystems) was added to avoid cell clumping. Recombinant TCR expression levels were measured by flow cytometry (see online supplemental methods and online supplemental table 3, 'TCR expression') and T cells tested for specificity: 150 000 mock or TCR-electroporated T cells were co-cultured with peptide-pulsed (10^{-5} M) APC in 96-well U-bottom plates (total volume: 200 µL). Target cells were patient-autologous B-LCLs (50 000/well) or dendritic cells (15 000/well, prepared from magnetically enriched $CD14^+$ monocytes (130-050-201, Miltenyi) differentiated for 5 days in X-Vivo20 containing 500 IU/mL interleukin-4 (130-093-921, Miltenyi) and 560 IU/mL granulocyte macrophage colony-stimulating factor (300-03-100, PeproTech)). Wells with T cells and unpulsed or control-peptide pulsed targets, or T cells and TransAct beads (130-111-160, Miltenyi) served as negative and positive controls, respectively. 5 µL anti-CD107a antibody (H4A3, 561343, BD Biosciences) was added directly into each co-culture well. After 1 hour of co-culture, GolgiPlug and GolgiStop (555029 and 554724, BD Biosciences, both at 1:1000 dilution) were added, and after four additional hours, cells were harvested and stained as described in online supplemental methods and online supplemental table 3 ('TCR testing').

TCRs were classified as reactive (R) if (*signal–background)>(background+2x SD of background*) was fulfilled for both CD107a and tumor necrosis factor (TNF)α. Percentages of $CD107a^+$ and $TNF\alpha^+$ cells were quantified in viable/singlet/$CD3^+$/$mTCR\beta^+$/$CD4^+$ or /$CD8^+$ cells, depending on the cell-of-origin of the TCR. Background was defined as the mean of signals measured in all control-stimulated co-culture wells for a particular patient and TCR-testing experiment. If two TCRα (TRA) chains were tested for a clonotype, data for the functional α/β pair are presented. Only TCRs with >2% mTCRβ expression were included in the analysis.

Statistical analysis

Data are represented as individual values or as mean±SD. Statistical tests are indicated in the figure legends. Statistics were calculated using GraphPad Prism V.10 or R package 'stats' V.4.3.2 for linear regression. Differential abundance and TCR repertoire overlap/Morisita-Horn index were determined using Adaptive Biotechnologies' immunoSEQ Analyzer, based on de Witt *et al*.[35] To compare TCR repertoire datasets, down-sampling was applied using VDJtools,[28] as indicated in text and figure legends.

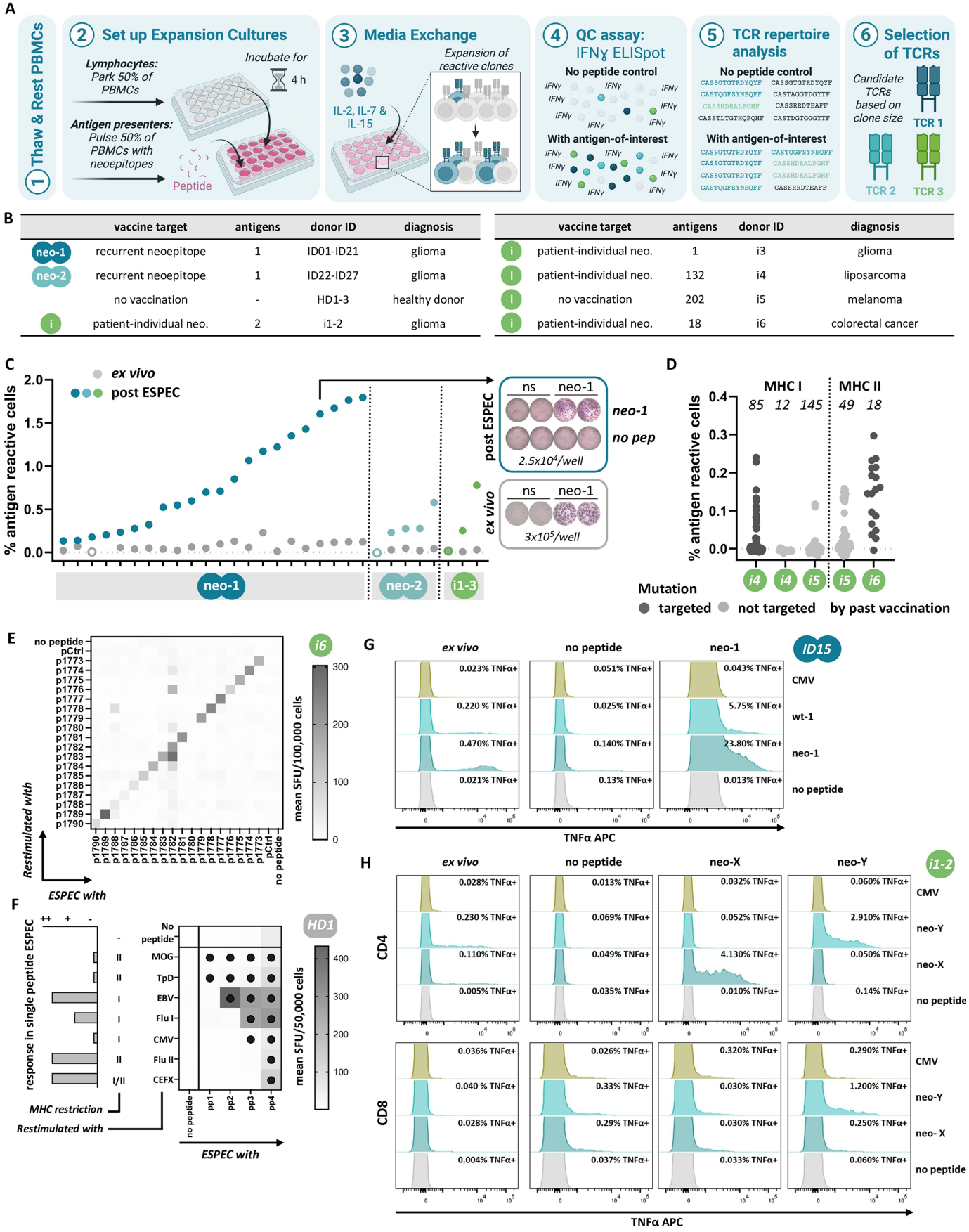
RESULTS

TCR repertoires are tremendously diverse, with at least 10^8 unique TRA-TRB combinations found in human peripheral blood.[36–38] In consequence, each clonotype can only occupy limited repertoire space. While polyclonal antigen-specific memory T cell responses can often be measured *ex vivo* with sensitive assays, the isolation of individual antigen-reactive TCRs remains challenging. To capture the diverse antigen-reactive TCR repertoire for a given antigen, we developed a protocol for epitope-specific expansion cultures (ESPEC) (figure 1A) involving re-stimulation of PBMC with the antigen-of-interest, supplemented (from day 4 on) with low cytokine concentrations. This method favors expansion of *in vivo* primed T cell populations, which can be confirmed by post-ESPEC functional readouts, and their receptors identified by TCR repertoire sequencing.

ESPEC robustly amplifies MHC I-restricted and MHC II-restricted neoepitope-specific T cell responses

We employed ESPEC in 32 patients with various tumor types (figure 1B). Twenty-nine had gliomas and at the time of blood sampling were undergoing long-peptide vaccination targeting either shared (neo1-2, n=27) or patient-individual (i1-3, n=2) neoepitopes (see online supplemental table 1 for patient details). With two exceptions, all patient T cell responses to the vaccine antigen were increased post-ESPEC compared with responses measured by *ex vivo* ELISpot (figure 1C, p<0.0001, Wilcoxon matched-pairs signed rank for pre-ESPEC/post-ESPEC comparisons). For one patient, no *ex vivo* ELISpot was available, but a significant post-ESPEC ELISpot response was observed. The mean increase in response was 15.5-fold (range 1.7-fold to 93-fold), yielding a mean response of 0.75±0.6%, compared with 0.06±0.05% *ex vivo*. Detection of antigen-specific responses after ESPEC is therefore substantially more robust than *ex vivo* ELISpot, where responses were often close to the limit of detection (0.005% for the reported ELISpot setup).

To validate ESPEC for the identification of patient-individual T cell responses, we included three additional patients with different tumor entities: liposarcoma (patient i4), melanoma (patient i5) and colorectal cancer (patient i6). Patients i4 and i6 had received long-peptide vaccination (16–20-mers) against individual neoepitopes in the past, between 3 and 7 months prior to blood sampling (online supplemental table 1). ESPECs were performed using peptides covering a range of mutations, including, but not limited to, those targeted by vaccination, to probe for endogenous versus vaccine-induced immune responses. Having established that ESPEC worked for individual peptides, we assessed



	vaccine target	antigens	donor ID	diagnosis
neo-1	recurrent neoepitope	1	ID01-ID21	glioma
neo-2	recurrent neoepitope	1	ID22-ID27	glioma
	no vaccination	-	HD1-3	healthy donor
i	patient-individual neo.	2	i1-2	glioma

	vaccine target	antigens	donor ID	diagnosis
i	patient-individual neo.	1	i3	glioma
i	patient-individual neo.	132	i4	liposarcoma
i	no vaccination	202	i5	melanoma
i	patient-individual neo.	18	i6	colorectal cancer

Figure 1 Epitope-specific expansion cultures (ESPEC) efficiently and specifically expand desired major histocompatibility complex (MHC) I-restricted and MHC II-restricted T cell responses. (A) Overview of workflow for ESPEC; (B) overview of analyzed patients and antigens used for ESPEC-SUIT; (C) frequency of antigen-specific T cell responses as determined by

interferon (IFN)γ Enzyme-Linked ImmunoSpot (ELISpot) in peripheral blood mononuclear cell (PBMC) (*ex vivo*, gray, 300 000 cells/well) or post-ESPEC samples (colored, 25 000–50 000 cells/well) for patients with glioma undergoing vaccination with shared (neo-1, n=22; neo-2, n=5) or individual MHC II-restricted neoepitopes (i1-3, n=2 patients, n=3 antigens); open symbols (gray/colored) indicate lack of antigen-specific T cell response (*ex vivo* or post-ESPEC); raw data inlays show well images of a representative neo-1 vaccinated patient post-ESPEC for no peptide ('no pep') and neo-1-stimulated cultures (top) versus *ex vivo* (bottom), re-stimulated without antigen (ns) or with neo-1 peptide; (D) frequency of antigen-specific T cell responses determined by IFNγ ELISpot in post-ESPEC samples (100 000–250 000 cells/well) for patients with liposarcoma (i4), melanoma (i5) or colorectal cancer (i6) not undergoing vaccination at time of blood sampling. i4 and i6 were in the past vaccinated with long peptides encompassing mutations also contained in short (9–11-mers, MHC I-presented) or long (>20 mer, MHC II-presented) peptides used in ESPEC ('targeted', dark gray). Long peptides used for stimulation contained amino acid sequences spanning point mutations or resulting from fusions/missense mutations; short peptides were mutation-derived epitopes predicted to bind the patient's MHC I molecules; ESPEC also included antigens not targeted by vaccination ('non-targeted', light gray); numbers above datasets indicate no. of antigens investigated; heatmap of IFNγ ELISpot spot-forming units (SFU) in (E) cultures stimulated with patient-individual (i6) 20-mer peptides (x-axis) after re-stimulation (y-axis) without antigen ('no peptide'), with the antigen-of-interest (diagonal) or control antigen (MOG), or (F) healthy donor (HD1)-derived cultures (x-axis) after ESPEC with four different peptide pools (pp1–pp4) of short (MHC I-restricted, '(I)') or long (MHC II-restricted, '(II)') recall antigens. Post-ESPEC, cells were re-stimulated (y-axis) with single antigens present (circles in heatmap) or absent from the pool; schematic graph to left of heatmap shows strength of the response to the antigens when used as single peptides in ESPEC; intracellular cytokine staining of tumor necrosis factor (TNF)α production in living/$CD3^+$/$CD4^+$ T cells after re-stimulation of 1×10^6 (*ex vivo*) or 0.4×10^6 (post-ESPEC) cells/condition in representative patients undergoing vaccination with (G) neo-1 or (H) two patient-individual neoepitopes (neo-X, neo-Y). Panels show the response pre-ESPEC ('*ex vivo*'), and post-ESPEC in control stimulated cultures ('no peptide') or cultures stimulated with antigen-of-interest ('neo-1/X/Y', as indicated above the panel), gated on $CD4^+$ T cells (ID15 and i1-2 top panel) vs $CD8^+$ T cells (i1-2, bottom panel). Histograms show responses to re-stimulation with various peptides: CMV (cytomegalovirus) peptide is included as a control for the unspecific expansion of endogenous T cell responses, wt-1 is included to assess cross-reactivity of neo-1-stimulated cells and neo-X/Y are specificity controls for recently stimulated T cells. APC, antigen-presenting cell; TCR, T cell receptor; neo, neoepitope.

whether multiplexed stimulation using peptide pools (pp) would also drive expansion and yield distinct responses to individual peptides contained within a pp. This is particularly relevant for high mutational load tumors with many potential antigens, such as melanoma patient i5, where from a total of 546 mutations, 194 neoepitope spanning peptides (and eight recall antigens) were selected for testing. Cultures of patient i4 and i5 were therefore performed using pp, each containing 2–14 short or long peptides, while for patient i6, 18 cultures stimulated with individual long peptides were set up. Of the 331 total antigens evaluated in these three patients (309 neoantigens and 22 recall antigen-derived peptides), 57 (19.4%) neoepitopes showed a positive post-ESPEC response in ELISpot assays, with a mean frequency of 0.095% (figure 1D). 49% of positive responses were reactive to antigens targeted by vaccination, but of 208 antigens not targeted by vaccination, 29 (14.1%) also yielded positive results of similar magnitude (mean frequency 0.072%). Importantly, antigens evaluated in i4-6 included epitopes with predicted binding to MHC I (9–11-mers), as well as mutation-spanning long peptides (≥20 amino acids) to evaluate MHC II-restricted responses. In unvaccinated patient i5, where both long and short antigens were evaluated, 25/49 (51%) long peptides induced a measurable T cell response (mean frequency 0.073%), while only 4/145 (2.8%) mutation-encoding short peptides yielded positive results (mean 0.067%). Three of the immunogenic short peptides were from mutations to which a parallel $CD4^+$ T cell response to the corresponding long mutation-spanning peptide was observed. In patient i4, 17/97 (17.5%) of predicted MHC I binding neoepitopes induced a T cell response and all underlying mutations had previously been targeted by long peptide vaccination.

The increase in T cell response is specific to the antigen used for expansion, as shown for the various neoepitopes evaluated in i6, which induced IFNγ production only in the culture stimulated with the relevant antigen, but not in all others, with the exception of a single culture that displayed broad reactivity to multiple peptides (figure 1E). This held true for peptide pools, where responses were observed only to antigens contained within the pool, as shown here in a healthy donor for a panel of MHC I-restricted and MHC II-restricted recall antigens (figure 1F).

Overall, we show that ESPEC amplifies both endogenous and vaccine-enhanced T cell responses to levels detectable by classical ELISpot assays with high sensitivity and specificity, for both $CD4^+$-mediated and $CD8^+$-mediated immunity. The functional assessment of post-ESPEC responses is particularly useful when working with predicted epitopes, which will normally not all be immunogenic (exemplified in i4-5), as it helps to narrow down the number of antigens for which to perform downstream investigations.

While we used ELISpot to quantify T cell reactivity, ESPEC can also be interpreted using flow cytometry, detecting cytokines such as TNFα and IFNγ (online supplemental figure 1A-B), exemplified here for ESPECs using a shared (figure 1G) or two distinct patient-individual neoepitopes (figure 1H). As an added benefit, flow cytometry-based methods give access to additional information, such as the cytokine-producing T cell subset, which is useful if the MHC restriction of a T cell response

is unclear. Of note, in the patient with glioma vaccinated with two patient-individual neoepitope-encoding long peptides, we observed a $CD8^+$ T cell response against one of the epitopes (figure 1H, bottom), in addition to the expected CD4 response (figure 1H, top).

ESPEC-SUIT results in antigen-driven clonal expansion of T cells

In ESPEC-SUIT, subsequent identification of antigen-reactive TCRs (SUIT) is based on repertoire sequencing. Depending on the antigen restriction, TCRβ deep-sequencing was preceded by $CD4^+$ or $CD8^+$ T cell enrichment to achieve improved cost-effectiveness and signal-to-noise ratios.

Each assay includes a culture exposed to cytokines but not antigen ('ctrl') to account for unspecific T cell expansion. Compared with 'ctrl', cultures exposed to antigen ('stim') show similar repertoire diversity (figure 2A, online supplemental figure 2A,B), and clonal expansion as well as contraction take place (online supplemental figure 2C,D). A significant increase in clonality of antigen-exposed cultures compared with 'ctrl' indicates antigen-driven expansion (figure 2B, online supplemental figure 2E,F) and highly expanded clonotypes account for a greater percentage of the overall repertoire in antigen-stimulated versus 'ctrl' post-ESPEC repertoires (figure 2C, online supplemental figure 2G). Importantly, this clonal expansion correlates with post-ESPEC ELISpot responses (figure 2D), with 4.8 times greater mean clonality of neo-1-stimulated cultures versus 'ctrl' in cultures with >10-fold increased post-ESPEC ELISpot response vs 1.6-fold difference for cultures with <10-fold increase.

Post-ESPEC repertoire sequencing revealed epitope-specific differences in cross-reactivity profiles: while neo-1 and its corresponding wild-type (wt) counterpart, at the supraphysiological peptide concentrations used *in vitro*, generated post-ESPEC repertoires with extensive clonotype overlap (online supplemental figure 3H,I), this was not observed for neo-2 (online supplemental figure 3J,K). Post-ESPEC repertoire analysis can thus be used to assess and avoid cross-reactivity, enabling selection of highly specific TCRs for safer transgenic therapies.

By studying the fold-expansion of the 10 largest clonotypes detected in antigen-stimulated post-ESPEC samples compared with their pre-expansion size, we found that general expansion capacity seemed to depend mostly on the individual, rather than on the T cell subset stimulated or the antigen, as shown for representative examples in figure 2E and all neoepitope-stimulated cultures in figure 2F. In this comparison, we did not detect differences in expansion against antigens applied as vaccines in the recent past (i3, 25 days ago) or the distant past (i4 and i6, 98 and 226 days ago, respectively), nor against viral recall antigens (figure 2E). To account for differences in sequencing depth, the data in figure 2F were down-sampled to 10 000 TCR counts, allowing for quantitative comparison. Here, we did not observe a correlation between maximal clonal expansion and size of the post-ESPEC ELISpot response (figure 2F). Of note, quantification of clonotype expansion is hampered by the fact that many expanded clones are below the limit of detection in the baseline sample (pre-ESPEC), which is further aggravated by down-sampling. On the other hand, this emphasizes the ability of ESPEC to expand clones that are elusive *ex vivo* due to their small size, but carry significant proliferative potential and, as will be shown later, antigen reactivity. Notably, the 10 largest post-ESPEC clones represent several per cent of antigen-stimulated cultures as shown for representative responses to MHC I-restricted and MHC II-restricted epitopes in figure 2G–H. Across all cultures, the sum of the 10 largest post-ESPEC clones averaged 24.4% (range 5%–68%, mean for CD4 enriched: 23.8%, mean for CD8 enriched: 16.9%).

ESPEC-SUIT enriches for functional, antigen-reactive TCR clonotypes

To further understand expansion behavior of relevant clonotypes in ESPEC-SUIT, we mapped functional parameters to TCRseq data for selected patients: here, we re-stimulated post-ESPEC samples with antigens-of-interest and enriched cells producing TNFα (figure 3A) or expressing the activation marker 4-1BB (figure 3B). TCR repertoires of enriched populations overlapped almost entirely with strongly expanded TCR clonotypes in unselected cultures stimulated with antigen-of-interest. Notable exceptions were clonotypes expanded in both antigen-stimulated and control-stimulated cultures, embodying clonotypes with high antigen-independent proliferative capacity (shaded areas in figure 3A–B). Each culture featured such a subset of clonotypes undergoing significant antigen-independent expansion, thus reaching large frequencies across multiple cultures stimulated with different antigens and/or in the control culture expanded without antigen.

After ESPEC-SUIT, we pursued validation of TCR reactivity for a subset of patients exhibiting strong post-ESPEC T cell responses to neo-1/2 (n=9) or patient-individual mutations (n=1) (figure 3C). Based on TCRβ repertoire results, we selected >380 TCRs, focusing on clonotypes strongly and exclusively expanded in response to antigen stimulation. We determined TRA for each candidate TCR by performing single-cell V(D)J sequencing of post-ESPEC antigen-stimulated cultures and cloned relevant TRA-TRB pairs. Recombinant TCRs were introduced by RNA-based electroporation into primary human T cells and co-cultured with antigen-pulsed target cells. Median TCR expression level on the day of co-culture was 64.8% (see online supplemental figure 3A). 246/389 (63.2%) of tested TCRs were validated to recognize the antigen used as stimulant in ESPEC (figure 3D; representative raw data of TCR expression and cytokine-based reactivity assays in online supplemental figure 3B,C). The validation rate, ranging from 29.7% to 100%, is biased by the fact that for some cultures, only very few highly expanded clones were tested, while for others, broad testing was performed to show distribution of reactive/non-reactive clonotypes across the post-ESPEC repertoire (figure 3E, online

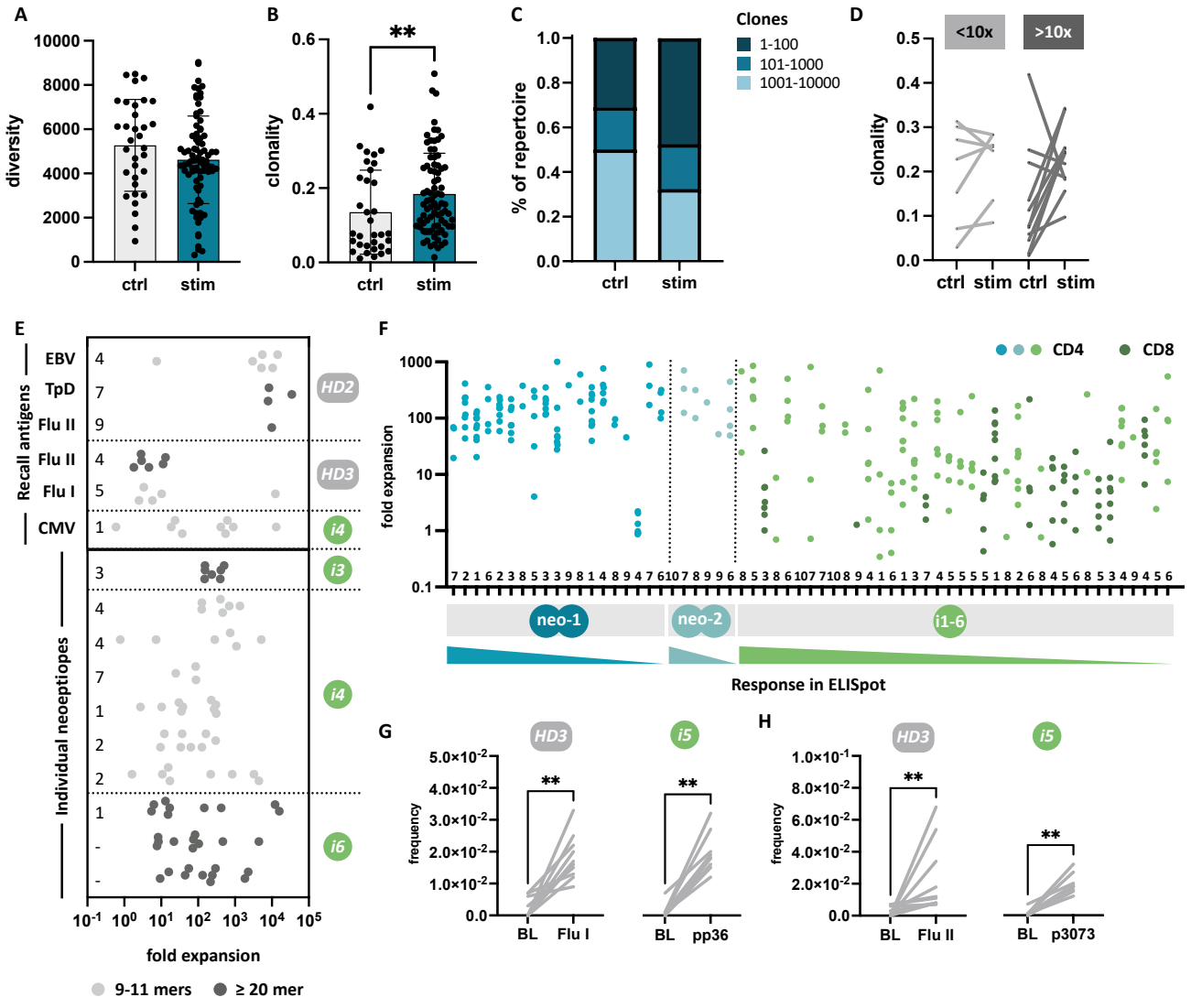


Figure 2 Epitope-specific expansion cultures (ESPEC)-induced changes in T cell receptor (TCR) repertoire composition. TCR repertoire (A) diversity (mean±SD) and (B) clonality (mean±SD) of post-ESPEC samples (n=80 from 32 individuals) expanded without peptide ('ctrl') or under antigen-of-interest ('stim'); note that some donors were stimulated with multiple different peptides or peptide pools in separate cultures, thus contributing multiple post-ESPEC samples and a single 'ctrl'. # **p≤0.01, Mann-Whitney U test; two-tailed; (C) mean percentage of repertoire occupied by clones with rank 1–100, 101–1000 or 1001–10 000 in post-ESPEC samples expanded without peptide ('ctrl', n=32) or with antigen ('stim', n=80, as some donors were stimulated with multiple antigens)#; (D) clonality in antigen-of-interest ('stim') or control stimulated cultures ('ctrl'), where ESPEC induced an increase in the ELISpot response to neo-1 stimulation of <10-fold (n=7) or >10-fold (n=11)#; (E) fold expansion of the 10 largest clonotypes post-ESPEC compared with their pre-ESPEC frequency in two healthy donors (HD2, HD3) and patient i4 stimulated with MHC I-restricted (light gray) or MHC II-restricted (dark gray) recall antigens (top panel), and three patients (i3–i4, i6) stimulated with short (light gray) or long (dark gray) peptides encoding patient-individual neoepitopes, including a patient-individual gene fusion targeted by peptide vaccination (i3). Full repertoire data were evaluated to avoid loss of small clones by down-sampling. Numbers next to the y-axis indicate number of top 10 post-ESPEC clonotypes that were below the limit of detection pre-ESPEC; (F) fold expansion of the 10 largest clonotypes post-ESPEC compared with their pre-ESPEC frequency in all neoepitope ESPEC cultures (n=63), sorted by antigen type and magnitude of the post-ESPEC ELISpot response#. Symbol hues indicate cultures enriched for $CD4^+$ (teal, mint, light green) or $CD8^+$ (dark green) T cells prior to repertoire sequencing. Numbers above the x-axis indicate the number of top 10 post-ESPEC clonotypes that were below the limit of detection pre-ESPEC; frequency of the 10 largest clonotypes post-ESPEC compared with their pre-ESPEC (baseline ('BL')) frequency for representative cultures exposed to (G) short and (H) long peptides and enriched for the relevant T cell subset#. **P≤0.01, Wilcoxon signed-rank test, two-tailed). #TCRβ deep-sequencing data were down-sampled to 10 000 TCR counts (panel A–D, F–H).

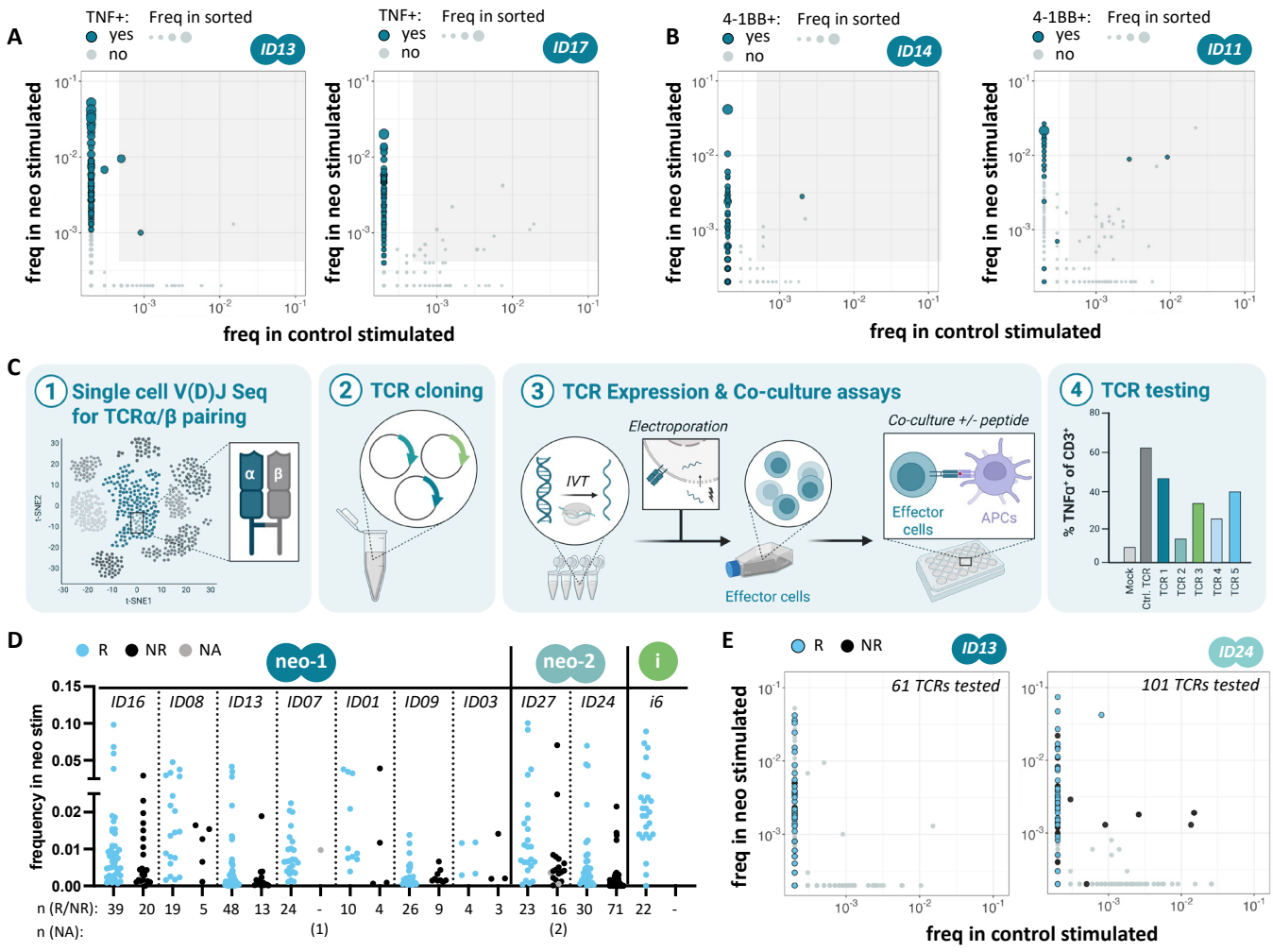


Figure 3 Functional validation of clonotypes expanded in epitope-specific expansion cultures (ESPEC). (A–B) Relative frequency of T cell receptor (TCR) clonotypes in neo-1-stimulated ESPEC (y-axis) versus control-stimulated cultures (no peptide, x-axis), highlighting clonotypes enriched for (A) secreting tumor necrosis factor (TNF)α (n=2 neo-1 vaccinated patients) or (B) expressing 4-1BB (n=2 neo-1 vaccinated patients) after *in vitro* re-stimulation with neo-1; bubble size indicates post-enrichment frequency of the highlighted clonotypes; in the enriched TCRβ-chain (TRB) dataset, a>0.002 cut-off was used to determine clones detected above background; areas of unspecific T cell expansion are shaded gray; TRB deep-sequencing data were down-sampled to 10 000 TCR counts; (C) workflow for validation of tumor-reactive TCRs; (D) relative frequency (y-axis) and number (below x-axis) of TCR clonotypes in antigen-stimulated ESPEC culture of 10 patients vaccinated with neo-1 (n=7), neo-2 (n=2) or patient-individual neoepitopes (i6) that were determined to be reactive ('R', blue) or non-reactive ('NR', black) to the antigen-of-interest based on *in vitro* functional validation; n=3 receptors could not be tested due to lack of expression ('NA', gray); for i6, 25 TCRs derived from 12 cultures stimulated with different patient-individual neoantigens were tested, value given for each clone refers to relative frequency of the tested clonotype in the relevant antigen-stimulated culture; (E) relative frequency of TCR clonotypes in antigen-stimulated ESPEC (y-axis) versus control-stimulated cultures (no peptide, x-axis) in n=2 representative patients vaccinated with neo-1 (left) or neo-2 (right), highlighting the distribution of clonotypes found to be reactive ('R', blue) or non-reactive ('NR', black) to the antigen-of-interest based on *in vitro* functional validation.

supplemental figure 3D). Of note, 219/357 (61.3%) clones from cultures where at least 10 candidate TCRs were tested (range 14–101) were confirmed to recognize the antigen used for expansion. There was a trend towards more reactive TCRs among the largest post-ESPEC clonotypes (figure 3D–E and online supplemental figure 3D), but highly expanded clonotypes were not enriched for TCRs with strong reactivity in *in vitro* assays (not shown). More importantly, data on post-ESPEC T cell functionality (figure 3A–B) and reactivity of ESPEC-SUIT-derived TCRs (figure 3E) underline the importance of excluding unspecifically expanded clonotypes.

ESPEC-SUIT detects large numbers of candidate antigen-reactive clonotypes

Large-scale functional TCR validation will not always be feasible and a statistical approach to TCR selection is hampered by the fact that many ESPEC-expanded TCRs are not found in pre-expansion samples. Instead, we established a set of heuristic rules to identify TCR clonotypes

of interest (also referred to as 'candidates'), which must be (i) frequent in the post-ESPEC culture stimulated with relevant antigen, (ii) rare in the post-ESPEC culture expanded without peptide and—optionally—(iii) larger in the culture stimulated with antigen-of-interest than with its wt counterpart. We provide TCRselect (https://tcrselect.dkfz.de), a tool to explore repertoire data and select cut-offs for candidate selection. TCRselect allows visualization of candidates and export of clonotype lists. In addition, it can be used to highlight clonotypes based on additional features such as reactivity.

The optimal cut-off values for a given experiment will be influenced by factors such as the number of cells analyzed and the sequencing depth. To allow for robust comparison across our large dataset (n=63 cultures), we applied a cut-off of $>10^{-3}$ in the antigen-stimulated culture and $<4\times10^{-4}$ in the control culture to post-ESPEC TCR repertoire data (down-sampled to 10 000 TCR counts to facilitate comparison between high-input and low-input samples). With very few exceptions, these heuristics yielded a large number of high-confidence candidate antigen-specific clonotypes (representative examples in figure 4A–B). The mean number of candidates across all patients was 73 (range 10–240) (figure 4C). Of 2200 candidates identified in the 10-patient cohort where we also performed TCR testing, 341 TCRs had undergone functional validation and 255 (75.6%, range 53.3%–100%) were reactive to their antigen-of-interest (figure 4D).

ESPEC-SUIT reproducibly expands candidate clonotypes

To assess assay reliability, we performed ESPEC-SUIT from two aliquots of the same blood sample, which were thawed and underwent peptide-driven expansion at independent time points. Across four paired patient samples, we observed good assay reproducibility, with candidate TCR overlap ranging from 31% to 71% overall (online supplemental figure 4A-D), and almost complete among the most expanded clonotypes (online supplemental figure 4E-H). Technical variation from TCRseq had little impact on reproducibility, as technical replicates generated highly similar results (online supplemental figure 4I). Of note, clones of small pre-expansion size were not under-represented among candidates shared between assays, suggesting that sampling is not a driver of variability (online supplemental figure 4E-H).

ESPEC-SUIT expanded clonotypes are antigen-specific and private

Candidate antigen-reactive TCRs from one culture were usually private, that is, not expanded in other cultures of the same patient (figure 4E–F), with occasional exceptions of TCRs expanded in response to the wt and mutant forms of a neoepitope, as previously discussed using the example of neo-1 and annotated in figure 4A. To further verify antigen-specific versus bystander expansion, we searched for known pathogen-specific clonotypes in neoepitope-stimulated cultures (figure 4A, blue dots and online supplemental table 4) and consistently found them outside the repertoire space which contained candidate antigen-reactive TCRs and TCRs with validated reactivity (figure 3E). Conversely, viral antigen stimulation produced candidates unique to these cultures and not expanded in neoepitope-stimulated cultures of the same individual (figure 4F), confirming ESPEC does not induce generalized expansion of bystander TCR clonotypes.

ESPEC-SUIT-derived candidate antigen-reactive clonotypes can be traced longitudinally and into tissue

Downstream of ESPEC-SUIT, the connection between TCR specificity and sequence gives insights into the *in vivo* behavior of antigen-reactive T cells. For example, tracing of candidate and validated clonotypes in peripheral blood revealed that neo-1-specific clonotypes were minute or undetectable prior to neo-1 vaccination, but achieved sizeable proportions after repeated subcutaneous administrations of neo-1 peptide combined with adjuvant montanide and local imiquimod (figure 5A–B, bars, left y-axis). Clonal expansion detected on the TCR repertoire level mirrored neo-1-specific T cell function as measured by *ex vivo* IFNγ ELISpot (figure 5A–B, dotted line, right y-axis). Where relevant tumor material was available, we could trace a subset of neoantigen-expanded clonotypes within TIL. For seven tumors available for analysis, we found 20–155 candidate clonotypes within the TIL, representing 19.5%–66.8% of all ESPEC-derived candidates (figure 5C). Overall, the candidates comprised 0.6%–9.2% of intratumorally detected TCRs (figure 5D) and patients with higher intratumoral frequency of candidate antigen-reactive TCRs exhibited higher frequencies of antigen-reactive cells in the peripheral circulation (online supplemental figure 5A,B).

In two patients with glioma where we were able to perform neo-1-specific ELISpot assays in minimally cultured TIL, we detected prominent neo-1-specific T cell responses (figure 5E–F). The frequencies of TIL producing IFNγ after neo-1 restimulation have similar magnitude as the sum of candidate neo-1 reactive clonotypes detected in matched tumor samples of these patients (figure 5G). Based on combined TIL single-cell RNA/V(D)J sequencing, we could further confirm that neo-1 expanded clonotypes were derived from $CD4^+$ T cells (not shown). The gene expression profiles of validated and candidate neo-1 reactive T cells were largely overlapping, confirming that clonotypes expanding in ESPEC were highly enriched in tumor antigen-reactive T cells (figure 5H–I). Taken together, we show that ESPEC-SUIT makes antigen-reactive T cell responses accessible with high specificity, depth and remarkable breadth.

CONCLUSIONS

With the advent of checkpoint inhibitory antibodies,[5] adoptive T cell transfer[8 11] and (individualized) antitumor vaccines,[6 7] the power of the immune system to mediate tumor control and rejection has become increasingly

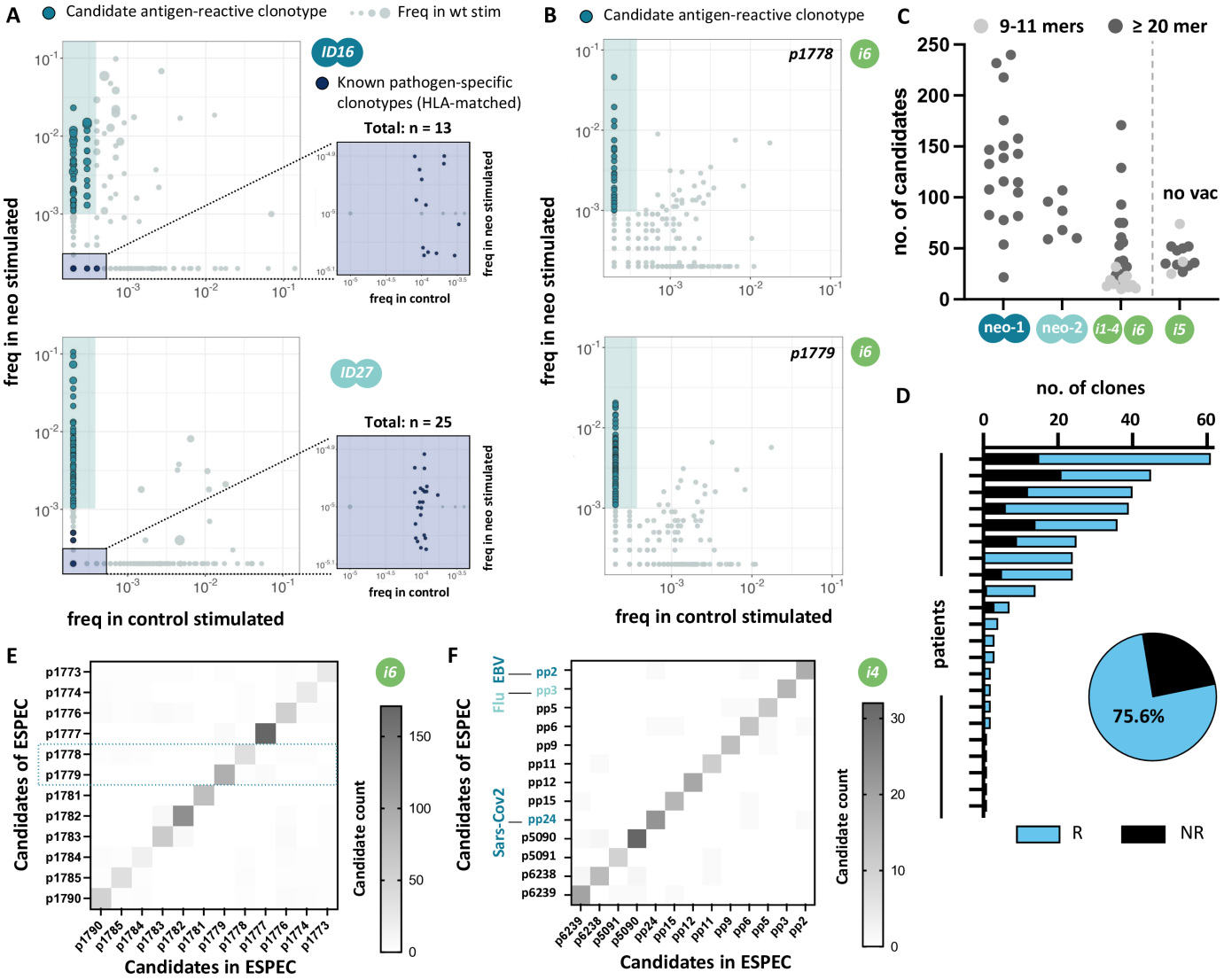


Figure 4 Epitope-specific expansion cultures (ESPEC)-based selection of antigen-reactive candidates. (A) Relative frequency of T cell receptor (TCR) clonotypes in antigen-stimulated ESPEC (y-axis) versus control-stimulated cultures (no peptide control, x-axis) in representative patients vaccinated with neo-1 (top) or neo-2 (bottom). Bubble size indicates frequency of the respective clone in the culture stimulated with the relevant wild-type (wt) variant; teal highlighting and shading indicate candidate antigen-reactive clonotypes, dark blue highlighting (zoom in for better visualization of small frequency clones) indicates clones with TCRβ-chain (TRB) CDR3 amino acid sequences reported as pathogen-reactive on the relevant human leukocyte antigen (HLA) background in a TCR reactivity database (VDJdb); (B) relative frequency of TCR clonotypes in antigen-stimulated ESPEC (y-axis) versus control-stimulated cultures (no peptide control, x-axis) in two representative neoepitope cultures of patient i6. Teal highlighting and shading indicate candidate antigen-reactive clonotypes; (C) number of candidate antigen-reactive clonotypes per culture identified based on ESPEC using neo-1 (n=19), neo-2 (n=6) or patient-individual antigens (i1-6, n=37). Cultures without significant post-ESPEC responses were excluded (n=1 of patient i1, n=2 of patient i4, n=4 of patient i5); candidate selection was performed on TCRβ deep-sequencing data down-sampled to 10 000 TCR counts. Colors distinguish short (light gray) and long (dark gray) peptide-driven cultures. For patient i5, the mutations have not been targeted by vaccination (no vac); (D) number of candidate antigen-reactive clonotypes derived from n=10 patients and n=22 cultures (each bar represents one culture) reactive ('R', blue) or non-reactive ('NR', black) to the antigen-of-interest based on *in vitro* functional validation; inlay depicts the frequency of reactive TCRs in all tested candidates (n=341); (E–F) heatmaps showing the number of candidate TCR clonotypes from a given antigen-stimulated culture (y-axis) and the number of times they were found as candidates across all other cultures of the same patient (x-axis); (E) patient i6; dotted-outlined box marks representative cultures of this patient shown in (B); (F) patient i4, colors highlight cultures stimulated with pools of viral peptides.

clear. While the prognostic relevance of tumor T cell infiltration had long been appreciated,[1 39 40] the mentioned therapeutic interventions provided vivid illustrations of T cell-mediated tumor eradication. What is still lagging behind is a detailed understanding of the characteristics exhibited by tumor-reactive T cells and the factors separating responders from non-responders.

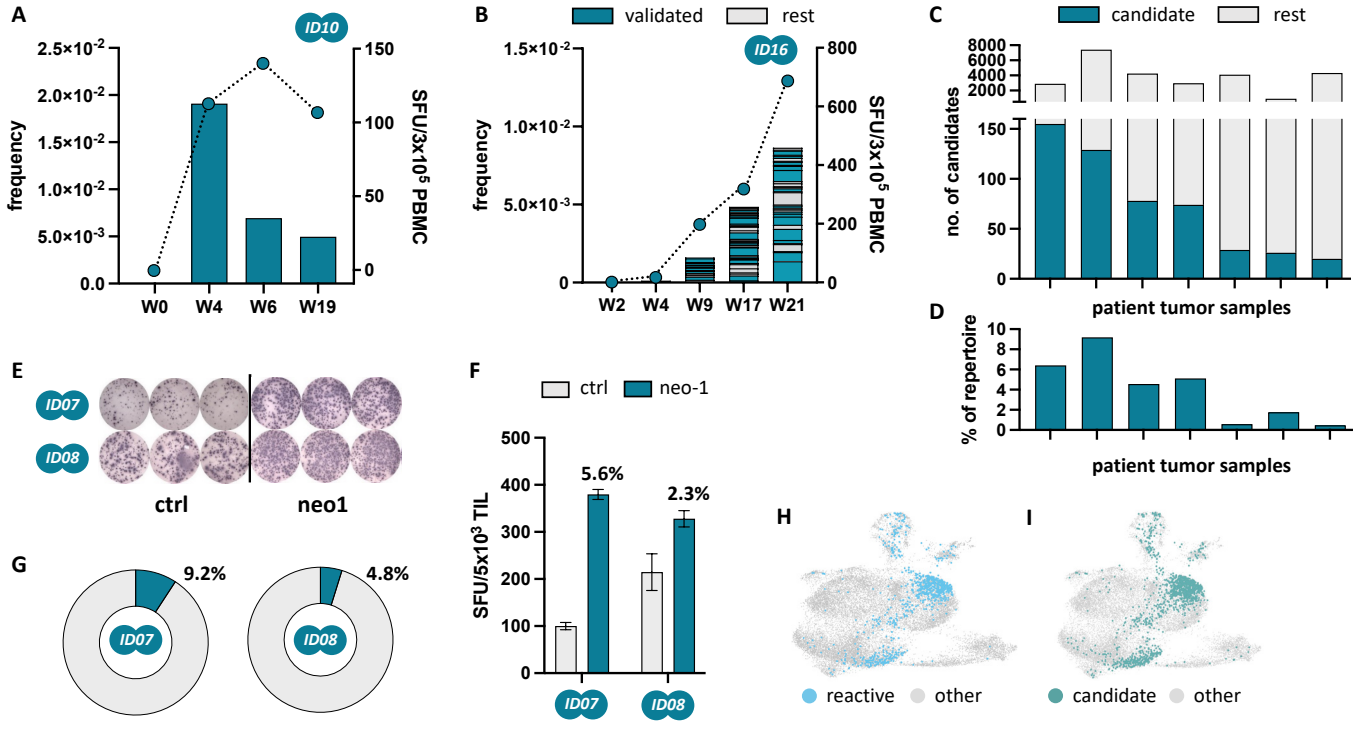


Figure 5 Tracing and characterization of antigen-reactive clonotypes in peripheral blood and tissue. (A–B) Representative data of the cumulative frequency of epitope-specific expansion cultures (ESPEC)-derived candidate T cell receptors (TCRs) (bars, left y-axis) and the *ex vivo* interferon (IFN)γ Enzyme-Linked ImmunoSpot (ELISpot) responses against neo-1 peptide (background subtracted, dotted line, right y-axis) at the relevant time points in peripheral blood of two neo-1 vaccinated patients. In (B), clonotypes with *in vitro* validated neo-1 reactivity are highlighted in teal, untested or unreactive candidates are shown in gray; for comparison between peripheral blood mononuclear cell (PBMC) samples, TCRβ deep-sequencing data was down-sampled to 50 000 counts; blood was collected before vaccination and after 1–7 administrations of long neo-1 encoding peptide (A: vaccination on week (W) 0, 2, 4, 10; B: vaccination on W0, 2, 4, 9, 17); number (C) and cumulative frequency (D) of ESPEC-derived candidate clonotypes (teal) versus all other clonotypes in tumors of neo-1 immunized patients (n=4, 5 tumors), and patient i3 (two tumors), who received a personalized, fusion-spanning peptide vaccine; tumor tissue was collected after eight vaccinations; raw (E) and quantified (mean±SD) (F) neo-1-specific IFNγ ELISpot response in minimally cultured tumor-infiltrating lymphocytes from two patients with glioma treated with neo-1 long peptide vaccine, after stimulation of 5000 cells/well with no peptide (ctrl) or neo-1 20-mer, and (G) cumulative frequency of neo-1 ESPEC-derived candidate clonotypes detected in the tumor-infiltrating lymphocyte (TIL) TCR repertoire of these patients as assessed by bulk (ID7) or single-cell (ID8) TCR sequencing; uniform manifold approximation and projection highlighting gene expression profiles of tumor-infiltrating lymphocytes in n=3 neo-1 vaccinated patients with TCRβ CDR3 sequences (H) derived from *in vitro* validated neo-1-reactive clonotypes (n=70 in n=827 cells) or (I) shared ('candidates', n=246 clonotypes in n=1270 cells) and not shared ('other', n=6519 clonotypes in n=11 779 cells) with ESPEC candidates selected from neo-1-stimulated cultures of the respective patient.

To gain insight into tumor-specific T cell repertoires, we established ESPEC-SUIT, a platform that enables identification of large numbers of tumor antigen-specific TCRs using expansion cultures from peripheral blood.

Based on TCR repertoire analysis, TCR cloning and *in vitro* functional assays in a dataset of n=63 expansion cultures from >30 patients, we find that ESPEC-SUIT induces expansion which is (i) antigen-specific, that is, no sharing of candidates between cultures stimulated with different antigens, (ii) pronounced, that is, greater in antigen than in control stimulated cultures, (iii) reproducible and (iv) yields high numbers of candidate antigen-reactive clonotypes, the majority of which recognize the antigen-of-interest when subjected to *in vitro* functional validation.

The high true-positive rate of antigen specificity of ESPEC-SUIT-derived candidates justifies tracing these clonotypes to peripheral blood and tumor tissue and thus provides rich information on kinetics and characteristics of tumor-reactive T cells. The ESPEC-SUIT platform further allows the discovery of many TCRs specific to antigens-of-interest, facilitating their characterization and, for example, the selection of lead candidates for clinical development.

Given the fact that peripheral blood is easily accessible, attempts have been made to extract tumor-reactive cells directly from peripheral repertoires.[2 15 16] Such efforts are hampered by the low frequency of circulating T cells reactive to single antigens. While we observe measurable neoantigen-specific responses in *ex vivo* assays using PBMC of vaccinated patients with glioma, these are

much increased after ESPEC. Of note, our 2-week expansion cultures revealed sizeable antigen reactivity even in patients where no *ex vivo* vaccine response was measurable by ELISpot or intracellular cytokine staining,[41] and patients with various tumor types that had not undergone neoepitope vaccination.

We optimized ESPEC-SUIT for simplicity, for example, circumventing initial T cell isolation or cell-sorting steps, and for the ability to expand both CD4 and CD8 T cell responses. In cases where large numbers of antigens need to be tested, expansion cultures can be stimulated with peptide pools, as also proposed by others.[21 42] We found that pooling increasing numbers of different MHC I and MHC II peptides led to higher background and decreased the response to individual peptides, but did not preclude our ability to detect responses, even when expanding multiple strong responses in parallel.

Beyond functional assessment of post-ESPEC T cell responses to confirm expansion of the desired T cell specificities, TCR repertoire analysis is the key readout to define candidate antigen-reactive clonotypes. While TCR diversity of the expansion cultures remained large, distinct antigen-driven clonal expansion was observed across various antigens and patients with diverse HLA types.

Taking cytokine-driven expansion from control cultures into account, we defined antigen-reactive candidates based on clonal expansion that was uniquely confined to cultures stimulated with the relevant antigen and made this available as an easy-to-use online tool (https://TCRselect.dkfz.de). This approach to candidate selection was supported by our extensive functional TCR validation of 341 TCRs selected from 2200 candidates across 10 patients, which showed that >75% of TCR candidates recognize the peptide used for expansion. Others have reported TCR selection using more sophisticated and more restrictive criteria and algorithms,[19 20] and one could also envision applying statistical models to determine TCR expansion.[43 44] While being highly user-friendly, the strength of our approach is the large number of candidate clonotypes identified, powering downstream analyses and comparisons. The mean number of candidates identified for antigens currently targeted by vaccination or expanded from the unperturbed repertoire is 81 and 42, respectively, while numbers reported by others in various settings mostly range from single-digit to low double-digit numbers.[20 22–24] The fact that ESPEC-SUIT successfully expands T cell responses to putative, patient-individual neoepitopes (see data from patients i4-6) highlights the suitability of the assay for the assessment of antigen immunogenicity. This can be used to assess the size and composition of the endogenous tumor-reactive TCR repertoire, as well as to guide selection of antigens for patient-tailored vaccination approaches.

Of note, data from post-ESPEC-SUIT isolation of antigen-reactive T cells and from broad TCR reactivity screens performed in selected patients would even suggest that the cut-off selected here for interpatient comparisons is rather conservative and might underestimate the number of antigen-reactive clonotypes. It has been suggested that the amount of relevant TCRs that can be discovered depends on the cellular input of the assay.[20] Importantly, while both cell input and sequencing depth are important factors, expanded clonotypes from our experiments include many examples that are below the limit of detection in the pre-expansion repertoire and could be functionally validated, while they would have been disregarded in other approaches. By performing replicate ESPEC-SUIT assays, we showed that clonotype expansion and candidate selection was highly reproducible for the top expanded TCRs and did not seem to be impacted by sampling.

Novel developments in single-cell sequencing and TCR cloning[12 45 46] make it possible to quickly identify TRA-TRB pairs for thousands of cells. In ESPEC-SUIT, we chose a two-step approach, basing candidate selection on bulk TCRseq and (optional) subsequent functional validation on single-cell TCRseq data. This results in TCR repertoire information that broadly and deeply interrogates the post-expansion repertoire and identifies many relevant TCRs supported with adequate sequence coverage. Despite the limited sensitivity of current single-cell sequencing approaches, it is in most cases sufficient to extract TRA-TRB pairing for candidates preselected based on TCR deep sequencing.

ESPEC-SUIT can thus be applied for discovering large numbers of antigen-reactive TCRs for further scrutiny with respect to, for example, affinity, avidity or HLA restriction, as well as to identify candidate TCRs to be tracked and characterized in patient blood or tissue. In our cohort of patients with glioma vaccinated with recurrent neoepitopes, we demonstrated that vaccine antigen-specific clonotypes were expanded in response to vaccination and were detectable in patient tumors, where they represented a significant percentage of the overall TIL repertoire, corresponding to the frequency of antigen-reactive T cells observed in TIL-based functional assays. We further showed overlapping gene-expression signatures of candidate clonotypes and TCRs with validated neoepitope reactivity within the TIL compartment. These two observations suggest that candidate antigen-reactive clonotypes identified in ESPEC-SUIT cover a large percentage of the true antigen-reactive TCR repertoire for a given antigen, with important implications for future insights into tumor-reactive TIL repertoires: we and others have shown that tumor-reactive TIL across multiple tumor entities exhibit distinct gene expression signatures,[4 14 22] but are the minority amid a multitude of bystander T cells infiltrating the tumor due to their activation status.[13 22 47] Methods to sensitively identify tumor-reactive T cells are thus paramount to further clarify their distinguishing features, and—equally important—identify cells carrying tumor-reactive receptors without exerting efficient effector functions.

Scheper *et al* sorted individual tumor-resident T cells and cloned >80 TCRs, but found few of them to carry

tumor-reactivity, despite high PD1 expression.[13] In an impressively scaled-up follow-up, thousands of TIL-derived TCRs were cloned from multiple patients, but only 2–34 candidate antigen-reactive TCRs were found, not all of which passed further validation.[12] Interestingly, even when screened TCRs were enriched for markers associated with tumor-reactivity, these numbers did not improve significantly. Beyond the technically advanced setup, these data illustrate the difficulty of finding relevant TCRs in unmodulated TIL populations. By foregoing TIL as input material and single-cell sequencing in initial steps, ESPEC-SUIT could provide a richer resource for discovering relevant TCRs.

In conclusion, ESPEC-SUIT represents a simple culture-based approach for the broad and sensitive discovery of tumor-reactive TCRs from $CD4^+$ and $CD8^+$ T cell subsets from peripheral blood samples. By enabling tracking and characterization of large numbers of tumor-reactive TCRs in patient blood and tissue, it has the potential to significantly advance our understanding of tumor rejection and therapy resistance.

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