Functional TCR Retrieval From Single Antigen-Specific Human T Cells Reveals Multiple Novel Epitopes

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Competing financial interests
U.S., Ö.T., P.S., T.O. are inventors on patents featuring TCRs described here. Based on the work described here, the company UNICELL (subsidiary of BioNTech Holding) has been founded of which U.S. is founder and management board member and P.S. and T.O. are employees. S.K., Ö.T., U.S. are inventors on patent applications featuring proprietary IVT RNA templates used in the process.

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ABSTRACT

The determination of epitope specificity of disease-associated T-cell responses is of relevance for the development of biomarkers and targeted immunotherapies against cancer, and autoimmune and infectious diseases. The lack of known T-cell epitopes and corresponding T-cell receptors (TCR) for novel antigens hinders efficient development and monitoring of new therapies. We developed an integrated approach for systematic retrieval and functional characterization of TCRs from single antigen-reactive T cells that includes identification of epitope specificity. This is accomplished through rapid cloning of full-length TCR-α and -β chains directly from single antigen-specific CD8⁺ or CD4⁺ T lymphocytes. Functional validation of cloned TCRs is conducted using in vitro transcribed RNA transfer for expression of TCRs in T cells and HLA molecules in antigen-presenting cells. This method avoids the work and bias associated with repetitive cycles of in vitro T-cell stimulation, and enables fast characterization of antigen-specific T-cell responses. We applied this strategy to viral antigens and tumor-associated antigens (TAA) resulting in the retrieval of 56 unique functional antigen-specific TCRs from human CD8⁺ and CD4⁺ T cells (13 specific for CMV-pp65, 16 specific for the well-known TAA NY-ESO-1, and 27 for the novel TAA TPTE), which are directed against 39 different epitopes. The proof-of-concept studies with TAAs NY-ESO-1 and TPTE revealed multiple novel TCR specificities. Our approach enables the rational development of immunotherapy strategies by providing antigen-specific TCRs and immunogenic epitopes.
INTRODUCTION

T cells determine the clinical outcome of various diseases, including infections, cancer and autoimmune diseases, through the recognition of antigenic peptides bound to HLA molecules on the surface of target cells (1). Recognition of HLA-presented α/β T-cell epitopes is exclusively mediated by the highly polymorphic heterodimeric complex of the TCR-α and -β chains. Strategies to decipher the fine specificity of antigen-specific T-cell responses against disease-associated antigens and identification of the corresponding TCR sequences on the single-cell level will promote basic research and broaden the scientific basis for the rational development of T cell-mediated targeted immunotherapies, including vaccination, modulation of aberrant T-cell reactivity associated with autoimmune disease and adoptive transfer of TCR-engineered T cells.

Antigen-specific T-cell responses are typically directed against a set of selected immunogenic peptides derived from a full-length antigen. The immunogenicity of a peptide is determined by several factors: it must be expressed, properly processed by proteases, efficiently loaded onto HLA molecules, and bind with sufficient affinity to form a stable peptide-HLA complex that is transported to the cell surface. Finally, surface-expressed peptide-HLA complexes must be able to trigger the activation of a T cell with a complementary TCR (2). Immunogenic epitopes for many well-known clinically relevant antigens have been characterized extensively (3, 4) (http://www.cancerimmunity.org/), and there is a growing list of novel antigens for which knowledge of confirmed epitopes is still scarce or lacking.
The presence of epitope-responsive T cells in the peripheral repertoire is dependent on the generation of appropriate TCR-α/β chains upon somatic recombination of V(D)J gene segments during T-cell development, survival of developing T cells during positive and negative selection in the thymus, and in the case of antigen-experienced T cells, on successful priming and proliferation of the mature T cells in the lymphatic system (5).

Several methods have been developed to track clonally expanded T-cell populations in order to identify protective or pathologic TCR clonotypes (6). One approach is the molecular analysis of TCR diversity by analyzing the TCR-β chain CDR3 repertoire using CDR3 spectratyping techniques (7). During the last years several PCR-based methods have been developed allowing TCR repertoire analyses at the single-cell level (8-12) as well as the in vitro reconstitution of full-length α/β-TCRs (13-16) from single cells. Likewise, next generation sequencing methods have evolved that generate millions of short sequence reads, which enable high-throughput profiling of TCR repertoires but have not addressed single T cells, thus precluding analysis of paired TCR-α/β chains (17-19). Only recently three different approaches addressing the latter limitation were reported. Linnemann and colleagues exploited the quantitative nature of TCR gene capture for frequency-based matching of TCR-α/β chains from oligoclonal T-cell populations (20). Turchaninova and colleagues (21) introduced an interesting cell-based emulsion RT-PCR technique for identification of TCR-α/β chain pairing. Most recently Han and colleagues (22) reported an elegant TCR profiling and phenotyping approach using the MiSeq platform. In parallel, many immunomics tools have evolved, including immunoinformatics enabling in silico prediction of epitopes for whole proteomes following in vitro validation of peptide candidates by HLA binding and cellular immune assays (23, 24). In summary, these
methods either focus on the identification of T-cell epitopes or the analysis of TCR repertoires, but none of them combines both, matching epitopes with repertoire. In this report, we introduce an integrated approach for rapid and efficient cloning of TCR-α/β chains directly from single antigen-specific CD8^+ or CD4^+ T lymphocytes of individual repertoires combined with rapid functional characterization of the cloned TCRs including definition of recognized epitopes. The technique was applied to identify TCRs and epitopes for the viral model antigen CMV-pp65, the well characterized tumor antigen NY-ESO-1, and the novel tumor antigen TPTE (Transmembrane Phosphatase with TEnsin homology). These proof-of-concept studies demonstrated that our technology enables the examination of antigen-specific TCR repertoires, the isolation of TCR genes for therapeutic or diagnostic use, and the identification of novel T-cell epitopes and epitope-cluster-regions for known and novel disease-associated antigens.
MATERIALS AND METHODS

Cell lines and reagents
The murine embryonal fibroblast cell line NIH-3T3, and the human chronic myeloid leukemia cell line K562 were obtained from American Type Culture Collection (ATCC) and cultured under standard conditions. K562 cells transiently or stably transfected with HLA allelotypes (referred to e.g. as K562-A*0201) were used for validation assays. The Jurkat76 T-cell line, deficient for both TCR-α and -β chains, was kindly provided by Dr. M. Heemskerk (25) and was also cultured under standard conditions.

The monospecific CTL cell line IVSB specific for the HLA-A*0201-restricted tyrosinase-derived epitope tyr368-376 (26) was cultured in AIM-V medium (Invitrogen) with 10% human serum type AB (Lonza), 350 U/ml IL2 (Richter-Helm BioLogics), 5 ng/mL IL7 (PeproTech), and 10 ng/ml IL15 (R&D Systems), and was stimulated weekly with irradiated SK29-Mel and AK-EBV cells. The cell lines IVSB, SK29-Mel and AK-EBV were kindly provided by Prof. Dr. T. Wölfel. All cell lines were tested and validated to be mycoplasma-free; no other authentication assay was performed.

Serotyping
An ELISA based on crude lysates of bacteria (CrELISA) expressing either full-length NY-ESO-1 or the N-terminus of TPTE (aa 1-51) was conducted as previously described (27). CMV-sero-positivity was analyzed by a standard ELISA for polyclonal CMV-specific IgG responses used for routine diagnostics.
Single-cell sorting of antigen-specific CD8\(^+\) or CD4\(^+\) T cells

Flow cytometric sorting of single antigen-specific CD8\(^+\) or CD4\(^+\) T cells was conducted either directly ex vivo from freshly isolated T cells or PBMCs or after one week of antigen-specific expansion. Prior to sorting, 2x10\(^6\) T cells or PBMCs were stimulated with 3x10\(^5\) autologous dendritic cells (DC) loaded with the peptide pool or transfected with in vitro transcribed (IVT) RNA encoding the antigen of interest or a control antigen, respectively, for 4 to 15 hours depending on the stimulation mode. Cells were harvested, treated with a PE/APC-conjugated anti-IFN\(\gamma\) antibody, a FITC-conjugated anti-CD8 and an APC- or PE-conjugated anti-CD4 antibody using the IFN\(\gamma\) secretion assay kit (Miltenyi Biotec). Sorting was conducted on a BD FACS Aria flow cytometer (BD Biosciences). One double-positive cell (IFN\(\gamma\)/CD8 or IFN\(\gamma\)/CD4) per well was harvested in a 96-well V-bottom-plate (Greiner Bio-One) containing NIH3T3 carrier cells, centrifuged at 4 °C and stored at -80 °C.

PCR amplification and cloning of V(D)J sequences

3 \(\mu\)l 5´RACE cDNA generated from mRNA of isolated T cells was subjected to 40 cycles of PCR in the presence of 0.6 \(\mu\)M V\(\alpha\)-/V\(\beta\)-specific oligo pool, 0.6 \(\mu\)M C\(\alpha\)- or C\(\beta\)-oligo, 200 \(\mu\)M dNTP and 5 U Pfu polymerase (cycling conditions: 2 min at 95 °C, 30 s at 94 °C, 30 s annealing temperature, 1 min at 72 °C, final extension time of 6 min at 72 °C). PCR products were analyzed using Qiagen’s capillary electrophoresis system. Samples with bands at 400-450 bp were size fractioned on agarose gels, the bands excised and purified using a Gel Extraction Kit (Qiagen). Sequence analysis was performed to reveal the sequence of the V(D)J domains. DNA was NotI-digested and cloned into IVT vectors containing the appropriate backbone for a complete TCR-\(\alpha/\beta\) chain.
ELISPOT assay

The ELISPOT used to analyze antigen-specific IFNγ secretion of TCR-engineered T cells was performed as described previously (28).

Fluidigm analysis

RNA isolation from tissues was conducted using the RNeasy Lipid Tissue Mini Kit procedure according to the instructions of the manufacturer (Qiagen). RNA was converted to cDNA by using the SuperScript II Kit (Invitrogen) according to the manufacturer’s instructions. For quantitative real-time (qRT)-PCR analysis using the Fluidigm detection system the samples and assays were prepared and analyzed according to the “Fast Gene Expression Analysis Using EvaGreen® on the BioMark™ or BioMark HD System Fluidigm® Advanced Development Protocol 37”. 96x96 Gene Expression Dynamic Array IFCs were loaded using the IFC Controller HX. Chip arrays were analyzed via a Fluidigm BioMark HD system. After normalization to the housekeeping gene HPRT1 (sense 5’-TGA CAC TGG CAA AAC AAT GCA-3’; antisense 5’-GGT CCT TTT CAC CAG CAA GCT-3’) the relative expression of TPTE (sense 5’-GAGTCTACAATCTATGCAGTG-3’; antisense 5’-CCATAGTTCTGTTCTATCTG-3’) was quantified using ΔΔCt calculation. A calibrator of 18.2 corresponding to 30 (maximal number of cycles used in the PCR) minus the mean of the HPRT1 values of the samples was used in this analysis.

Additional methods

Detailed methodology is described in the Supplementary Methods.
RESULTS

Design and set up of the TCR retrieval and characterization strategy

To obtain functional antigen-specific TCR molecules from natural T-cell repertoires, we developed a three-step procedure comprising isolation of single antigen-reactive T cells from patients (Fig. 1A top), cloning of TCR-α/β chains from sorted cells (Fig. 1A middle), followed by functional validation of complete TCRs (Fig. 1A bottom).

For isolation of single antigen-reactive T cells, we selected donors with pre-existing immune responses against the selected antigens. The presence of CD8^+ and CD4^+ T-cell responses against an antigen is known to correlate with measurable titers of circulating IgG antibodies (29, 30). Consequently, we included only PBMCs of donors that had antibody responses against the selected antigens. We isolated single antigen-reactive T cells from the peripheral blood of seropositive donors either ex vivo or after one week of antigen-specific expansion. Stimulation prior to sorting was conducted with autologous DCs either pulsed with a pool of overlapping peptides or transfected with IVT RNA encoding full-length antigens (31, 32). Activated antigen-specific T cells were detected by an IFNγ secretion assay (Fig. 1A top). We sorted single CD8^+/IFNγ^+ or CD4^+/IFNγ^+ T cells by flow cytometry, extracted RNA, generated first-strand cDNA and performed global PCR amplification by a modified SMART protocol (33) (Fig. 1A middle). Prior to amplification of variable regions, we verified the presence and integrity of TCR-encoding cDNA by amplification of a TCR-β chain constant region sequence.

A particular challenge was the amplification of unknown full-length V(D)J regions of TCRs, including leader sequences without prior knowledge of the variable domain subtype. To achieve this, we designed and applied sets of sequence-specific but
partially degenerated primers covering all functional Vα and Vβ genes (Supplementary Table S1, S2 A,B).

Amplified V(D)J sequences were then cloned into vectors containing TCR-α/β constant region cassettes providing full-length templates for immediate in vitro transcription. Discovery of new (‘unique’) TCRs was confirmed by direct sequencing of the V(D)J amplification products prior to cloning, since dominant TCR clonotypes were repeatedly retrieved. The frequency of discovery for each clonotype was documented to identify dominant clonotypes.

To assess surface expression of cloned TCR chains we transfected the TCR-deficient cell line Jurkat76 (25) with TCR-coding IVT RNA and detected recombinant TCRs by flow cytometry (Fig. 1B). To be independent of patient cells, we conducted immunologic validation of identified TCRs using IVT RNA transfer for rapid expression of TCRs in lymphocytes and HLA molecules in antigen-presenting cells (APC) (Fig. 1C). For functional validation TCR-α/β chain IVT RNA was transferred into the tyrosinase-specific IVSB T-cell line (26) or into primary CD8+ or CD4+ T cells from healthy donors. As APCs, either autologous DCs or K562 cells expressing the HLA class I and II molecules of the respective donor either stably or transiently after IVT RNA transfer (Fig. 1C) were applied. TCR function and epitope specificity were evaluated by IFNγ-ELISPOT and luciferase-based killing assays (Fig. 1A bottom).

Identification of TCRs recognizing HLA class I- and II-restricted epitopes of the viral antigen CMV-pp65

To assess whether the method recovers immunologically relevant TCRs, CMV-pp65 phosphoprotein, the major target of human CMV-specific T-cell responses, was used as a model antigen (34). In HLA-A*0201-positive individuals the bulk of CMV-specific
T-cell responses is directed against epitope pp65_{495-503}, which confers immunoprotection against the virus (3). We pre-sensitized CD{8}^{+} T cells of donor ID1, a healthy HLA-A*0201-expressing CMV-seropositive individual, with autologous DCs pulsed with this epitope and sorted for IFNγ^{+}/CD{8}^{+} T cells. We isolated and cloned six unique TCRs from single cells. Four of them reprogrammed IVSB cells to recognize specifically pp65_{495-503}-pulsed K562-A*0201 cells, while a control TCR that we cloned randomly from a seronegative person did not (Fig. 2A, Table 1). IFNγ secretion in response to both pp65_{495-503} and tyr_{368-376}-pulsed K562-A*0201 targets suggested appropriate signaling via the recombinant TCR and maintenance of specificity of the endogenous TCR. Remarkably, all four TCRs differed in their V{α} and V{β} rearrangements (Supplementary Table S3).

To test whether TCRs against the immunodominant peptide pp65_{495-503} can also be recovered upon stimulation with the whole pp65 antigen, CD{8}^{+} T cells of HLA-A*0201-expressing donors ID2, ID3 and ID4 were pre-sensitized with pp65 RNA-transfected DCs followed by sorting for CD{8}^{+}/IFNγ^{+} T cells (Supplementary Fig. S4). T cells of donor ID2 and ID3 underwent one week of antigen-specific expansion before we started our procedure, whereas cells from donor ID4 were sorted directly ex vivo (Table 1). Six of the twelve cloned unique CD{8}^{+} T cell-derived TCRs from these three donors were found to reprogram IVSB cells to recognize DCs loaded with pp65 peptide pool or pp65 RNA, the latter confirming recognition of naturally processed epitopes (Fig. 2B, Table 1). As exemplified for TCR_{CD{8}}-CMV#4, four TCRs were directed against pp65_{495-503} in the context of HLA-A*0201. Testing of K562 target cells expressing individual HLA class I alleles of the patient revealed HLA-B*3501 as the restriction element for TCR_{CD{8}}-CMV#1 (Fig. 2C top). Analysis of individual 15-mers of the pp65 peptide pool mapped recognition to the region of aa
117-131 of pp65, suggesting its identity with the previously reported HLA-B*3501-
restricted epitope pp65_{123-131} (IPSINVHHY) (35) (Fig. 2C bottom).

TCR_{CD8-CMV#1} and TCR_{CD8-CMV#14} were transferred into IVSB cells to further test
their ability to confer specific killing of pp65_{117-131}-pulsed K562-B*3501 and pp65_{495-
503}-pulsed K562-A*0201 target cells, respectively. Remarkably, for both TCRs,
specific killing was comparable to the killing of tyr_{368-376}-pulsed K562-A*0201
mediated by the endogenous TCR at all tested E:T ratios (Fig. 2D).

To evaluate the suitability of the approach for cloning of HLA class II-restricted TCRs
from CD4^{+} T cells, PBMCs of donor ID1 were stimulated ex vivo with a pp65 peptide
pool followed by sorting for single CD4^{+}/IFN\gamma^{+} T cells. Three of the five cloned TCR-
\alpha/\beta chain pairs were capable of reprogramming autologous CD4^{+} T cells for specific
recognition of autologous monocytes pulsed with pp65 peptides (Table 1). Using
pp65 peptide pool-pulsed K562 cells expressing individual HLA class II alleles of the
donor as targets, we identified HLA-DRB1*0701 as the restriction element for all
three TCRs (Fig. 2E). Single peptide-pulsing localized the epitope of TCR_{CD4-CMV#1}
to aa 117-139 of pp65, in which no HLA-DRB1*0701-restricted T-cell epitope has
been reported to date (Table 1). The epitopes of TCR_{CD4-CMV#3} and CMV#5 were
mapped to aa 337-359 of pp65, a region in which a HLA-DRB1*0701-restricted
epitope has been described previously (36).

In summary, our single-cell TCR cloning and validation procedure successfully
retrieved functional TCRs against a viral model antigen from CD8^{+} and CD4^{+} T cells.

**Cloning of TCRs directed against HLA class I- and II-restricted epitopes of the
tumor-associated antigen NY-ESO-1**
As compared to T cells recognizing viral antigens, precursor frequencies of T cells recognizing tumor-associated self-proteins are usually low even in primed seropositive patients. To evaluate the capability of our approach to clone functional TCRs from antigen-specific T-cell populations of low abundance, we used tumor-associated antigen (TAA) NY-ESO-1, one of the best characterized members of the cancer/germline antigen family (37). In cancer patients NY-ESO-1 frequently elicits spontaneous CD4⁺ and CD8⁺ T-cell responses (29, 38, 39), whose specificities have been mapped over the last years (40, 41).

Due to its natural immunogenicity it is a prototype for immunotherapy strategies and many clinical trials targeting NY-ESO-1 either by vaccination or transfer of TCR-engineered T cells are ongoing.

We selected non-small cell lung cancer (NSCLC) patient ID5 based on his confirmed autoantibody reactivity against NY-ESO-1. Bulk PBMCs from this patient were pulsed with NY-ESO-1 peptide pool and expanded in vitro for one week. Approximately 0.3% specific CD8⁺/IFNγ⁺ T cells were obtained by exposure to autologous DCs transfected with RNA encoding NY-ESO-1. We identified a total of 16 different TCRs from the sorted single cells (Table 1). As shown for TCRCD8-NY#2 and TCRCD8-NY#5, seven TCRs conferred specific recognition of autologous DCs presenting epitopes endogenously processed after transfer of NY-ESO-1 RNA (Fig. 3A). To determine restriction elements we tested the reactivity of TCR-transfected IVSB effectors against NY-ESO-1 peptide pool-pulsed K562 cells expressing single HLA class I alleles of the donor (Fig. 3B). Remarkably, epitopes of all seven TCRs localized between aa 85-111 (NY-ESO-1 peptides P22 to P25) of the NY-ESO-1 protein (Fig. 3C,D), a hydrophobic region known to undergo efficient proteosomal cleavage giving rise to multiple epitopes with various HLA restrictions (41). To our knowledge, this is the first time that these restriction elements have been associated with this region. By
screening serial nonamers (Supplementary Table S5), we narrowed down the HLA-
B*3508-restricted epitope of TCR<sub>CD8</sub>-NY#5, -NY#6, -NY#8 and -NY#15 (all differing in
their Va/β usage, Supplementary Table S3) to NY-ESO-1<sub>192-100</sub> (LAMPFATPM) (Fig.
3D).

Activated CD8<sup>+</sup> T cells from a healthy donor were engineered to express TCR<sub>CD8</sub>-
NY#5 efficiently and specifically lysed autologous DCs pulsed with decreasing
amounts of NY-ESO-1<sub>192-100</sub> peptide (Fig. 3E left). Cytotoxicity was dose-dependent
with an IC<sub>50</sub> at a peptide concentration of 10<sup>-10</sup> M. In analogy, TCR<sub>CD8</sub>-NY#2-
reprogrammed IVSB effectors were also capable of killing peptide-loaded K562 cells
transfected with the appropriate restriction element (Fig. 3E right).

It is noteworthy that transfer of TCR<sub>CD8</sub>-NY#5 successfully reprogrammed both CD8<sup>+</sup>
and CD4<sup>+</sup> T cells for specific recognition of NY-ESO-1 indicating that this TCR is
functionally independent of the CD8 coreceptor (Supplementary Fig. S6).

CD4<sup>+</sup>/IFNγ<sup>+</sup> T cells obtained from patients ID6 and ID7 (Fig. 4A) were used to clone a
total of 9 different NY-ESO-1-specific TCRs (Table 1). Mapping of restriction
elements (Fig. 4B) and epitopes (Fig. 4C, D) revealed that almost all specificities of
these T-helper cell-derived TCRs are novel (Table 1). Seven of these TCRs
recognized epitopes in a peptide stretch comprising aa 117-147 in the context of
different HLA class II elements, suggesting a hot spot for T-helper cell epitopes (Fig.
4D).

Notably, nearly all NY-ESO-1-specific TCRs isolated from CD4<sup>+</sup> T cells (6 of 7 tested)
were also functional in CD8<sup>+</sup> T cells indicating that these TCRs are coreceptor-
independent as well (Supplementary Fig. S7).
Isolation of TCRs recognizing epitopes derived from cancer/germline gene TPTE

Whereas NY-ESO-1 is known to be highly immunogenic, multiple tumor-associated genes are still not characterized on the level of T-cell epitopes. One example is TPTE, a germline-specific protein that is aberrantly transcribed in human liver, prostate and lung cancers (42-44).

We found that TPTE mRNA expression in healthy tissues is in fact confined to the testis, epididymis and placenta, while transcript amounts were below the quantification limit of qRT-PCR in all other normal tissue specimens (Fig. 5a). TPTE expression was detected in 22 of 111 (20%) lung tumor samples (Fig. 5b).

TPTE-reactive serum autoantibodies have been reported in patients with hepatocellular carcinoma (43). Recognition of TPTE by T cells, however, has not been shown so far. We pulsed bulk PBMCs of three TPTE-seropositive NSCLC patients (ID8 to ID10) with a TPTE peptide pool and expanded them in vitro for one week. Expanded TPTE-specific T cells were visualized by IFNγ secretion assay after pre-sensitization with autologous, antigen-pulsed patient-derived DCs and used for single-cell TCR retrieval (Fig. 6A). We identified 27 functional TPTE-reactive TCRs (Table 1) from which one (TCR<sub>CD8</sub>-TPT#35) was derived from a CD8<sup>+</sup> T cell. We revealed that this TCR recognized an HLA-B*0702-restricted, endogenously processed peptide in the region of aa 185-199 and is capable of mediating lysis of TPTE peptide-pulsed target cells (Fig. 6B). By analyzing serial nonamers covering this region, we determined the novel epitope TPTE<sub>188-196</sub> (PRWTHLLRL).

The other 26 TCRs isolated from single-sorted CD4<sup>+</sup>/IFNγ<sup>+</sup> T cells were confirmed to recognize HLA class II-derived epitopes on TPTE peptide-pulsed K562 cells transfected with one of the patient’s restriction elements (Fig. 6C, Table 1,
Supplementary Table S3). Fine mapping using single peptide-pulsed HLA allele-expressing K562 target cells disclosed that the epitopes were distributed widely over the TPTE protein sequence (6D,E, Table 1).
DISCUSSION

The identification of epitopes that evoke protective or pathologic T-cell responses as well as the genetic composition of antigen- or epitope-specific TCR in immune responses have important implications for pathogenesis studies, diagnosis, therapy development and monitoring of infections, malignancies and autoimmune diseases. In this study, we introduce a technology platform that combines the systematic analysis of functional antigen-specific TCR repertoires with the identification of corresponding T-cell epitopes. This is accomplished through rapid and efficient cloning of TCR-α and -β chains directly from single antigen-specific CD8+ or CD4+ T lymphocytes of individual repertoires followed by functional characterization of cloned TCRs via IVT RNA. Notably, our process is not only efficient but also allows cloning and functional immunologic characterization of new TCRs within less than 2 weeks.

By applying our newly designed primer sets, we covered 80% of all known Vα/Vβ gene family members (Supplementary Table S2 A, B). In contrast to established primer sets for the amplification of TCR CDR3 regions from single T cells (10, 13, 14, 45), our primers amplify full-length variable regions. In the elegant study by Ozawa and colleagues, they were able to generate matching TCR α/β cDNAs from ~20% of processed single cells using the 5’RACE technique (9). Depending on the quality of the patient’s material up to 50% paired TCR chains can be obtained using our method. Several groups observed dual TCR expression (9-12), raising the need for functional analysis. Not only are we able to study dual TCR expression, using this method, we also can delineate the different (productively rearranged) TCR-α chains of a single T cell. Compared to other strategies that aim to validate functional TCRs from single T cells (15, 16) our approach includes the directed cloning step of
amplified variable regions into IVT vectors, thus providing the TCR-α/β constant regions for extremely rapid generation of full-length TCRs for validation assays that include the determination of epitope specificity for any antigen. While Next-generation Sequencing-based methods and the use of barcodes allows for identification of potentially hundreds of α/β-TCRs in a single experiment (22), validation of these TCRs still requires subsequent cost-intensive gene synthesis and cloning.

In total we identified 398 TCRs representing 189 different clonotypes. Among these, 56 TCRs were shown to be antigen-specific corresponding to 30% of uniquely identified TCRs. The number of non-specific TCRs identified depends on the background IFNγ secretion by non-specific T cells after restimulation. This background is in part donor-specific but also depends on the target cells that are used for restimulation. Furthermore, the given frequency of antigen-specific T cells influences how good the antigen-specific cells can be separated from bystander T cells. The usage of other activation-induced molecules such as CD137 may increase the percentage of antigen-specific TCRs of uniquely identified clonotypes.

Ex vivo as well as in vitro expanded T cells stimulated either with defined epitopes or whole antigens were exploitable for the isolation of entire TCR panels from individuals. Poly-epitopic stimulation of antigen-specific CD4+ as well as CD8+ T cells prior to flow cytometric single-cell sorting without prior knowledge of T-cell epitopes or the HLA allelotype of the respective donor was achieved by transfecting autologous APCs with IVT RNA encoding the respective antigen. Moreover, IVT RNA with optimized translation efficiency and stability was used for analysis of surface expression and function of recombinant TCRs as well as for expression of HLA molecules in target cells for epitope and restriction mapping. In summary, these technical developments enabled an exceptionally fast, flexible, and robust process
for discovery and validation of antigen-specific TCRs with need for minimal amount of patient material.

We established the platform using CMV-pp65 as a model antigen and retrieved 13 different CMV-pp65-specific TCR clonotypes (Table 1). These TCRs isolated from CD4+ and CD8+ T cells not only recognized known CMV-pp65-derived epitopes, but also one novel epitope. This proves that even in extensively characterized antigens, our platform can still discover novel epitopes and identify corresponding novel TCRs. The proof-of-concept study with the highly immunogenic tumor antigen NY-ESO-1 resulted in retrieval of 16 NY-ESO-1-specific TCRs from single T cells of three different NSCLC patients, and which recognized endogenously processed NY-ESO-1 epitopes. Immunologic validation assays revealed novel and known HLA class I- and II-restricted epitopes, which clustered in previously described immunogenic regions of the NY-ESO-1 protein and thus confirmed the validity of our study (41, 46, 47).

We then addressed TPTE, a tumor antigen that was not yet described as a target for antigen-specific T cells. First we confirmed the ectopic expression of TPTE in lung cancer tissues by qRT-PCR. Expression of TPTE mRNA was detected in 20% of lung cancer tissues analyzed, but not in healthy tissues. From three seropositive NSCLC patients TPTE-specific T cells were isolated and a total of 27 TPTE-specific TCRs were cloned from these cells. Immunologic validation assays revealed multiple HLA class I- and II-restricted epitopes distributed over the entire sequence of the antigen. To our knowledge this is the first report that describes TPTE-specific T-cell responses in cancer patients. Furthermore, we identified multiple novel HLA class I- and II-restricted TPTE-derived T-cell epitopes.

The effectiveness of the platform that we developed is illustrated by the great number of functional TCRs identified. We isolated 56 TCRs specific for CMV-pp65, NY-ESO-
1, and TPTE. Furthermore, upon TCR characterization we found multiple novel epitopes for the viral antigen, the well-studied tumor antigen, as well as a novel cancer antigen.

In order to assess the biological relevance of individual epitopes comprehensive analyses of epitope-specific T-cell responses in larger groups of cancer patients sharing at least one HLA allele are necessary.

The provision of characterized TCRs with different tumor antigen specificities and HLA restrictions may fuel the development of therapies based on vaccination or adoptive transfer of TCR-engineered T cells that have recently led to promising results (48-50).

This approach can be applied for the isolation of high-affinity TCRs, e.g. from \textit{in vitro} primed allo-restricted T cells or from autologous mutation-specific T cells. The latter is particularly interesting as cancer mutanome approaches based on identification of nonsynonymus mutations are emerging for individualized cancer therapy. Furthermore, adaption to liquid handling stations will enable the high-throughput process that is needed to identify immunodominant epitopes shared by different cancer patients as well as appropriate lead structures for personalized TCR gene therapy.

In summary, we successfully developed and validated an approach for the rapid cloning and functional testing of antigen-specific TCRs from single T cells with broad applicability for research and development of T cell-based immunotherapies. Most importantly, by applying this technique we have extended the universe of TAA epitopes and TCRs considerably.
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We thank Dr. Martin Sebastian (Dpt. of Medicine III, Johannes Gutenberg-University, Mainz) for providing the blood samples of NSCLC patients and Prof. Thomas Wölfel (Dpt. of Medicine III, Johannes Gutenberg-University, Mainz) for providing the cell lines SK29-Mel, AK-EBV and IVSB as well as Dr. Mirjam Heemskerk (Dpt. of Hematology, University Medical Center, Leiden) for providing the cell line Jurkat76. We thank Magdalena Brkic and Monika Schmidt-Habrich for technical assistance, Michael Schäfer and the Stefan-Morsch-Stiftung for HLA typing and Uli Luxemburger for guiding the ELISA screenings.
REFERENCES


Table 1. Study synopsis of all investigated individual repertoires and identified TCRs

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aHLA haplotypes are listed in Supplementary Table S8
bPrevious reports on epitopes of the respective restriction located in the recognized region
cTCR recognizes an epitope in combination with more than one HLA allele.
n.r., no report; pept., peptide pool; iDC, immature DC; fDC, fast DC; CD14, monocytes.
FIGURE LEGENDS

FIGURE 1. Technology platform for cloning and characterization of antigen-specific TCRs. (A) T cells of cancer patients seropositive for the antigen of interest (1) are stimulated with antigen-loaded autologous DCs (2). IFNγ secreting CD4⁺ or CD8⁺ T cells are isolated by flow cytometry (3). Single cells are harvested in multiwell plates for RNA extraction and 5'-RACE PCR (4). Full-length TCR-α/β variable regions are amplified (5) and cloned into vectors containing TCR-α/β constant region cassettes (6) for in vitro transcription (IVT). For specificity testing T cells are co-transfected with IVT RNA encoding the identified TCR-α/β chains (7). Autologous DCs or K562 cells expressing the respective antigen/HLA combination are used as antigen-presenting cells (8). Confirmed TCRs are subjected to further characterization including definition of epitopes (9). (B) For flow cytometric verification of TCR expression Jurkat76 cells electroporated with TCR-α/β chain RNA or without RNA were stained with a pan TCR antibody. (C) IVT vectors used for expression of antigens (top), HLA molecules (middle) and TCR-α/β chains (bottom). The vector backbone is derived from the pST1-sec-2βgUTR-A(120)-Sap1 plasmid featuring a T7 site and 3'-modifications for increased RNA stability and translational efficacy (3'-untranslated region of the human β-globin gene (3’βgUTR)). Vector templates for antigens contain sequence modules (leader peptide (sec), MHC class I trafficking signal (MITD)) to increase presentation of epitopes. TCR V(D)J sequences were inserted upstream of the TCR constant region cassettes.

FIGURE 2. TCRs cloned from CMV-pp65-specific CD4⁺ and CD8⁺ T cells. (A) TCRs cloned from ex vivo isolated CD8⁺ T cells of donor ID1. IVSB cells were transfected with TCR-α/β chain RNAs, stimulated with K562-A*0201 pulsed with
pp65_{495-503} and analyzed by IFNγ-ELISPOT. Negative controls: unrelated peptide SSX-2_{241-249}, an irrelevant TCR cloned from a CMV-seronegative donor. Positive control: the tyrosinase-derived tyr_{368-376} epitope. (B,C) TCRs obtained from donor ID2 pre-stimulated with the whole pp65 antigen. (B) TCR-engineered IVSB cells were tested on autologous iDCs or K562-A*0201 cells loaded with the pp65 peptide pool, pp65_{495-503} or pp65 IVT RNA. Negative controls: TPTE peptide pool; unrelated peptide SSX-2_{241-249}; irrelevant TCR. Positive control: tyr_{368-376}. (C) HLA restriction was analyzed by testing IVSB cells transgenic for TCR_{CD8-CMV#1} for recognition of K562 expressing HLA class I alleles of the donor pulsed with pp65 overlapping peptides or without antigen as a control (top). Fine specificity was determined with K562-B*3501 targets pulsed with individual pp65-derived 15-mer peptides (bottom). (D) Specific killing of peptide-pulsed HLA-transfected K562 cells by TCR-engineered IVSB cells analyzed by luciferase cytotoxicity assay. Killing through the endogenous receptor was determined with tyr_{368-376}-pulsed targets. Negative control: irrelevant TCR. (E) Determination of HLA restriction of TCRs from CD4+ T cells of donor ID1. TCRs were transferred into CD4+ T cells of a CMV-seronegative donor and tested against K562 target cells expressing single HLA class II alleles loaded with pp65 peptide pool by IFNγ-ELISPOT.

**FIGURE 3. TCRs from CD8+ T cells directed against the tumor antigen NY-ESO-1.** (A) Specificity testing of TCRs isolated from NY-ESO-1-specific CD8+ T cells of NSCLC patient ID5. TCR-transfected IVSB cells were tested for recognition of autologous iDCs loaded with NY-ESO-1 RNA or peptide pool by IFNγ-ELISPOT. Negative controls: iDCs pulsed with TPTE peptide pool; an irrelevant TCR. Positive control: tyr_{368-376}-pulsed K562-A*0201. (B) Identification of HLA-restricting elements. TCR-engineered IVSB cells were analyzed by IFNγ-ELISPOT for recognition of K562
cells transfected with individual HLA class I alleles and pulsed with NY-ESO-1 peptide pool. Negative controls: HIV-gag peptide pool; K562 electroporated without RNA (mock). Positive control: tyr368-376 peptide (C,D) Testing of TCR-transfected IVSB cells for specific IFNγ secretion in response to K562 cells expressing the appropriate HLA class I allele and pulsed with (C) overlapping 15-mer peptides representing the whole antigen or (D) 9-mer peptides covering aa 85-101 of NY-ESO-1. (E) Specific killing of TCR-transgenic T cells analyzed by luciferase cytotoxicity assay. (Left) OKT3-activated CD8+ T cells from a healthy donor were transfected with TCRCD8-NY#5 and co-cultured with autologous iDCs loaded with decreasing amounts of NY-ESO-192-100 or SSX-2241-249 as control (E:T = 60:1). (Right) K562-A*6801 cells pulsed with NY-ESO-1 peptide pool were used as targets for IVSB cells transfected with TCRCD8-NY#2 at different E:T ratios. Control: TPTE peptide pool.

FIGURE 4. TCRs from CD4+ T cells directed against the tumor antigen NY-ESO-1. (A) Flow cytometric sorting of NY-ESO-1-specific CD4+ T cells from NSCLC patient ID7 after one week of expansion. IFNγ-secreting T cells were isolated after stimulation with autologous NY-ESO-1 RNA-transfected iDCs. Control: iDCs transfected with eGFP RNA. (B) Identification of HLA-restricting elements for TCRs isolated from CD4+ T cells of donor ID6. TCR-engineered CD4+ T cells were analyzed by IFNγ-ELISPOT for recognition of K562 cells expressing individual HLA class II alleles of the donor and pulsed with NY-ESO-1 peptide pool. Negative controls: HIV-gag peptide pool; irrelevant TCR. (C,D) Mapping of HLA class II-restricted epitopes recognized by TCRs obtained from patient ID6 and ID7. CD4+ T cells transfected with NY-ESO-1-specific TCRs were tested for recognition of K562
cells expressing the appropriate HLA class II allele and pulsed with partially overlapping 15-mers representing the whole NY-ESO-1 protein.

FIGURE 5. Ectopic expression of TPTE in lung cancer tissues

Analysis of TPTE mRNA expression by qRT-PCR of reverse-transcribed mRNA obtained from (A) normal or (B) lung cancer tissues. The threshold used to define positive normal and tumors tissues is indicated as a line.

FIGURE 6. TCRs from tumor antigen TPTE-specific CD4⁺ and CD8⁺ T cells. (A) TPTE-specific T cells of patient ID9 after one week of expansion. CD8⁺ and CD4⁺ T cells secreting IFNγ in response to TPTE peptide-pulsed fastDCs (fDCs) were sorted by flow cytometry. Control: fDCs pulsed with HIV-gag peptide pool. (B) Specificity and function of CD8⁺ T cells transfected with TCRCD8-TPT#35 obtained from patient ID10. (Top) TCR-transfected CD8⁺ T cells in response to autologous mature DCs (mDCs) either pulsed with TPTE peptide pool or transfected with TPTE RNA were analyzed by IFNγ-ELISPOT. Control: an irrelevant TCR-α chain RNA. (Middle, bottom) TCR-transgenic IVSB cells were tested for recognition of K562-B*0702 cells pulsed with TPTE₁₈₅-₁₉₉ containing the epitope of TCRCD8-TPT#35 by (middle) IFNγ-ELISPOT and (bottom) luciferase cytotoxicity assay (E:T = 40:1). Control: target cells pulsed with TPTE₁₉₃-₂₀₇. (C) Determination of HLA class II restriction of TCRs isolated from TPTE-specific CD4⁺ T cells of patient ID8 by analyzing TCR-transfected CD4⁺ T cells for recognition of K562 cells expressing single HLA class II alleles of the donor pulsed with TPTE or HIV-gag peptide pool. (D) Epitope mapping for TCRCD4-TPT#87 obtained from patient ID10 by IFNγ-ELISPOT testing of TCR-engineered CD4⁺ T cells for recognition of K562 cells expressing the appropriate HLA class II antigen.
pulsed with individual TPTE 15-mer peptides. (E) Localizations of all newly identified epitopes (black loops). Dark green: transmembrane domains.
Figure 1

A

1. Autoantibody ELISA
2. Antigen-Specific T cell Stimulation
3. Single Cell Sorting
4. RNA Extraction
5. TCR-Vα/Vβ PCR
6. IVT TCR Vector
7. TCR Cloning
8. Specificity Testing
9. Cytotoxicity Assay

B

Jurkat76 control
Jurkat76 TCR-α/β RNA

C

EcoRI
sec Antigen MITD 3'βgUTR 3'βgUTR polyA(120)

EcoRI
NotI

NotI

TCR variable region

NotI

NotI + EcoRV and ligate

TCR constant region

TCR constant region

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Figure 5

A

Normal tissues

Relative expression

B

Lung cancer tissues

Relative expression

Lung cancer
Functional TCR Retrieval From Single Antigen-Specific Human T Cells Reveals Multiple Novel Epitopes

Petra Simon, Tana Omokoko, Andrea Breitkreuz, et al.

Cancer Immunol Res  Published OnlineFirst September 22, 2014.

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