ARTICLE

Rapid cloning, expression, and functional characterization of paired αβ and γδ T-cell receptor chains from single-cell analysis

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Transgenic expression of antigen-specific T-cell receptor (TCR) genes is a promising approach for immunotherapy against infectious diseases and cancers. A key to the efficient application of this approach is the rapid and specific isolation and cloning of TCRs. Current methods are often labor-intensive, nonspecific, and/or relatively slow. Here, we describe an efficient system for antigen-specific αβ TCR cloning and CDR3 substitution. We demonstrate the capability of cloning influenza-specific TCRs within 10 days using single-cell polymerase chain reaction (PCR) and Gibson Assembly techniques. This process can be accelerated to 5 days by generating receptor libraries, requiring only the exchange of the antigen-specific CDR3 region into an existing backbone. We describe the construction of this library for human γδ TCRs and report the cloning and expression of a TRGV9/TRDV2 receptor that is activated by zoledronic acid. The functional activity of these αβ and γδ TCRs can be characterized in a novel reporter cell line (Nur77-GFP Jurkat 76 TCRαβ) for screening of TCR specificity and avidity. In summary, we provide a rapid method for the cloning, expansion, and functional characterization of human and mouse TCRs that can assist in the development of TCR-mediated therapeutics.

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INTRODUCTION

T cells play a vital role in the control of viral infections and tumors. T cells are activated by antigen-presenting cells via interactions between peptide-major histocompatibility complex and T-cell receptors (TCRs). This interaction can induce proliferation and the development of effector functions, including cytokine production and cytotoxic activity. T cells can also infiltrate infected or transformed tissues, e.g., as tumor-infiltrating lymphocytes, to perform these effector functions.1,2 However, in some chronic viral infections and tumors, responding effector T cells progressively get exhausted and become dysfunctional.3–5 In addition, control of tumors and/or infection may require large numbers of highly reactive lymphocytes that cannot be achieved due to normal tolerance mechanisms. One effective method to overcome this barrier is the use of therapeutic adoptive transfer of lymphocytes.2,6,7

Adoptive transfer of lymphocytes such as in vitro-expanded or TCR-engineered antigen-specific T cells has been successfully used to control viruses and tumors in patients.8–12 In vitro expansion of viral or tumor-specific T cells require significant time to prepare and the targets are not usually fully characterized. Lymphocytes expressing engineered TCRs and chimeric antigen receptors target specific antigens, with chimeric antigen receptors recognizing surface antigens through immunoglobulin-type interactions6,10,11 and TCRs recognizing tumor-associated peptide-major histocompatibility complexes. Chimeric antigen receptor therapy directed against surface antigens requires a tumor-associated antigen that can be universally targeted (even on healthy, nontumor tissue) without significant toxicity. Tumor-specific antigens that are targeted by TCRs represent an attractive alternative that can provide greater specificity and reduce nontumor-associated toxicities.13–15 Additionally, engineered T cells expressing high-affinity antigen receptors can be conditioned to overcome immune tolerance, which has been a major limitation for immunotherapy.14,15,16 Apart from the clinical applications, a robust system for the cloning and expression of TCRs is a valuable tool for the investigation of TCR structure and functions.17–20

Techniques to rapidly profile and clone antigen-specific TCRs have improved and shortened the process of TCR-engineered immunotherapy.2,11,12 These approaches are useful contributions to the field and are able to handle large cell inputs very effectively. However, for certain applications, the reported methods still have some limitations. First, approaches that rely on deep sequencing and cloning of bulk sorted cells can still be limited by target cell numbers. In contrast, single-cell approaches can utilize input sizes starting with a single cell but are less efficient at dealing with high cell number inputs (greater than 10,000 cells). As a result, single-cell methods are best directed at defined samples such as antigen-specific responses or tissue-associated infiltrating cells. Second, for bulk sorting, pairing of TCR chains requires algorithmic imputation, which can have difficulty dealing with cells expressing two distinct TCR chains of one type (e.g.,...
two TCRα chains), which are quite common. A recently reported algorithm has addressed this concern and efficiently pairs bulk processed TCRs, using barcoded pools of cells. This method, though, requires relatively large inputs to successfully pair and would likely not be appropriate for very small sample sizes as might be obtained from tissue biopsies or tetramer sorting of small populations.

Third, while currently described methods are able to generate full-length receptors either by synthesis or by 5’ rapid amplification of cDNA ends (RACE)-associated approaches at the single cell level, these methods often are reported to require expansion of the isolated cells prior to TCR isolation. Lastly, the majority of antiviral and antitumor adoptive therapy has focused on αβ T-cell clones due to their exquisite antigen specificity. However, γδ T cells have also been shown to mediate antiviral and antitumor effects and are novel candidates for therapeutic development. To date, there is little research about profiling and utilizing the TCRγδ repertoire for therapeutic purposes, and as such applying γδ T cells for immunotherapeutic applications may be a promising future approach in conjunction with traditional TCRαβ techniques. Therefore, it is important to establish a system to define the repertoire and functional activity for γδ T cells. Additionally, improving efficiencies for cloning αβ TCRs from single cells may have complementary uses in the lab and in the clinic.

To overcome these limitations, we developed a rapid cloning and expression system for specific TCRs. In conjunction with single-cell multiplex polymerase chain reaction (PCR) techniques for TCRαβ or TCRγδ profiling, and Gibson Assembly cloning of synthesized DNA, we were able to rapidly sequence and clone specific TCRs in appropriate expression vectors in as little as 5 days after cell isolation with highly robust, inexpensive methods. Thus, this protocol provides an efficient and relatively high-throughput means for TCR engineering for therapeutic or research applications.

RESULTS
Paired TCRγδ analysis of human PBMC samples at the single cell level
Following on our previously reported single-cell PCR protocols for TCRαβ, we developed a similar strategy to characterize the paired TCRγδ repertoire in humans (Figure 1a). The primers were designed for all nonpseudogene TRGV and TRDV regions along with antisense primers for their respective constant regions. Two sets of primers (external and internal) were designed in order to perform a nested PCR (Table 1). The PCR products were examined by agarose gel electrophoresis before sequencing (Figure 1b). The average success rate for obtaining paired CDR3γ and CDR3δ sequences at the single cell level from human peripheral blood mononuclear cell (PBMC) samples is 71.25 ± 18.75%. The TRGV/TRDV family usage was determined from the multiplex PCR products (Figure 1c). On average, 20% of the sequences from our analysis of 14 human PBMCs were TRGV9/TRDV2. This technique along with the established mouse and human αβ single-cell multiplex PCR offers a rapid method (turnaround time ~3 days per 160 cells) for characterizing paired TCRγδ chains at the single cell level. The data of paired TRGV/TRDV family usage percentage in each human sample are shown in Supplementary Table S1.

Establishment of human TCRαβ and TCRγδ retroviral expression clones
Many of the downstream applications of paired TCRαβ or TCRγδ sequence analysis require cloning and expression of the

Table 1 Primers targeting human T-cell receptor-γ (TRGV) and δ (TRDV) genes

| TRGV gene(s) targeted by primer | External primer sequence | Internal primer sequence |
|--------------------------------|--------------------------|-------------------------|
| HuTRGV3.5                     | 5’TCTTCACACTTGGAAGGG3’   | 5’GTCATCATGCTGAATC3’    |
| HuTRGV7                       | 5’TCTTCACACTTGGAAGGG3’   | 5’GTCATCATGCTGAATC3’    |
| HuTRGV4                       | 5’GGGTACCTCTGTGTAAGGG3’  | 5’TACCTATGGCTGTAGGAG3’  |
| HuTRGV8                       | 5’GGGCTCTCAAAGATCTG3’    | 5’GCGCTCTGCTACCCCAGG3’  |
| HuTRGV9                       | 5’CAAACCTGGAGGAAC3’      | 5’AAAGCGGTACCCAC3’      |
| HuTRGV10                      | 5’TATACCAAAGTGACCGATTG3’ | 5’CAGCTACCCAAGGCTC3’    |
| HuTRGV11                      | 5’GAACAAACCTGAAATCTTCC3’ | 5’CATACCTGAAAGAGC3’     |
| HuTRGV1.2.4.6                 | 5’GGGCTACCTGTGGAATC3’    | 5’CAGGAGGGAAGGC3’       |
| HuTRGC                        | 5’GGGCTTCCCTCCTCGT3’     | 5’CAGGAATGTGTTGCT3’     |
| TRDV gene(s) targeted by primer | External primer sequence | Internal primer sequence |
| HuTRDV1                       | 5’GCCGAGGTTACTCAAG3’     | 5’AGCAAGAAGATTTTTTCTTA3’|
| HuTRDV2                       | 5’ATGATGGCTGCGCTGAC3’    | 5’TATACCTGACGCGGACC3’   |
| HuTRDV3                       | 5’TGTGGACAAATGGGAGGTC3’  | 5’GTTCTGGCTGCGCTGAC3’   |
| HuTRDV4/TRAV14                | 5’CAAAACCAAAGGGAATG3’    | 5’AGGAAAGGAGGCTGAC3’    |
| HuTRDV5/TRAV29                | 5’GCAGTTATACAAAATTTCC3’  | 5’CTGTGAGTCCTTCTAC3’    |
| HuTRDV6/TRAV23                | 5’TGAGTATGCCGAAAAGGAC3’  | 5’CGTGGACTTCTTTTATG3’   |
| HuTRDV7/TRAV36                | 5’GACAGGAGGGTACAAAGCC3’  | 5’ATCTCTGAGGCTCCAC3’    |
| HuTRDV8/TRAV38-2              | 5’CGGCTACCTGCTCAGCAGC3’  | 5’TCTGGATACGCGCTC3’     |
| HuTRDC                        | 5’CTTCATTATTCAAGCCTGAC3’ | 5’GATGACAAATACGAGATAC3’ |

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Figure 1  Unbiased single-cell amplification of paired T-cell receptor (TCR) CDR3 regions. (a) Overview of the multiplex polymerase chain reaction (PCR) protocol to amplify and sequence paired TCR CDR3 α/γ and CDR3 β/δ. After sorting single human αβ or γδ T cells into a 96-well plate, reverse transcription is performed to obtain single-cell cDNA. Taking human γδ T cells as an example, a first round of PCR is performed by using an external primer mixture of nine TRGV and eight TRDV sense and single TRGC and TRDC antisense primers following reverse transcription (RT) PCR. The first-round PCR products are subjected to two separate second-round PCRs using a corresponding internal primers mix (nine sense TRGV, single antisense TRDC, and eight sense TRDV, single antisense TRGC, respectively). The timeline of this process is shown on the left. (b) An agarose gel electrophoresis image of TCR segments containing CDR3γ and CDR3δ is shown. Paired CDR3γ and CDR3δ products from the same cell were loaded in adjacent lanes. Negative control PCR reactions are shown in the boxed region and in the ladder lane, a 500 bp label is shown. (c) Paired TRGV/TRDV usage is determined by multiplex PCR and sequencing (n=14 human apheresis rings). The percentage of different TRGV/TRDV usage in each sample was assessed (mean ± standard error of the mean).
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Cloning and expressing TCRαβ/γδ genes in retroviral vectors

1. Synthesis of the target genes by gBlock and vector linearization
   - 3–5 days
   - 8 days in total

2. Construction of target genes and linearized vector
   - 2 days

3. Expression of TCR genes in TCR-null human T cell line (Jurkat 76 TCRαβ/γδ-null cells)
   - 2 days

4. Expression of TCR clones in human T cell line to test functions

5. Establish the human TCRαβ and TCRγδ clone library

Search the human TCRα/β and TCRβ/δ gene sequences in IMGT website

Synthesize human TCRα/β and TCRβ/δ gene by g-blocks

Linear pMICherry Vector 6.3kb

Human TCRα/β and TCRβ/δ genes with 15–20bp overlapping ends

γδ-chain 2A
β-chain

Construct target genes and linearized vector

Figure 2 Rapid cloning and expression of human TCRαβ or TCRγδ in a retroviral vector. (a) A schematic diagram of T-cell receptor (TCR) cloning using gBlock synthesized DNA fragments and a linearized retroviral vector (pMICherry) is shown. Family-specific TRGV and TRDV full-length TCR chains were synthesized with a 15–20bp overlap sequence (purple) in the 2A region (pink). Together with a linearized pMICherry expression vector, a three-way ligation is performed by using Gibson Assembly Cloning. The timeline of this process is presented on the left. (b) Expression of TCR constructs in the Jurkat 76 TCRαβ cell line. The flow cytometry results of transfected cells are shown. The flow cytometry results of transfected cells are shown in silico. 2A oligopeptides can interact with the ribosomal exit tunnel to terminate sequence translation at the final codon (Pro) of the 2A sequence. Recently, multi-cistronic 2A-based retroviral vectors have been widely used for TCR:CD3 structural and functional studies. The entire sequence of TCRγ2A-TCR8 along with an 25bp overhang complementary to the ends of the linearized pMICherry vector were constructed full-length TCRγ and δ chains and TCRα and β chains joined by the 2A "self-cleaving" site in silico. 2A oligopeptides can interact with the ribosomal exit tunnel to terminate sequence translation at the final codon (Pro) of the 2A sequence, and reinitiate translation of the following sequence. Thus, we sought to develop a rapid cloning method to improve on conventional restriction enzyme-mediated ligation techniques, which can be cumbersome and time consuming. In addition, use of restriction enzymes for cloning becomes problematic because of the potential for restriction sites to appear in some variable regions and the nongermline CDR3 sequences of the TCR chains. The vector that we chose for TCR expression is pMICherry, which has been successfully used to construct TCR clones for the generation of retrogen mice. A schematic diagram of the cloned TCR chains in the pMICherry vector is shown in Figure 2a.

To clone full-length TCR chains, as a proof of principle, we chose a TRGV9/TRDV2 clone to demonstrate the feasibility of our cloning system, since the TRGV9/TRDV2 clonotype is dominant in the TCRγδ repertoire analysis from human PBMCs (Figure 1c). Similarly, a human TCRαβ pair was chosen derived from an influenza-specific CDB8 T cell from an infected individual (unpublished data). Using the IMGT-reported human TRGV, TRDV, TRGC, and TRDC sequences for TCRγδ or human TRAV, TRBV, TRAC, and TRBC sequences for TCRαβ and our single-cell CDR3γ and δ or CDR3α and β sequence data we constructed full-length TCRγ and δ chains and TCRα and β chains joined by the 2A "self-cleaving" site in silico.
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**Figure 2a.** After this cloning procedure, an average of 70.9% of the colonies picked after transformation were entirely matched with target sequences. The others contained either point mutations resulting from the cloning process or no inserts. To date, we have cloned more than 30 different TCR constructs by using this system, including, mouse and human TCR γδ and αβ. The cloning system is highly reproducible and we have succeeded in generating clones in all attempts.

To test the functionality of the TCR clones that were made following the method described in Figure 2a, we transfected the human TRGV9/TRDV2 construct into the Jurkat 76 TCRαβ cell line and checked for the cell surface expression by anti-TCRγδ and anti-CD3 antibody staining and flow cytometry. Although Jurkat cells have endogenous CD3, the expression of TCRγδ was not robust. Since γδ T cells do not develop in CD3-deficient mice and patients,38 we cloned the human CD3 complex into an MSCV vector (pMIAmetrine) and cotransfected it with our human TCR constructs. mCherry and ametrine, 16.1% of which were positive for tetramer staining (lower panel, Figure 2a). We analyzed the expression of the influenza virus-specific TCRαβ by staining the transfected cells with allophycocyanin (APC) conjugated influenza-M1 tetramer (HLA-A*0201, GILGFVFTL) and CD3 antibody. The FACS plot shows that 5.03% of the transfected cells were double positive for mCherry and ametrine, 16.1% of which were positive for tetramer staining (bottom panel, Figure 2a).

Effective TCR activation reporting by Nur77-GFP Jurkat 76 TCRαβ cells

An important application of TCR cloning and expression is to screen molecules that can activate or inhibit TCR signaling. Thus far, the common methods to detect TCR activation are using ELISA to detect cytokines (e.g., IFNγ) in the cell culture medium, or intracellular staining to report cytokine production by flow cytometry, or qRT-PCR to quantify the mRNA expression of cytokines, which
Table 2  CDR3 amino acid sequences of paired human TRGV9/TRDV2 cells isolated from peripheral blood mononuclear cells (n = 14)

| Paired amino acid sequence in TRGV9-CDR3 region | Paired amino acid sequence in TRDV2-CDR3 region | Frequency |
|-------------------------------------------------|-------------------------------------------------|-----------|
| ALFQIELGKKIKV                                  | ACDVLGDTEDRKL                                  | 2         |
| ALWDPYKKKL                                      | ACDTVFTGIGSWSWDRQMF                            | 2         |
| ALWDIPQELGKKIKV                                 | ACDTGLGETSWDRQMF                               | 2         |
| ALWAEOELGKKIKV                                  | ACDSGGYSWDRQMF                                 | 2         |
| ALWAERQELGKKIKV                                 | ACDTLFPGSATDKLI                                | 2         |
| ALWEGTQGLGKKIKV                                 | ACDTVGAHDTKL                                   | 2         |
| ALWEGTVQGGLKIKV                                 | ACDPLMGTGSFLYTDKL                              | 2         |
| ALWEGTVHSGGLKIKV                                | ACDTGGFDSWDRQMF                                | 2         |
| ALWEGTVHSGGLKIKV                                | ACDTGGFDSWDRQMF                                | 2         |
| ALWEGTVHSGGLKIKV                                | ACDTVGMRGDLKL                                  | 2         |
| ALWEGTVGELGKKIKV                                | ACDILGINTDKL                                   | 2         |
| ALWEGTVGELGKKIKV                                | ACDRLGDWPDKL                                   | 2         |
| ALWEGTVQELGKKIKV                                | ACDTVAPRIGGLKYTDKL                             | 2         |
| ALWEGTVQELGKKIKV                                | ACDTVGGYTDKL                                   | 2         |
| ALWEGTVQELGKKIKV                                | ACDTVGTAQ                                      | 2         |
| ALWEGTVQELGKKIKV                                | ACDTVGGSTPYTDKL                                | 2         |
| ALWEGTVQELGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEGTVQELGKKIKV                                | ACDTLIIFSGTDPTDKL                              | 2         |
| ALWEGTVQELGKKIKV                                | ACDVPLGWDTKL                                   | 2         |
| ALWEGTVQELGKKIKV                                | ACDTGILDFDKL                                   | 2         |
| ALWEGTVQELGKKIKV                                | ACDLGSTDGVKL                                   | 2         |
| ALWEANGLKL                                      | ACDLRLGAGQIDKL                                 | 1         |
| ALWAEQELGKKIKV                                  | ACDGKKTODTKL                                   | 1         |
| ALWAEQELGKKIKV                                  | ACDGLGGDRTYDKL                                 | 1         |
| ALWAEQELGKKIKV                                  | ACDGVTGTDTKL                                   | 2         |
| ALWEAQELGKKIKV                                  | ACDSTGSGTVKL                                   | 1         |
| ALWEAQELGKKIKV                                  | ACDGVDGDSTVKL                                  | 1         |
| ALWEAQELGKKIKV                                  | ACDGVDGDSDVKL                                  | 1         |
| ALWEAQELGKKIKV                                  | ACDGVDGDSDVKL                                  | 2         |
| ALWEDLQGLGKKIKV                                 | ACDLRLGAGQIDKL                                 | 1         |
| ALWEDLEQGLGKKIKV                                | ACDTGGSTPYTDKL                                 | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |
| ALWEDLEQGLGKKIKV                                | ACDTVSIFTGDPTDKL                               | 2         |

are time-consuming, labor-intensive, and expensive. Hence, we established a TCR activation reporter cell line, Nur77-GFP Jurkat 76 TCRβ-β (NJ76 cells). The Nur77-GFP reporting system has been demonstrated to reflect specific TCR signal strength by GFP expression.9,41 Here, we established the NJ76 cell line by stably transducing Nur77-GFP BAC DNA into Jurkat 76 TCRβ-β cells.

To test the functionality of NJ76 cells in reporting TCR activation, we transfected NJ76 cells with a murine K bPB1703-specific TCR αβ derived from influenza-infected mice along with mouse CD3. The K bPB1703 TCRαβ+ NJ76 cells (PB1-NJ76) were incubated with mouse splenocytes, the influenza PB1703 peptide, splenocytes and peptide, or mouse α-CD3/human α-CD28 as a positive control for 4 hours and GFP expression in transfected NJ76 cells was detected by flow cytometry (Figure 3a). The quantification of GFP levels is shown in Figure 3b. The results show that PB1-NJ76 cells can robustly express GFP after specific peptide stimulation (PB1) with antigen-presenting cells. The gating strategy for GFP detection is shown in Supplementary Figure S2.

This TCR-activation reporting system has also been tested for TCRγδ signaling. Zoledronic acid (Zometa, Novartis, Basel, Switzerland) is an amino-bisphosphonate that has demonstrated antitumor effects via inhibition of tumor growth and angiogenesis and induction of malignant cell apoptosis in humans.42–44 In addition, zoledronic acid can specifically stimulate and expand human TRGV9/TRDV2 cells.45–48 Since it can result in the accumulation of upstream metabolites in the mevalonate pathway, e.g., IPP, which induce the expansion of γδ T cells in vitro and in vivo, zoledronic acid pretreatment can increase the cytolyis of some cancer cell lines by γδ T cells.49 After transfection of the human TRGV9/TRDV2 vector and human CD3 vector into NJ76 cells, we pulsed the transfected TRGV9/TRDV2-NJ76 cells with 50 μg/ml of zoledronic acid for 3 hours, and washed and incubated the cells at 37 °C for 12 hours. We quantified the GFP expression level in transfected TRGV9/TRDV2-NJ76 cells and control cells by flow cytometry. The Nur77-GFP expression level is shown in Figure 3c (top panel). The transfected TRGV9/TRDV2-NJ76 cells showed a significantly higher level of GFP expression, which demonstrates that zoledronic acid can trigger γδ TCR signaling for the stimulation and expansion of γδ T cells. The fold change of GFP expression over time in stimulated TRGV9/TRDV2-NJ76 cells and nonstimulated TRGV9/TRDV2-NJ76 cells (red line) and nonstimulated TRGV9/TRDV2-NJ76 cells (black line) is shown in Figure 3c (bottom panel).

Rapid TCR cloning by CDR3 substitution using overlap extension PCR and TCR library

For TCRs, the only hypervariable regions are the CDR3 regions. Thus, cloning full-length TCRs de novo for each application may expend unnecessary resources. To improve on this, we have generated a library of potential TRGV and TRDV “backbone” combinations that only require the swapping of individual CDR3 regions directly from PCR products. For example, in TRGV9/TRDV2 cells from PBMCs of healthy donors, the CDR3s of both γ and δ chains were found to be highly diverse (Table 2). To rapidly generate a library of diverse TRGV/TRDV clones, we designed DNA linkers, whose ends overlap with the TRGC and TRDV in our single-cell PCR products. These DNA linkers contain the TRGC region, 2A and one of the TRDV regions, as is shown in Figure 4. By overlapping PCR with the single-cell PCR products, DNA linkers, TRGV sense primers, and TRDC antisense primers, we can connect any pair of TCR from our library as a template for the second-step overlap extension PCR. By using this substitution method, we have
Successfully cloned different γδ TCRs with matched CDR3s from our human single-cell PCR products. In principle, the same approach could be used with αβ TCRs, although the clone library would be larger. As an estimate, this CDR3 substitution approach can shorten the cloning process to within 5 days (Figure 4).

**DISCUSSION**

Here, we report several useful techniques for the analysis of TCR biology, including a single-cell–based protocol for γδ TCR amplification, a rapid protocol for TCR cloning and expression, and a novel platform for functional characterization of TCR clones. Our system provides an accurate and efficient method to approach rapid cloning at the single cell level, which can improve the development of multiple applications, including TCR-mediated immunotherapy.

The most prominent recent immunotherapy approaches involve T-cell checkpoint blockade inhibitors. However, these therapies depend on the presence of significant numbers of antitumor T-cell responses. The ex vivo expansion of tumor-infiltrating lymphocytes has also been successful, but it is time consuming. Our protocol could significantly accelerate the amount of time needed to generate large numbers of antitumor T cells, by allowing the efficient transduction of identified antitumor receptors. The key to the application of this form of directed T-cell immunotherapy is the rapid and accurate isolation and cloning of paired TCRs. Thus far, various methods have been developed for the cloning of TCR genes by traditional PCR, but the acquisition and expression of TCRs is often labor-intensive, time-consuming, expensive, and nonspecific. The system we have described provides efficient acquisition of TCR gene products for cloning based on single-cell isolation, with an amplification success rate of isolated paired single-cell TCRγδ CDR3 products of 71.25 ± 18.75% based on total sorted single cells in each sample. We have also developed a platform for screening TCR activation after cloning. By inserting the Nur77 reporter into our TCR cloning library (pMIC-TCRαβγδ-2AC-TCRβδn) with the same TRGV and TRDV family usage but different CDR3s by overlap extension PCR. The timeline of the whole process is on the left.
ligands have been identified and confirmed. 18,60 The GFP reporter
This is particularly useful in the context of
transduced cell lines can be used for the screening of novel antigens.
interaction, or, in the case where ligands have not been identified,
for study in vitro. This can include the characterization of biochemi-
cloning and it provides a relatively high-throughput, accurate, and
efficient method of TCR engineering for therapeutic or research
APPLICATIONS.

MATERIALS AND METHODS

Subjects and PBMC samples

Samples were obtained on research protocols approved by St. Jude Children's Research Hospital's institutional review boards (Memphis, TN). Peripheral whole blood was collected from heparinized apheresis rings from healthy immunocompetent individuals not taking immunomodulatory pharmaceutical agents. PBMCs were isolated via density-gradient centrifugation (GE Healthcare Ficoll-Paque PLUS, Marlborough, MA), and red blood cells (RBCs) were removed using RBC lysis buffer (8.3 g NH4Cl, 1 g KHCO3, and 1 ml 0.1% Phenol Red in 1 l distilled water). Isolated PBMCs were frozen in −80 °C for future use. All PBMCs used in the paper were stored frozen. Compared to fresh PBMC data from healthy apheresis rings, our frozen PBMCs did not have a significantly lower success rate for single-cell amplification (data not shown).

Single-cell sorting and staining

PBMCs were treated with human FcR blocking reagent (Miltenyi Biotec, Auburn, CA) on ice for 20 minutes. Human TCRβ cells were isolated by staining with PE-conjugated anti-human TCRβ (Biolegend, San Diego, CA), clone: B1), fluorescein isothiocyanate (FITC) conjugated anti-human CD3 (Biolegend clone: ICRF44), and CD3ε (Biolegend clone: OKT3), a dump gate consisting of APC-conjugated anti-

TAB 3 Types and sequences of 2A regions

| 2A type                  | 2A amino acid sequence | Separation |
|-------------------------|------------------------|------------|
| F2A (foot-and-mouth disease virus) | VKQTLNFDDLKLKAGD VESNPAGP | CD3δ and CD3γ |
| T2A (Thosea asigna virus) | EGRGSLTCGDVEENPGP | CD3γ and CD3ε |
| P2A (porcine teschovirus-1) | ATNFSLLKQAGDVEENPGP | CD3ε and CD3ζ |

rapid approach for immunotherapy are obvious. Tumor-specific
T cells have been characterized by broad nonspecific surface
phenotypes that can be used to isolate, clone, and express potential
tumor-targeted clones.53 The recent advancement of tumor
sequencing has allowed for identification of tumor neoantigens and
overexpressed self-antigens.54–57 Combining these technologies will
allow for promptly characterized and tailored antitumor therapy.

T-cell transfers have also been used for the treatment of opportu-
nistic infections in immunosuppressed patients, particularly after
hematopoietic stem cell transplant. The reactivation of herpes
viruses like human cytomegalovirus and Epstein-Barr virus is a clini-
cal dilemma that cannot always be addressed with antivirals.24,58,59
Analogous to tumor-infiltrating lymphocyte therapies, ex vivo
expansion of antiviral T-cell specificities can be clinically useful, but
suffers from similar workflow limitations. By generating a library of
specific TCR constructs reactive against a range of viruses and HLA
types, TCR-directed therapies could be used prophylactically or
immediately at the earliest signs of reactivation.

In addition to these therapeutic applications, our protocol sig-
nificantly improves the workflow for cloning and expressing TCRs
for study in vitro. This can include the characterization of biochemi-
features of the TCR-peptide-major histocompatibility complex
interaction, or, in the case where ligands have not been identified,
transduced cell lines can be used for the screening of novel antigens.
This is particularly useful in the context of γδ T cells, where very few
ligands have been identified and confirmed.19,60 The GFP reporter
line we have engineered can be used directly in high-throughput
screening platforms; alternative reporters (such as luciferase) can be
easily substituted as well.

In conclusion, we introduce a novel method to rapidly clone,
express, and characterize the function of paired αβ and γδ TCR
chains from single cells. Our platform addresses the nonspecific,
labor-intensive, and time-consuming issues of traditional PCR-based
cloning and it provides a relatively high-throughput, accurate, and
efficient method of TCR engineering for therapeutic or research
APPLICATIONS.

Table 3 2A primers targeting human CD3δ, γ, ε, and ζ genes

| TRGV gene(s) targeted by primer | Primer sequence |
|---------------------------------|-----------------|
| CD3δ sense                      | 5’CCCTACACTTCTCTCTTACGGGCGGGAATTCGCCAGAGATGGAAACATAGCACG3’ |
| CD3δ antisense                  | 5’CCACGTCCTCGGCACTTTGGAAGGTCAAATTCGAAATGCTGTTCACCCTGTCCTGGACCC3’ |
| CD3γ sense                      | 5’GAATTGTGACCTTCTCAAGTTGGGCGAGTGCAGATCCAAACCAGGCCCCATGAAAGGCGG3’ |
| CD3γ antisense                  | 5’CTCTCGAGCTACAGCAGTAGTCAGACTCTTCCTGCCCTGAGATCTTTATCTCCTTCAAC3’ |
| CD3ε sense                      | 5’CAGAGGAAGCTCTGCTCAATACCTGCGGTTGAGAATCTGCGGGGCCCAATCAGCTGGACCTC3’ |
| CD3ε antisense                  | 5’GGTTCTCTCACTGTCCTGCTGTGCTTTAAACAGAGAAGATTGCTGCGGGATCTCCGATGCTCTG3’ |
| CD3ζ sense                      | 5’CTCTGTGTTAAAAACGACGAGACGGGAAAGAACCCTGGTCTCATGAAGTGGAAAG3’ |
| CD3ζ antisense                  | 5’GAGGAGAGAGGGCCGGAATTTCTCAGGCAATTGTTAGCAAGGAGGCGG3’ |

Table 4 Types and sequences of 2A regions

| 2A type                  | 2A amino acid sequence | Separation |
|-------------------------|------------------------|------------|
| F2A (foot-and-mouth disease virus) | VKQTLNFDDLKLKAGD VESNPAGP | CD3δ and CD3γ |
| T2A (Thosea asigna virus) | EGRGSLTCGDVEENPGP | CD3γ and CD3ε |
| P2A (porcine teschovirus-1) | ATNFSLLKQAGDVEENPGP | CD3ε and CD3ζ |

Reverse transcription, multiplex, nested single-cell PCR and sequencing

Complementary DNA (cDNA) from TCRβ and TCRδ mRNA was reverse tran-
scribed directly from the sorted and stored single cells in the PCR plate
without any RNA extraction step using the iScript cDNA Synthesis Kit (Bio-Rad)
in a 2.5 μl reaction mix as per the method described previously.28 The cDNA
synthesis was carried out by incubating at 25 °C for 5 minutes, 42 °C for 30
minutes, and 80 °C for 5 minutes. Alternatively, we used the SuperScript Vilo
cDNA synthesis kit (Invitrogen), which produces a higher success rate for single-
cell PCR by incubating the reaction mixture at 25 °C for 10 minutes, 42 °C
for 60 minutes, and 80 °C for 5 minutes. The TCRβ transcripts from each
Figure 5  Human TCRγδ linker DNA library. Yellow color indicates TC sequence; pink color indicates 2A sequence; and gray color indicates TRDVx sequence.
cell were amplified by a multiplex nested PCR strategy as described previously. For amplification of TCRp transcripts, the overall strategy was simi-
lar to the published TCRαβ amplification, except for the primers described in Table 1. We designed nine TRGV external sense primers, nine TRGV internal sense primers, eight TRDV external sense primers, and eight TRDV internal sense primers targeted for individual TRGV and TRDV families based on the sequences derived from the IMGT database (http://www.imgt.org/geneddb/; ref. 61). For the antisense primer, we designed single TRGC external, TRGC internal, TRDC external, and TRDC internal primers complementary to the published TRGC and TRDC sequences in IMGT. Human TRAV14/DV4, TRAV23/DV6, TRAV29/DV5, TRAV36/DV7, and TRAV38-2/DV8 are shared primers in TRAV and TRDV primer sets. The primers were synthesized by Integrated DNA Technologies and stored at −20 °C at a stock concentration of 100 μmol/l in TE with low EDTA (pH 8.0). The primers for each category (sense external, sense internal of TRGV and TRDV) were combined so that the final concentration of each primer in the mixture was 10 μmol/l. The antisense primers were diluted to 10 μmol/l. The PCR conditions for the TCRαβ nested PCR were 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 20 seconds, 53 °C for 20 seconds, and 72 °C for 45 seconds, followed by final extension of 72 °C for 7 minutes. The PCR products were run on a 2% agarose gel to check for the success rate of the PCR as well as contamination, following which the products were purified by a modified Exonuclease I - Shrimp alkaline phos-
phatase (ExoSAP-IT, Bio-Rad) method to eliminate unincorporated primers and dNTPs for high-quality DNA sequencing. One microliter of the single-cell PCR product was added into the mixture of 4.6 μl of Tris-CI (50 mmol/l, pH 8.0), 0.2 μl of Exonuclease I and 0.2 μl of Shrimp alkaline phosphatase and was incubated at 37 °C for 15 minutes and 80 °C for 15 minutes. The purified PCR products were sequenced using the relevant TRAC, TRBC, TRGC, or TRDC primer. A schematic of the PCR strategy is shown in Figure 1a.

Generation of human CD3 construct

Human CD3 δ, ε, and ζ genes were amplified from human PBMC cDNA using the primers in Table 3. All the genes were linked together by overlap PCR with the TCRαβ 2A regions inserted in a double digested EcoR I (20 units) and Xho I (20 units) restriction enzymes (New England Biolabs) at 37 °C for 3 hours as per manufacturer’s instruction. Agarose gel purified-linearized pMChierry vector (100 ng) and 2× TCR G blocks were ligated in a two-way ligation, including the TCRγ gene, TCRδ gene, and linearized vector by using the Gibson Assembly Cloning kit (New England Biolabs) per manufacturer’s instructions. Two microclusters of the ligation mixture were transformed into DH5α Competent Escherichia coli (New England Biolabs) per manufacturer’s instructions.

DNA isolation, cell culture, and transfection

Recombinant pMChierry plasmids with full-length TCRαβ or TCRγδ inserts were isolated in small scale by using a NucleoSpin Plasmid kit (Clontech, Mountain View, CA) and in large scale for transfection using a Plasmid Midi kit (Qiagen, Hilden, Germany) per manufacturer’s instructions. The instruction Transfection System was used to transfect 10 μg TCRαβ or γδ DNA in the pMChierry vector into the human Jurkat 76 TCRαβ cell line (2×10⁶ cells/ml, 100 μl) followed by three pulses with a voltage of 1,350V and a width of 30ms. The transfection was cultured for 48 hours before being assayed for TCRαβ or TCRγδ expression on the surface by FACS analysis. The human Jurkat 76 cells TCRαβ cell line was cultured in complete-RPMI 1640 medium, which is RPMI 1640 with 10% of fetal bovine serum, 1% Penicillin-Streptomycin, and 1% l-glutamine at 37 °C and 5% CO₂.

Immunofluorescent and flow cytometric analysis

For surface staining, cells (1×10⁶) were harvested from culture and washed with FACS buffer (PBS with 1% of BSA and 0.1% sodium azide) prior to staining. The cells were treated with human FcR blocking reagent (Miltenyi Biotech) on ice for 20 minutes, and cells were then treated with various fluorescent conjugated antibodies against cell surface markers in FACs buffer. Human γδ T cells were stained with APC-conjugated anti-human TCRγδ (Biolegend, clone: B1) or APC-conjugated anti-human TCRαβ (Biolegend, clone: IP26) and Pacific Blue-conjugated anti-human CD3 (Biolegend, clone: OKT3). For influenza-specific tetramer staining, cells (1×10⁶) were stained with APC-conjugated Influenza-M1 tetramer (Beckman Coulter, Brea, CA, HLA-A*0201, GILGFVFTL) in FACs buffer at room temperature for 1 hour prior to surface staining with the same staining antibodies described.

The modification of the CDR3 region by two-step overlap extension PCR cloning

The substitution of the CDR3 was carried out by an overlap extension PCR cloning protocol. A schematic diagram of the procedure is shown in Figure 4. Briefly, we generated a library of linker DNA by gBlock syn-
thesis at Integrated DNA Technologies (Figure 5). The linker DNA consists of TRGC-2A-TRDVx (X represents the TRDV family) sequence. Using the single-cell PCR products of γ and δ chains of the desired clonotype and the relevant linker gblock DNA, we carried out an overlap PCR. The PCR reaction was set up and carried out as follows: 12.5 μl 2x Phusion high-fidelity DNA polymerase (New England Biolabs), 0.25 μl of 100x DMDSO, 1 μl of 10 μmol/l TRGV internal sense primer, 1 μl of TRDV internal antisense primer (Table 1). 1 μl of linker DNA, and deionized H₂O up to 25 μl. The PCR program was 98 °C for 30 seconds; 34 cycles of each at 98 °C for 10 seconds, 58 °C for 30 seconds, 72 °C for 1 minute; then finally 72 °C for 10 minutes. The PCR products were visualized on a 1% agarose gel, and purified from the gel for cloning into the existing construct with the same TRGV and TRDV family usage. The reaction conditions used were as follows: 20 ng of a TCR construct in pMChierry vector with identical TRGV and TRDV but an irrelevant CDR3γ and δ, with 50 ng of the first-step PCR products, 12.5 μl of 2x Phusion high-fidelity DNA polymerase, 0.25 μl of 100x DMDSO, and deionized H₂O up to 25 μl. The PCR conditions used were 98 °C for 30 seconds; 17 cycles of each at 98 °C for 10 seconds, 65 °C for 30 seconds, 72 °C for 4 minutes; then finally 72 °C for 10 minutes. The PCR products were incubated with 1 μl Oligo enzym (New England Biolabs, Ipswich, MA) at 95 °C for 1 hour, and 1 μl of the digested products transformed into NovaBlue Supercompetent cells (EMD Millipore, Darmstadt, Germany).

Nur77-GFP Jurkat 76 TCRαβ cell line

To characterize the functionality of TCRαβ or γδ clones, we established the Nur77-GFP Jurkat 76 TCRαβ cell line (NJ76 cells). After linearization of a Nur77-GFP recombinant Bac clone (constructed based on pTARBAC) by using BAC DNA, 2 μl 10x reaction buffer, 10 units of Pi-Scel restriction enzyme (New England Biolabs), and nuclease-free water to make the volume up to 20 μl with incubation at 37 °C for 3 hours and inactivation at 65 °C for 20 minutes, we added 80 μl of nuclease-free water, 15 μl of sterile sodium acetate (3M, pH 7.0), and 300 μl of ethanol to the reaction mixture, and centrifuged at 12,000g for 30 minutes at 4 °C. The resulting DNA pellet was washed with 75% ethanol, dried in the air, and resuspended by Tris-EDTA buffer (pH 8.0). We used the Neon Transfection System (Invitrogen) following the manufacturer’s instruction to transfect the linearized BAC DNA into the human Jurkat 76 TCRαβ cell line (2×10⁶ cells/ml, 100 μl), with three pulses with a voltage of 1,350V and a width of 10 ms. Cells were then cultured in complete-RPMI 1640 medium containing 300 μg/ml Genetin (Invitrogen) for selection.

Stimulation of KPB1703 TCRγδ NJ76 cells (PB1-NJ76) by flupeptide PB1

NJ76 cell transfected with a murine KPB1703-specific TCRγδ derived from influenza-infected mice and transfected cells were incubated with mouse splenocytes (cell number ratio of PB1-NJ76/splenocytes is 2:1), the influenza PB1703 peptide (1 μmol/ml), mouse splenocytes and peptide, and mouse γ-CD (Biolegend, 2C11; 10 μg/ml) and human γ-CD28 (Biolegend, CD28:2; 10 μg/ml) in c-RPMI 1640 medium at 37 °C for 4 hours. The GFP expression in the mouse TCRγδ CD3 cell population was quantified by flow cytometry.

Stimulation of TRGV9/TRDV2-NJ76 cells by Zoledronic acid

NJ76 cells transfected with a TRGV9/TRDV2 clone were incubated with 50 μg/ml zoledronic acid (Zometa) in c-RPMI 1640 medium at 37 °C
for 3 hours, washed three times and incubated for 12 hours. The GFP expression in the TCRβγ CD3 cell population was quantified by flow cytometry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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