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Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Measuring T cell receptor and T cell gene expression diversity in antigen-responsive human CD4⁺ T cells

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ARTICLE INFO

Article history:

Received 2 August 2013

Accepted 11 November 2013

Available online xxx

Keywords:

Human

T cells

T cell receptor

Gene expression

ABSTRACT

T cells have diversity in TCR, epitope recognition, and cytokine production, and can be used for immune monitoring. Furthermore, clonal expansion of TCR families in disease may provide opportunities for TCR-directed therapies. We developed methodology for sequencing expressed genes of TCR alpha and beta chains from single cells and applied this to vaccine (tetanus-toxoid)-responsive CD4⁺ T cells. TCR alpha and beta chains were both successfully sequenced in 1309 (43%) of 3038 CD4⁺ T cells yielding 677 different receptors. *TRAV* and *TRBV* gene usage differed between tetanus-toxoid-responsive and non-responsive cells ($p = 0.004$ and 0.0002), and there was extensive TCR diversity in tetanus-toxoid-responsive cells within individuals. Identical TCRs could be recovered in different samples from the same subject: TCRs identified after booster vaccination were frequent in pre-booster memory T cells (31% of pre-booster TCR), and also identified in pre-booster vaccination naïve cells (6.5%). No TCR was shared between subjects, but tetanus toxoid-responsive cells sharing one of their TCR chains were observed within and between subjects. Coupling single-cell gene expression profiling to TCR sequencing revealed examples of distinct cytokine profiles in cells bearing identical TCR. Novel molecular methodology demonstrates extensive diversity of Ag-responsive CD4⁺ T cells within and between individuals.

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1. Introduction

Antigen (Ag)-directed adaptive immune responses are a pathogenic feature of immune mediated disorders including allergy and autoimmunity. Thus, quantification and characterization of Ag- or peptide-specific T cells are an important part of immune monitoring of patients (Maecker et al., 2012). Ag-specific T cells are usually identified with functional assays

such as cell proliferation, or Major histocompatibility complex (MHC)–peptide multimer binding assays (Mannering et al., 2010). These assays are challenging (Herold et al., 2009), and with recent exceptions (Su et al., 2013; Varadarajan et al., 2012) provide relatively limited information with respect to diversity and phenotype of the response. The specificity of the adaptive response is provided by Ag receptors. Diversity of the T cell Ag receptor is high and results from combinatorial rearrangements of variable (V), joining (J) and diversity (D) gene segments. The resulting V-D-J sequence constitutes the hyper-variable complementary determining region 3. The T cell receptor (TCR) Complementarity determining region (CDR3) alpha and beta loops provide the major contact point with the Ag peptide and determine Ag specificity (Bentley and

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Mariuzza, 1996). Detailed CDR3 nucleotide sequence information is, therefore, important for understanding the diversity of T-cell responses and repertoire against a given Ag or peptide, and may permit follow-up of individual clones in a molecular fashion (Codina-Busqueta et al., 2011). TCR repertoire analysis has been mostly conducted by staining responder cells with TRBV or TRAV specific monoclonal antibodies (Reijonen et al., 2004) or by amplifying TCR sequences by PCR on cell pools or clones (Codina-Busqueta et al., 2011; Kent et al., 2005). Such analyses suggest that Ag- and peptide-specific T cells induced by infection, transplantation, autoimmunity or hypersensitivity have biased TCR profiles selected from the naive repertoire (Argaet et al., 1994; Godthelp et al., 2001; Kolopp-Sarda et al., 1999; Miles et al., 2005, 2011; Prisco et al., 1997; Quinn et al., 2006; Wucherpfennig et al., 1990). Most studies have been carried out on one TCR chain only, and mostly on the β chain (Douek et al., 2002; Li et al., 2011; Price et al., 2004; Reijonen et al., 2004). Efficient single cell analysis of the complete CDR3 region would increase information and allow TCR reconstruction. Parallel amplification of α and β chains from single cells has been described, albeit favoring amplification of certain subgroups, or of size limited pools and relatively low output (Arakaki et al., 2010; Kurokawa et al., 2001; Ozawa et al., 2008; Seitz et al., 2006). Recently, single-cell based strategies to uncover TCR alpha and beta chain repertoires have been developed and used to examine virus responsive, multiple sclerosis- and Psoriasis-associated T cells (Kim et al., 2012; Su et al., 2013; Wang et al., 2012). These strategies provided novel insight into the diversity of TCRs of T cells recognizing Ag or associated with disease. However, relatively few cells were analyzed and methods developed did not couple TCR analysis with functional or transcriptional analysis of cells.

Here we describe methods that permit higher throughput of Ag-specific TCR repertoire analysis based on alpha and beta CDR3 sequencing information from single CD4⁺ T cells. We exemplarily applied the method to describe the diversity of CD4⁺ T cell responses to the model vaccine Ag tetanus toxoid (TT). The method also successfully incorporated cytokine gene expression analysis from single cells.

2. Material and methods

2.1. Subjects

Sequential peripheral venous blood samples were obtained from four adults and two children with informed consent and ethical committee approval. Subject characteristics are described in S Table 1.

2.2. Isolation of tetanus toxoid-responsive CD4⁺ T cells

Fresh PBMC from heparinized peripheral blood were isolated over Ficoll gradient. CD4⁺ T cells (>96% purity) were first enriched by negative isolation using magnetic beads according to manufacturer's instructions (Miltenyi Biotec Inc, Auburn, CA). Subsequently, CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells were separated using magnetic anti-CD45RO beads. The CD4⁺ fraction was also collected and mixed 1:1 with the CD4⁺CD45RO⁺ or CD4⁺CD45RO⁻ fraction. 10⁷ mixed cells/ml in PBS were labeled with 0.5 μ M CFSE (Invitrogen, Carlsbad, CA) for 10 min at 37 °C. Staining was terminated with cold RPMI 1640 containing 5%

human serum (PAA Laboratories, Pasing, Austria), followed by an additional PBS wash. Stained mixed cells (10⁵ CD4⁺ T cells/well) were cultured in each of 10 wells of round-bottom 96-well microtiter plates in RPMI 1640 containing 5% heat-inactivated human AB serum without or with 1 μ l/ml TT (Sanofi Pasteur MSD, Leimen, Germany) for 4 days or 6 days. Cells were harvested and stained using the following monoclonal antibodies from BD-Pharmingen (San Diego, CA): anti-CD4 PB (clone RPA-T4), anti-CD25 APC-Cy7 (clone M-A251), anti-CD45RA APC (clone HI100), anti-CD45RO PE-Cy7 (clone UCHL1) and anti-CD134 PE (clone ACT35). 7-AAD (BD Pharmingen) was used to exclude dead cells. Cells were washed twice with PBS with 1% HS and acquired on a Becton Dickinson FACS Aria II flow cytometer with FACS Diva software and analyzed using FlowJo software version 7.6.1 (TreeStar). CD4⁺ T cells that had proliferated as determined by CFSE dilution and which had up-regulated CD25, CD45RO and CD134 were identified as responding cells and single cell sorted directly into 96-well PCR plates containing 5 μ l PBS (prepared with DEPC H₂O). Non-proliferating cells (CFSE^{high}, CD25⁻) and non-stimulated cells were also sorted for some experiments.

2.3. TCR alpha and beta chain PCR

Reverse Transcription (RT) was performed for 60' at 37 °C using reverse primers (0.13 μ M final concentration) for the α and the β chain constant regions (C-region 3' TCR A3, C-region 3' TCR B5; for all primer sequences see Table 1) and MuLV (Applied Biosystems, Life Technologies, NY, USA) in 15 μ l with 3.3 mM MgCl₂, 1 mM dNTPs, 40 units rRNasin® (Promega, Madison, Wisconsin, USA). A first Polymerase chain reaction (PCR) round was performed on the RT product using a mix of 17 and 12 tagged (HTSP-“tag” (HT)) variable region primers covering all subgroups of TCR alpha and beta, respectively, and constant region primers C-region 3' TCR A4 and C-region 3' TCR B4 (final primer concentrations: 0.003 μ M for TCR alpha and 0.015 μ M for TCR beta), with AmpliTaq Gold360 (New England Biolabs Inc., Ipswich, MA, USA) in 85 μ l (200 μ M dNTPs, 3.3 mM MgCl₂, 5% DMSO) with 30 cycles (95 °C 45"; 49 °C with 0.3 °C increment/cycle 1'; 72 °C 1'30"). Note that replacing MuLV by the qScript™ cDNA SuperMix (Quanta BIOSCIENCES, Inc., Gaithersburg, MD, USA) and AmpliTaq Gold360 by the TATAA PreAmp GrandMaster® Mix (TATAA Biocenter AB, Göteborg, Sweden) in recent experiments improved results. A second round nested PCR was performed for each chain separately on 3 μ l of the first round product, using the HTSP-“tag”-primer and constant region primers C-region 3' TCR B1 or C-region 3' TCR A1 to amplify 300 bp fragments containing 40 bp of the variable, the complete J and D genes and 20 bp of the constant regions with TaKaRa Ex Taq™ (TAKARA BIO INC., Japan) in 15 μ l (0.4 μ M Primer, 0.2 mM dNTPs) with 40 cycles (94 °C 1'; 56 °C 40"; 72 °C 30"). PCR products were Sanger-sequenced using HTSP-“tag” primer.

2.4. Cytokine and transcription factor real time PCR

The experimental strategy was based on a previous report (Peixoto et al., 2004). All gene sequence data was obtained from the Ensemble Project database. Primers for *IFNG* are described (Peixoto et al., 2004). Primers for other genes were

Table 1

Primers used for TCR amplification and qPCR.

TCR alpha	
C-region 3' TCR A1	TCGGTGAATAGGCAGACAGA
C-region 3' TCR A3	CACCTGTTGCTCTTGAAGTCC
C-region 3' TCR A4	AGACCTCATGTCTAGCACAG
Vregion 5' TCR A– 36/39/27/35–HT	GAAACAAGAATAGAAGGAGATATTGTAAGCCTACWWTGGTAC ARGCAGGA
Vregion 5' TCR A– 24–HT	GAAACAAGAATAGAAGGAGATATTGTATTACACTGGTACAGATGGGAAC
Vregion 5' TCR A– 5/13/34/20/10–HT	GAAACAAGAATAGAAGGAGATATTGTAACACTGGT ATARGCAARADHHTGG
Vregion 5' TCR A– 3n–HT	GAAACAAGAATAGAAGGAGATATTGTACTTTTTTGGT ATGTTCAATACCCCAAC
Vregion 5' TCR A– 40/9/18–HT	GAAACAAGAATAGAAGGAGATATTGTACTTTTTCTGGT ATG T SS AA TA YC
Vregion 5' TCR A– 6/17–HT	GAAACAAGAATAGAAGGAGATATTGTAACCTACAG TGG T AYMGAC AA
Vregion 5' TCR A– 8/16–HT	GAAACAAGAATAGAAGGAGATATTGTATTCTGGT ATGTCC AGT AC Y C
Vregion 5' TCR A– 25–HT	GAAACAAGAATAGAAGGAGATATTGTACTCTGGTGG ACATCCCG
Vregion 5' TCR A– 2n–HT	GAAACAAGAATAGAAGGAGATATTGTATTG TGGC ACCTTCACTTC
Vregion 5' TCR A– 26–HT	GAAACAAGAATAGAAGGAGATATTGTAAGTATGTGATTGGT ATCG ACAG ATT
Vregion 5' TCR A– 4/23–HT	GAAACAAGAATAGAAGGAGATATTGTATGGTACCAA CAR TT Y CCY RG
Vregion 5' TCR A– 29–HT	GAAACAAGAATAGAAGGAGATATTGTATCCTATGGT ACAAAAAA TACCC TGC
Vregion 5' TCR A– 7/14/19–HT	GAAACAAGAATAGAAGGAGATATTGTARYWSTGGT ACA R G CA RMMYA
Vregion 5' TCR A– 1n–HT	GAAACAAGAATAGAAGGAGATATTGTAGTTTTATGGGCTGTCTGGT
Vregion 5' TCR A– 41–HT	GAAACAAGAATAGAAGGAGATATTGTACTTACACTGGCTGCAACA
V–region 5' TCR A–1–HT	GAAACAAGAATAGAAGGAGATATTGTANTGGTACMRRYARRA
V–region 5' TCR A–3–HT	GAAACAAGAATAGAAGGAGATATTGTATGGTTTCATCAAAACCTT
TCR beta	
C–region 3' TCR B4	GTCCGGGTAGAAGCCTGT
C–region 3' TCR B1	CTTTTGGGTGTTGGGAGATC
C–region 3' TCR B5	ACTTGACAGCGGAAGTGGTT
VAR–B1	GAAACAAGAATAGAAGGAGATATTGTATGTWYTGTAHMRWCAG
VAR–B2	GAAACAAGAATAGAAGGAGATATTGTAGTWYTGATCRACAAG
VAR–B3	GAAACAAGAATAGAAGGAGATATTGTASTYWWCTGGTACMDRSAG
VAR–B4	GAAACAAGAATAGAAGGAGATATTGTAATTGGTATCGACGTGTTATG
VAR–B5	GAAACAAGAATAGAAGGAGATATTGTATTTWTTGGTAYCRACAG
VAR–B6	GAAACAAGAATAGAAGGAGATATTGTAGTTTACTGGTATCRKMG
VAR–B7	GAAACAAGAATAGAAGGAGATATTGTATTGGTATCAGCAGAATCAG
VAR–B8	GAAACAAGAATAGAAGGAGATATTGTATGCTCGGTATCGACAAG
VAR–B9	GAAACAAGAATAGAAGGAGATATTGTATRTMCTGGTAYMRACARG
VAR–B10	GAAACAAGAATAGAAGGAGATATTGTAGTATTGTACAAAGCARARWGC
VAR–B11	GAAACAAGAATAGAAGGAGATATTGTATGTTCTGGTACYGTACAGC
VAR–B12	GAAACAAGAATAGAAGGAGATATTGTACTATTGGTACAGACAAATC
HTSP – (“tag”)	GAA ACA AGA ATA GAA GGA GAT ATT GTA
Cytokines and Transcription Factors	
RT Primers	
IL10n_1-3'	CACGGCCTTGCTCTTGTTT
INFg-1_3'	TGGATGCTCTGGTCATCTTT
IL4_1_3'	CTCTGGTTGGCTTCCCTCAC
IL17f_1-3'	GGGGACAGAGTTCATGTGGT
Foxp3n-1_3	CACAGATGAAGCCTTGGTCA
SRP14_3'	GCTGCTGCTTTGGCTTCTT
PCR1	
IL10n2_1_5'	TGAAGGATCAGCTGGACAAC
IL10n_1-3'	CACGGCCTTGCTCTTGTTT
INFg-1_5'	CTGTACTGCCAGGACCCAT
INFg-1_3'	TGGATGCTCTGGTCATCTTT
IL4_1-5'	GGCTCCAAGAACACAACCTG
IL4_1_3'	CTCTGGTTGGCTTCCCTCAC
IL17f_1-5'	GCCCAAAATCTGAGGACAAG
IL17f_1-3'	GGGGACAGAGTTCATGTGGT
Foxp3n-1_5'	CAAAATGGTCTGCAAGTGG
Foxp3n-1_3	CACAGATGAAGCCTTGGTCA
SRP14_3'	GCTGCTGCTTTGGCTTCTT
SRP14_5'	TATGACGGTCAACCAAAACC
Real time PCR Primers	
IL10n2_2_5'	AGCCTTGCTGAGATGATCC
IL10n_1-3'	CACGGCCTTGCTCTTGTTT
INFg-2_5'	GGTCAATTCAGATGTAGCGGA
INFg-1_3'	TGGATGCTCTGGTCATCTTT
IL4_2-5'	GCCAGTTCACAGCCACCAT
IL4_1_3'	CTCTGGTTGGCTTCCCTCAC
IL17_2-5'	ACCAATCCAAAAGTCTCTC
IL17f_1-3'	GGGGACAGAGTTCATGTGGT
Foxp3n-2_5'	CCAGAGGACTTCTCAAGCA

(continued on next page)

Table 1 (continued)

TCR alpha	
Foxp3n-1_3	CACAGATGAAGCCTTGGTCA
SRP14_3'	GCTGCTGCTTTGGTCTTCTT
SRP14_5'-2	TATGACGGTCAACCAAACC

designed with CLC DNA workbench 5.6 (CLC bio A/S) and Primer3 (Rozen and Skaletsky, 2000) to span exon boundaries (Table 1). For all primer pairs used, verification that no competition between primer pairs was taking place during amplification was performed by amplification with either the primer pairs for each gene individually or multiplexed. The corresponding gene specific primers were added to the RT (0.13 μ M) and into the first round PCR reaction (0.015 μ M) as described above in addition to the TCR alpha and TCR beta primers. To quantify expression, real time PCR was carried out for each gene separately using 0.8 μ l of the first round PCR reaction and specific primers with AccuPrime™ Taq DNA Polymerase (Life Technologies) on a LightCycler®480 (Roche, Basel, Switzerland) in 10 μ l (0.2 μ M primer, 1x EvaGreen® Biotium Inc., CA), with a hot start (1 \times 94 °C, 2') and 45 cycles (94 °C 15"; 60 °C 15"; 68 °C 20").

Reproducibility of the data was assessed for the qPCR and for the first PCR round (PCR1). CPs obtained for SRP14, IFN γ , IL4, IL10, IL17f and FOXP3 from experiments for 41 stimulated cells and 36 resting cells were compared over three PCR1 experiments and 3 independent qPCR experiments. CP profiles were similar in the replicates for each cell (data not shown). Single cell CP values were processed using the GenEx Pro 5.3.6 Software (Copyright © MultiD 2006–2013).

2.5. Cloning of PCR fragments

PCR fragments obtained from single cell amplifications were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and cloned into vector pGEM®-T Easy (Promega). For reconfirmation of alpha or beta chains shared between TCRs, PCR1 and PCR2 were repeated from the remaining RT, products, subcloned, and 6–10 clones Sanger-sequenced.

2.6. Sequence analysis, CDR3 identification and TCR comparison

Analysis of TCR alpha and beta sequences was conducted with reference to the IMGT database (Lefranc, 2008). Junction peptide amino acid sequences were extracted and analyzed using KNIME 2.5.2 (Berthold et al., 2007). Up to two amino acid mismatches were allowed for TCR chain identity. Traces of sequences that looked very similar but did not follow this rule or sequences containing uncertainties were analyzed manually and if possible, corrected. Low quality sequence traces were disregarded.

2.7. Statistical analysis

TRAV and TRBV gene usages in cell populations were compared using Fisher's Exact test for count data. Recovery of TCR from naïve or memory T cells in post-booster vaccination memory T cells was compared using the Chi-squared test. Statistical analysis was conducted using R64 (2.15.0 2012-03-30; The R Foundation for Statistical Computing).

3. Results

3.1. TCR sequences from single CD4⁺ T cells

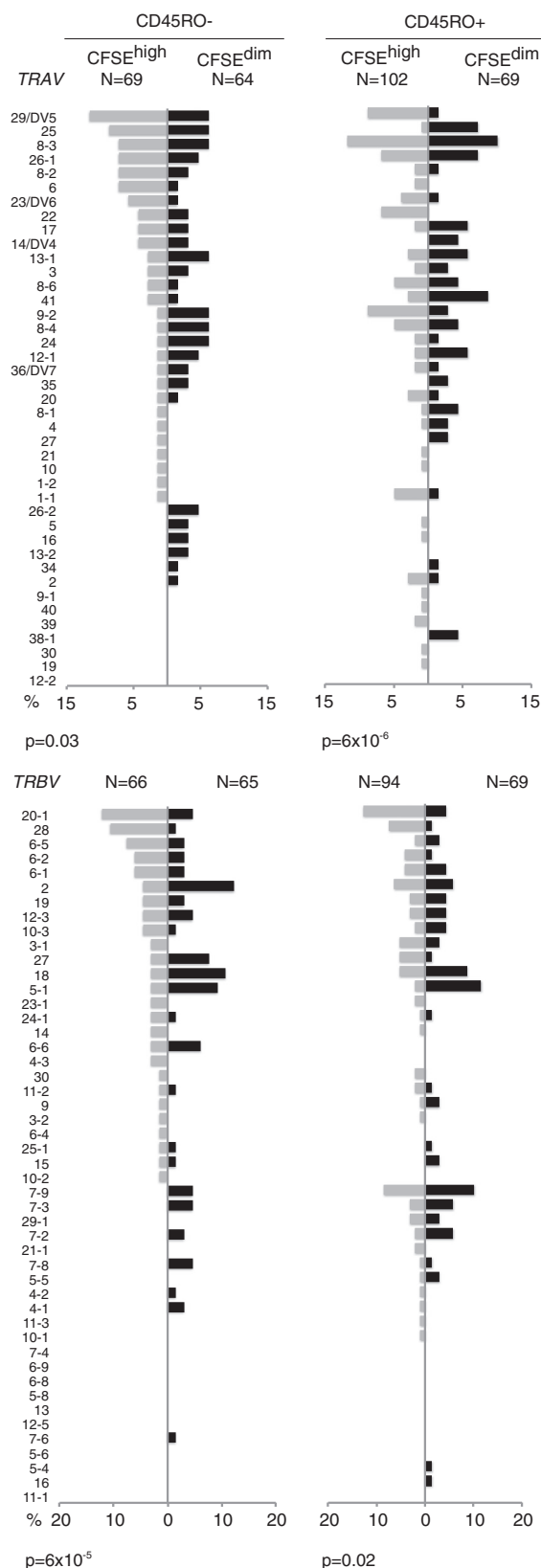
In total, 2523 TT-responsive and 515 non-responsive cells obtained from four adults and two children were processed for single cell TCR sequencing (S Table 2). Beta chain sequences were obtained for 2041 cells (67.2%), alpha chain sequences for 1839 (60.5%) cells, and sequences of both chains for 1309 (43.1%) cells, thereof 1120 (36.9%) sequences were of a quality allowing unambiguous reading. In total, 538 distinct Ag-responsive and 139 non-responsive TCRs were observed. All functional TCR alpha and beta subgroups were found, except for TRAV7 and 11, but these were amplified from another set of cells (not shown) (S Table 3). All non-responsive CD4⁺CD25⁻ T cells examined had unique TCRs (not shown).

3.2. TCR variable gene usage

As expected, usage of TRAV and TRBV genes in TT-responding (CFSE^{dim}) cells differed from that in the corresponding non-responder (CFSE^{high}) cells in the same subject (TRAV: $p = 0.03$ for CD45RO⁻, $p = 6 \times 10^{-6}$ for CD45RO⁺; TRBV: $p = 6 \times 10^{-5}$ for CD45RO⁻ cells, $p = 0.02$ for CD45RO⁺ cells; Fig. 1). Encouragingly, variable gene usage did not differ in unstimulated CD45RO⁺ CD4⁺ T cells of two samples taken 1 year apart from the same individual (TRAV, $p = 0.05$; TRBV, $p = 0.16$; not shown), and in CD45RO⁺ TT responder cells from two post-booster vaccination samples taken from the same individual (TRAV, $p = 0.86$; TRBV, $p = 0.14$; not shown). TRAV and TRBV gene distributions of the CD45RO⁺ non-responding cells differed slightly to that of CD45RO⁻ non-responding cells ($p = 0.006$ and $p = 0.02$).

3.3. Tracking Ag-responsive CD4⁺ T cells across cell subsets and over time

Vaccination is a useful setting to follow the conversion of naïve to memory Ag-responding T cells. TT was used as a model Ag in an attempt to validate the TCR sequencing methods (Fig. 2). A previously immunized adult (A1), was given a TT booster vaccination, and blood drawn at -10 (pre-booster), +18 (post-booster 1) and +180 days (post-booster 2). Increased antibody responses to TT were observed after vaccination (data not shown). CD45RO⁻ and CD45RO⁺ CD4⁺ T cells were isolated from the pre-booster sample and CD45RO⁺ CD4⁺ T cells from post-booster samples. Each of the 4 isolated cell populations was stimulated with TT in 10 wells each containing 10⁵ CD4⁺ T cells for 4 days. TT-responsive cells (CFSE^{dim}, CD25⁺) from each well were single cell sorted and processed. Purity of sorted TT-responsive cells was 99% (data not shown). Responding cells at the end of culture were lower in the CD4⁺CD45RO⁻ cell population (mean, 0.22 \pm 0.13) than



the CD4⁺CD45RO⁺ cell population (mean, 13.4 ± 3.0; p = 2.3 × 10⁻⁷).

High quality sequences for both TCR alpha and beta chain sequences were obtained for 571 (47.8%) of the processed TT-responsive T cells. Overall, 259 different TCRs were identified in the four TT-responsive populations from subject A1. This includes 61 different TCRs in the pre-booster CD45RO⁻ TT-responders, 77 in the pre-booster CD45RO⁺ TT-responders, 93 in the first post-booster CD45RO⁺ TT-responders, and 82 in the second post-booster CD45RO⁺ TT-responders, with 64 TCRs observed in more than one cell representing enriched TCRs (Fig. 3A; TCR sequences given in S Table 2). Many enriched TCRs were observed in multiple cells from a single well, and several were observed in multiple wells from the same donor T cell source, or wells corresponding to different cell sources (CD45RO⁻ and CD45RO⁺ CD4⁺ T cells) or from the different samples taken from donor A1 (Fig. 3A). Recovery of TCRs in multiple wells was low for CD45RO⁻ CD4⁺ TT-responsive cells (1 of 61 TCRs was found in multiple wells), consistent with an expected low precursor frequency in naïve CD4⁺ T cells. In contrast, TCRs of the memory CD45RO⁺ TT responder cells were often seen in multiple wells (29 of 218 TCRs, p = 0.03), and some were observed in 10 of the 10 wells sampled.

Amongst the 61 TCRs identified in the pre-booster CD45RO⁻ TT-responsive cells, 4 (6.5%) were also found in the post-booster CD45RO⁺ TT responsive pools (Fig. 3B). Higher recovery of TCRs after booster vaccination was observed from the pre-booster CD45RO⁺ TT responders (24 of 77, 31%; p = 0.006). Two TCRs were observed in all four CD45RO⁻ and CD45RO⁺ TT-responsive pools and a further 13 were present in all three CD45RO⁺ TT-responsive pools suggesting that they were relatively abundant and persistent in this subject. These data show the ability to track vaccine responsive T cell clones over time.

3.4. Tetanus-toxoid responses to primary vaccination

We subsequently examined responses in 2 children before and after their first exposure to TT vaccine. TCR alpha and beta chain sequences were obtained from 38 (infant 1) and 44 (infant 2) pre-vaccination TT-responsive CD4⁺ T cells, and from 102 (infant 1) and 89 (infant 2) post-vaccination TT-responsive CD4⁺ T cells (S Table 2). In total, 82 TCRs were observed in TT-responsive cells from infant 1 and 92 from infant 2. Few were observed in multiple wells or samples: 3 (4%) of 68 TCRs from the pre-vaccination responder cells and 22 (21%) of 106 TCRs from the post-vaccination responder cells (p = 0.02 pre- vs post-vaccination; Fig. 3C). No TCR was found in both pre- and post-vaccination responding cells.

3.5. Ag-responsive TCR sharing between subjects

We next asked whether some of the TCRs of the TT-responsive cells were shared between subjects. The HLA type of the hyperimmunized adult A1 included the HLA DRB1*0401 allele. This was common to one of the children

Fig. 1. TCR variable gene usage in CD4⁺ T cell responses to Ag. *TRAV* (upper panels) and *TRBV* (lower panels) frequency in TCRs of responsive (CFSE^{dim}) and non-responsive (CFSE^{high}) CD45RO⁻ or CD45RO⁺ CD4⁺ T cells to tetanus-toxoid in subject A1.

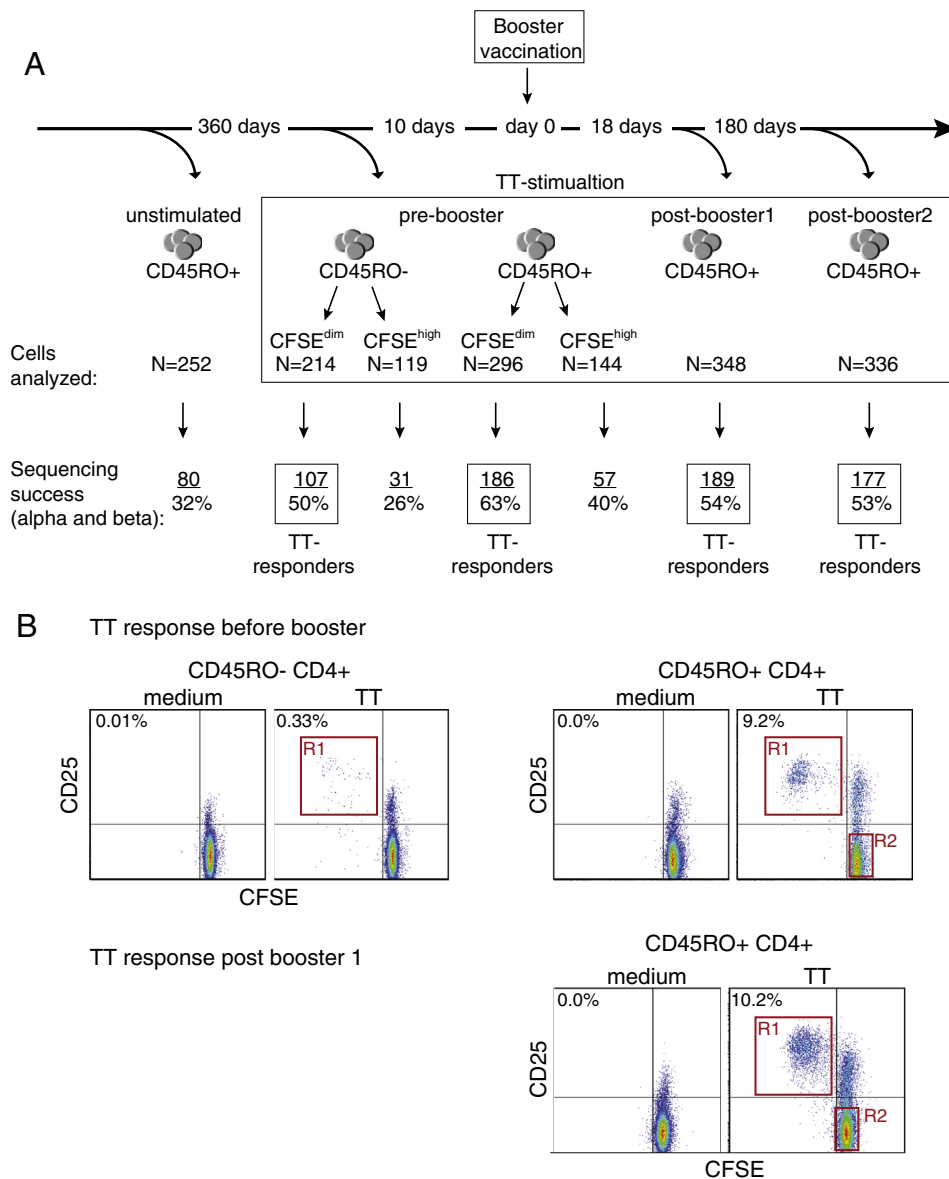


Fig. 2. Isolation of antigen responding and non responding cells. A. Schematic showing time between samples, cell type, numbers of single cells processed for sequencing, and sequencing success. B. Representative FACS plots of CD4⁺ T cells responses to medium or TT showing gates for sorted responding (R1) and non-responding (R2) CD4⁺ T cells.

and also to another adult, A4, in whom 44 TCRs from TT-responsive CD45RO⁺ T cells were identified. None of the TCRs or their CDR3 sequences from TT-responsive CD4⁺ T cells was identical at both alpha and beta chains between subjects. However, some TCRs identified from the responsive cells shared single chains (Table 2). TCRs grouped in S α 1 and S α 2 are mostly derived from unique precursor cells in subject A1 and share their alpha chains with multiple beta chains. The two TCRs grouped in S β 1 have the same beta chain with different alpha chains and are derived from two different subjects. All nucleotide sequences of these examples of shared alpha or beta chains were identical.

3.6. Cells with identical TCRs can differ in gene expression phenotype

We observed that the same TCR could be obtained for multiple cells within or between wells and samples suggesting that these may be clones. We therefore developed methods that allowed us to examine both TCR and mRNA expression of IFN γ , IL-4, IL-10, IL17f, FOXP3 and housekeeping genes, SRP14 and RPL13A in single cells and analyzed selected TT-responsive cells from three adult donors (Fig. 4). In A1, TCR55 showed evidence of clonality of the multiple cells with all but one cell expressing IFN γ . However, for

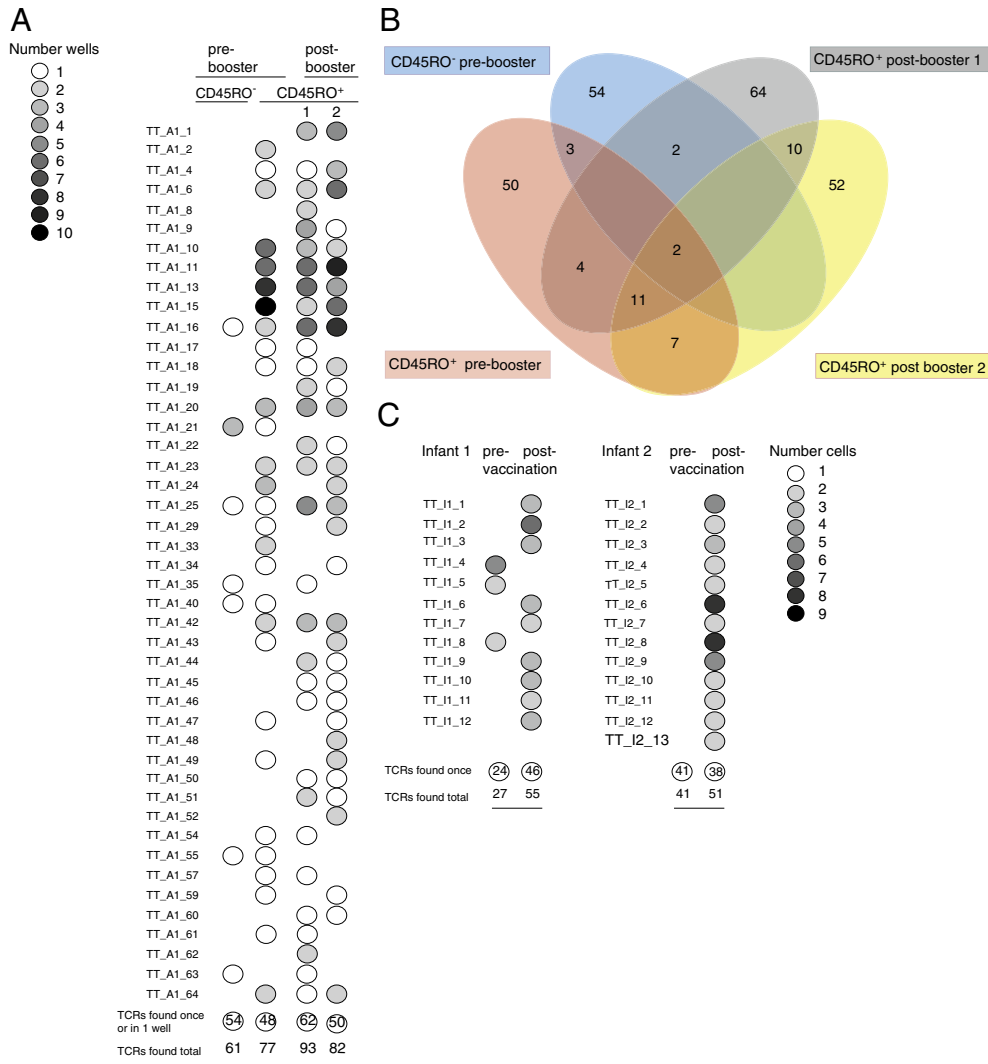


Fig. 3. TCR recovery in TT responsive CD4⁺ T cells. A. TCRs of A1 TT-responding cells in pre-boost CD45RO⁻ and CD45RO⁺ and post-boost CD45RO⁺ CD4⁺ T cells. TCRs found in multiple cell pools or wells together with the number of wells harboring each TCR are shown. Numbers of TCRs found once are also indicated. B. Venn diagram of different TCRs. C. TCRs TT-responding CD4⁺ T cells obtained pre- and post-vaccination in two children around their first vaccination. TCRs found in multiple cell pools or wells together with the number of cells harboring each TCR are shown. Numbers of TCRs found once are also indicated.

TT15, 12 cells were examined, of which 4 cells had an IFN γ phenotype, 1 cell an IL-4 phenotype, 1 cell a FOXP3 phenotype, 2 cells a mixed phenotype and 4 cells showed no mRNA

Table 2
TCRs of TT-responsive CD4⁺ T cells in which alpha or beta chain sharing was observed.

TCR group	Beta CDR3	Alpha CDR3	Subject	Cells (n)
TT S α 1	CASSQDLGGNTEAFF	CAGSGGYQKVTF	A1	1
	CSAGLGLGEQYF	CAGSGGYQKVTF	A1	1
	CASRSQQGGTDTQHF	CAGSGGYQKVTF	A1	1
	CASSVAIGMSSYNEQFF	CAGSGGYQKVTF	A1	1
TT S α 2	CASNGPGQIVTFF	CAAHFGNEKLTF	A1	1
	CASSGPGQGGTFF	CAAHFGNEKLTF	A1	2
TT S β 1	CASTRDSYEQYF	CATGNNTGTASKLTF	I1	1
	CASTRDSYEQYF	CIVLTGTASKLTF	A1	1

expression of any of the genes. For this TCR, all cells had identical amino acid sequences with no amino acid uncertainty. For Adults 2 and 3 similar observations were made. For example, TCR5 in adult 2 had a clonal IL-4 phenotype, while TCR4 and 6 from adult 2, and TCR1 from adult 3 had evidence of cells where phenotype differed in the presence of identical TCR. The remaining TCRs examined were uninformative with respect to gene expression diversity.

4. Discussion and conclusions

We have developed an efficient approach to characterize the molecular signature of single Ag-responsive T cells that provides the sequence of the TCR alpha and beta chain and quantitative expression of T cell phenotype signature genes. Examining several thousand Ag-responsive CD4⁺ T cells, we show marked diversity in TCRs used for Ag responses. Despite this, it was

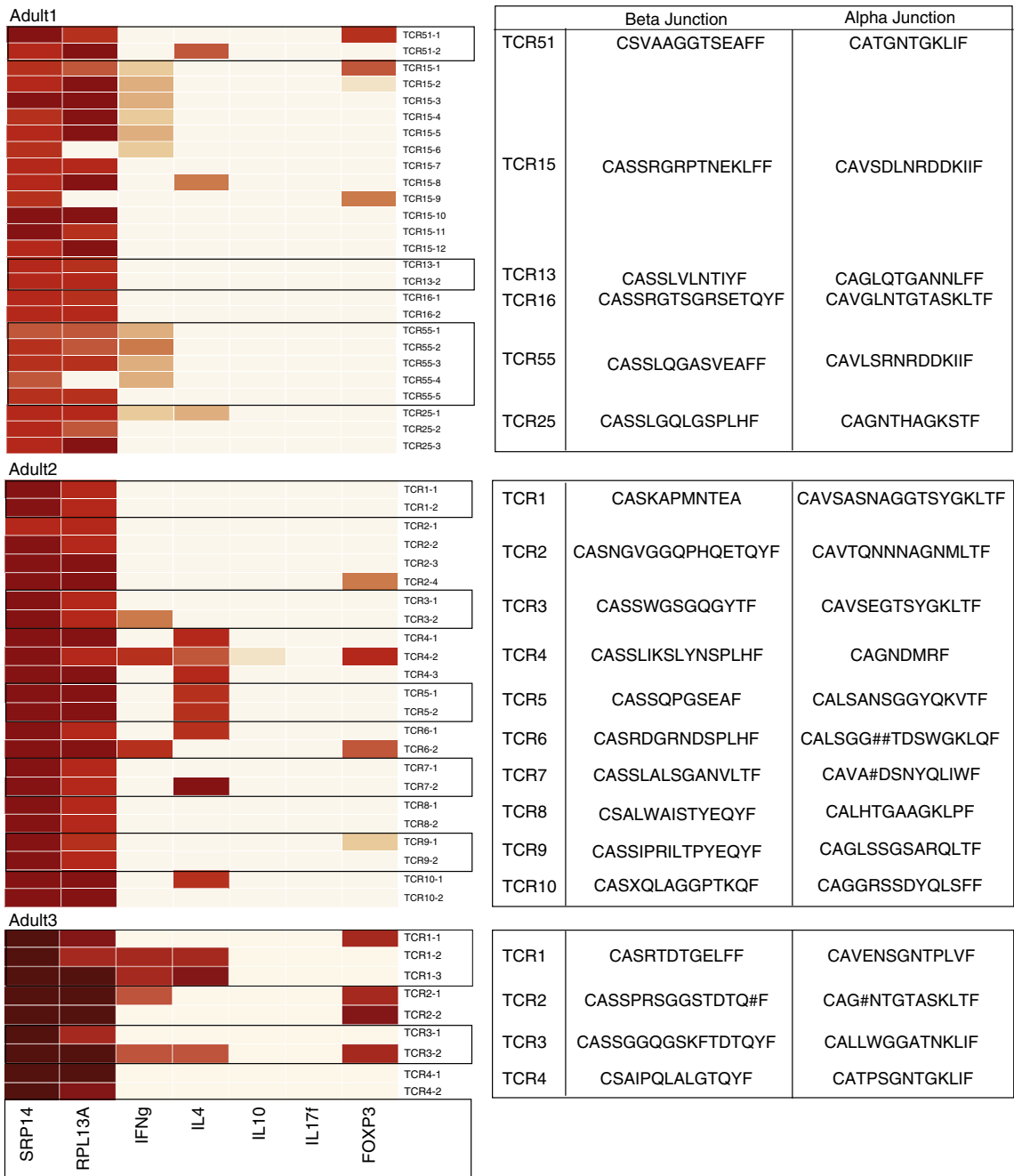


Fig. 4. Differential gene expression phenotype in T cells with identical TCR. Heat map of qPCR expression levels for IFN γ , IL4, IL10, IL17f, FOXP3 and for 2 housekeeping genes, SRP14 and RPL13A in individual cells from three subjects (Adults 1, 2, 3). Amino acid translation of the beta and alpha junctions for each TCR is shown in the table.

possible to track CD4⁺ T cells with identical TCRs from naïve through to memory CD4⁺ T cells and in multiple samples.

Apart from the strengths of this study in providing validated methods to obtain the largest annotation of TCRs from human Ag-responsive T cells, some limitations are acknowledged. First, although TCR alpha and beta sequencing efficiency from single cells was consistent, the overall efficiency with high quality sequences was still less than 50%, and thus TCR in over

half of the Ag-responsive T cells could not be surveyed. The failure of TCR sequences in a little over half of the CD4⁺T cells could be due to a lower efficiency for certain alpha or beta genes or poor mRNA quality. We have noted that efficiency is affected by the amount of care taken to avoid RNase contamination at all cell isolation steps. Second, the manuscript reports methodology and shows an example of how it can be applied to obtain information on T cell diversity and phenotype. Thus, a number of the findings may be biased by

the methods used to identify and isolate TT-responsive cells. For example, although 10^6 CD4⁺ T cells were tested in most samples (10 wells at 10^5 cells/well), not all responding cells were sorted and processed, and it is likely that more identity between wells and samples would have been seen if all cells were analyzed. Furthermore, it is likely that a portion of the Ag-responsive T cells examined is responsive because of bystander or other non-cognate Ag-specific stimulation (Novak et al., 1999, 2001). Validation of Ag specificity of the TCRs will require their expression and testing in secondary stimulation assays. We used Ag stimulation to recover Ag-responsive cells and findings may have differed if tetramer positive cells were isolated directly from unstimulated PBMC. Of note, the efficiency of single cell TCR sequencing was also high when applied to unstimulated *ex vivo* multimer isolated CD4⁺ and CD8⁺ T cells (data not shown). Indeed, as a further validation, *ex vivo* stained DRB1*0401-tetanus toxoid₅₀₅₋₅₂₅ tetramer positive cells obtained 540 days after booster vaccination from Adult 1 were sorted and TCR sequenced. Of these, over 10% had sequences that were also found in the whole antigen stimulated cells (data not shown). Moreover, TCR alpha chain deep sequencing on one million fresh CD4⁺ T cells from Adult 1 recovered 83 of the TCR alpha chain sequences identified from TT-responsive single cells. It is also noted that the findings were generated using PBMC, and it is possible that the diversity will differ if other Ag-presenting cells such as dendritic cells are used. Finally our approach has only examined proliferating Ag-responsive T cells, and therefore does not include all Ag-responsive T cells.

An ability to obtain full TCR sequence information and product for subsequent cloning from almost 50% of single T cells is an important achievement. As compared to previous methods that rely on polyclonal expansion cloning of antigen-specific cells for sequencing (Kent et al., 2005), single cell TCR sequencing markedly reduces time and labor required to obtain and isolate TCR. Previous efforts at single cell TCR sequencing have typically provided less efficient TCR alpha and beta sequencing with information on hundreds of cells at most (Kim et al., 2012). We expect that the relatively high and consistent efficiency of TCR sequencing is partially due to primer selection, and the optimized RT-PCR protocol. Although the study was restricted to CD4⁺ T cells, similar TCR sequencing efficiency was obtained for single CD8⁺ T cells (not shown). A second advantage of the protocol is that it is one of the first studies to combine TCR sequencing with quantitative gene expression. We showed that cytokine gene expression can be informative to distinguish diversity. Moreover, we have now extended gene expression to substantially more genes. Of note, despite >95% efficiency for the housekeeping genes, a number of the responsive cells examined did not express a signature gene of typical CD4⁺ T cell phenotypes. This may in part be due to periods of decreased expression during cell proliferation and pulsed transcriptional bursts (Bengtsson et al., 2005; Chubb et al., 2006; Raj et al., 2006) rather than inefficient qPCR or true null phenotype cells. Further assessment of the time course of expression during cell cycle is required. Further improvements in gene expression from single cells are likely to be achieved with improved RNA seq methodology.

Diversity of TCRs against Ag is well documented (Kedzierska et al., 2006; Miconnet et al., 2011; Selin et al.,

2004). Nevertheless, we had not expected to find such a plethora of TCRs against any single Ag in the peripheral blood. The findings emphasize that while sampling a relatively small volume of blood from the total circulating volume may be able to consistently detect Ag-responsive cells, these responsive T cells are infrequently derived from the same T cell clone. These data highlight limitations of TCR repertoire analyses from less than 1% of the blood volume, and suggest caution when interpreting findings of infrequent Ag-responsive T cells.

In summary, we provide methodology for analyzing clonal diversity of Ag-responsive CD4⁺ T cells and show that diversity is marked within and between individuals. These methods which can be applied to T lymphocytes isolated by diverse strategies, can be used to help track T cell changes over time during disease, remission, and immunotherapy.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2013.11.003>.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

We are grateful to D. Walther, D. Kühn, V. Splith and A. Borrmann for technical assistance and thank M. Bickle (MPI, CBG, Dresden) for initiation of the development of KNIME workflows. P. van Endert provided helpful suggestions for the development of single cell RT-PCR protocol. The work was supported with funds from the Deutsche Forschungsgemeinschaft-Center for Regenerative Therapies Dresden, Cluster of Excellence (FZ 111), the Deutsche Forschungsgemeinschaft (DFG ZI-310/14-1 to 4), and the German Ministry for Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.).

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