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ORIGINAL ARTICLE

T-cell receptor excision circles and repertoire diversity in children with profound T-cell immunodeficiency

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T-cell receptor (TCR) repertoire

Background/Purpose(s): Approximately 40% of patients with profound T-cell immunodeficiency have no identified molecular basis. Early assessment of T-cell impairment is vital for medical intervention, if hematopoietic stem cell transplantation is needed. The dynamics of T-cell receptor excision circles (TRECs) revealing recent thymic output of naïve T cell and T-cell receptor (TCR) repertoire diversity reflecting broader responses to multiple antigens, are both important in resisting infections.
Methods: The TRECs value and TCR repertoire diversity were evaluated from peripheral blood mononuclear cells in patients with primary severe T cell immunodeficiency, to elucidate the T-cell response.
Results: In seven children with <30% of normal phytohemagglutinin (PHA)-stimulated lymphocyte proliferation, including two IL2RG (Try74Gly and Arg226Lys, X-linked) and one RAG2 mutations [(Ser205Tyr) and (del 1366T, frameshift, 484stop); autosomal recessive], lower TRECs

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value and oligo- and restricted TCR diversity patterns, were associated with increased susceptibility to opportunistic infections, but not inversely correlated to the severity and frequency of infections. Three patients had successful cord blood stem cell transplantation which reconstructed the T cell immunodeficiency, with normalized TRECs value and TCR repertoire diversity at 6 months post-transplant, without clinical events.
Conclusion: Low TRECs value and restricted TCR repertoire diversity can help in the early diagnosis of T cell immunodeficiency before irreversible sequelae and in the monitoring of post-transplantation T-cell immune reconstruction.

Introduction

Primary immunodeficiency diseases (PIDs) are natural knockout human models for investigating complex immune mechanisms of increased susceptibility to infections.[1,2] Identified genetic defects elucidate causal molecules in the host immunity's fight against pathogens. To date, at least 206 kinds of PIDs have been recognized and almost 110 genetic defects explored.[3] The clinical manifestations of PIDs with identified genetic defects are more predictable, because a relationship between genotype and phenotype exists despite rare exceptions.[4–6] In cases still without identified genetic defects, immune functional analysis has become more important and practical for aggressive intervention, like hematopoietic stem cell transplantation (HSCT), which is mandatory for rescuing PIDs with severe T and phagocytic disorders.[7–11] In patients with profound T-cell immunodeficiency of <30% mitogen [e.g., phytohemagglutinin (PHA)] proliferation function in healthy individuals,[12] often presenting as severe combined T and B immunodeficiency (SCID)], >95% of SCID patients have successful HSCT and excellent prognosis if diagnosed and transplanted before the age of 3 months.[9,13]

Patients with profound T-cell immunodeficiency and identified genetic defects are candidates for pre-natal diagnosis for early HSCT of the children if parents are carriers. However, in profound T-cell immunodeficiency like SCID, approximately 40% of patients (858/2157) remained without identified genetic defects, even after the end of the human genome projects in 2000.[14] Sporadic and/or *de novo* mutation cases are diagnosed only after severe and/or opportunistic infections and irreversible sequelae. Despite HSCT for such situations, the success rate of engraftment is reduced to <75% and the life quality is not as well tolerated.[11,13] To predict the phenotypic severity of profound T-cell immunodeficiency for early HSCT if indicated, T-cell receptor excision circles (TRECs) and T-cell receptor (TCR) repertoire are used to investigate T-cell production, development, and maturation if with severe and/or refractory infections before 3 months of age.

The majority of T cells generated in the thymus express TCR composed of alpha-(A) and beta-(B) chains, which are encoded by variable (V), diversity (D), and junctional (J) gene segments rearranged to form the highly diverse hypervariable TCR regions during the process of V(D)J recombination. These regions are largely responsible for the antigenic specificity of the individual TCR and are called "TCR repertoire".[15] Rearrangement of both the TCRA and TCRB genes during T-cell maturation produces TRECs, formed when the intervening stretches of DNA are excised as the "coding" ends which are joined to form a functional TCR gene in chromosomal DNA.[15,16] Such TRECs can be used to detect and quantify thymic output of alpha-beta ($\alpha\beta$)-T cells.[17]

Like conventional lymphocyte proliferation function to evaluate T cell immunity, TRECs and TCR repertoire in this study were used to qualify T-cell function and elucidate the relationship between phenotype and these functions on patients with profound T-cell immunodeficiency, but unknown genetic defects.

Materials and methods

Patients

The Human Investigation Committee approved this study and the participant's parents or guardians provided informed consent. Excluding those with malignancy, zinc deficiency, malnutrition, and human immunodeficiency virus (HIV) infection, patients with profound primary T-cell immunodeficiency characterized by lymphopenia, chronic diarrhea, failure to thrive, and/or opportunistic infections, with <30% normal PHA-stimulated proliferation function were enrolled.[12,18]

Heparinized venous blood samples (10—15 mL) from patients and healthy controls were sent to the laboratory within 72 hours. Serum immunoglobulins and lymphocyte subsets, including T-, B-, and NK-cells, activated lymphocytes, and memory cells were evaluated. Candidate genes were further sequenced as in a previous study.[19]

Lymphocyte proliferation function

To induce lymphocyte proliferation, peripheral blood mononuclear cells (PBMCs; 10^5/well) were incubated *in vitro* with concentrations of PHA, concanavalin A (ConA), and pokeweed mitogen (PWM) for 3 days, or the Candida antigen and BCG vaccine for 7 days. Stimulation was then incubated with ^{3}H-thymidine as previously described.[20]

T-cell receptor excision circles (TRECs) analysis

Real-time quantitative polymerase chain reaction (PCR) for detecting TRECs and β-actin was performed in a total volume of 20 mL containing 1X TaqMan Gene Expression

Master Mix (Applied Biosystems, Foster city, CA, USA), 0.5 mmol/L TREC primers, 0.25 mmol/L β-actin primers, 0.15 mmol/L TaqMan probes, and 0.8 mL 1% BSA (New England Biolabs, Ipswich, MA, USA). The DNA sequences of the primers and probes are listed in Table 1. The reactions were carried out on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems), with one cycle at 5°C for 2 minutes, one cycle at 95°C for 5 minutes, and 40 cycles at 95°C for 30 seconds and at 60°C for 60 seconds.

For data collection, a fixed cycle threshold (Ct) was set at the point when PCR amplification was still in the exponential phase. The Ct was the number of cycles at which the amplification plot, representing the fluorescence emission of the report dye, passed a fixed threshold. Serially diluted plasmids containing a known copy number of TRECs and β-actin were used to generate a calibration curve. The copy numbers of TRECs and β-actin were automatically determined based on this curve (a representative TRECs standard curve is demonstrated in Fig. 1). The PCR amplification of the β-actin gene was used as a reference gene to assess successful DNA extraction. The β-actin and Cα gene primers were also designed to generate amplicons flanking an intron/exon junction.

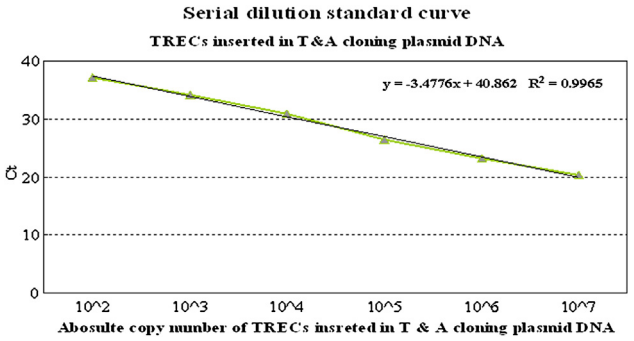

Figure 1. Real-time PCR amplification demonstrated a linear relationship between detectable PCR cycle number (Ct; Y axis) and serial log dilution of TREC copy number (X axis).

T-cell receptor (TCR) repertoire size (CDR3) spectra-typing

Total RNA was prepared from $CD4^+$ T cells after sorting as described.[21] Total RNA (2 ug) was synthesized as cDNA using

Table 1 Sequence of primers used for TREC and TCR repertoire spectra-typing

TRECs	Forward (5′-3′ sequence)	Backward (5′-3′ sequence)
TRECs	CCA TGC TGA CAC CTC TGG TT	TCG TGA GAA CGG TGA ATG AAG
β-actin	ATT TCC CTC TCA GGC ATG GA	CGT CAC ACT TCA TGA TGG AGT TG
Cα:	CCT GAT CCT CTT GTC CCA CAG	GGA TTT AGA GTC TCT CAG CTG GTA CA
TREC probe	6FAM-CAC GGT GAT GCA TAG GCA CCT GC-TAMRA	
β-actin probe	6FAM-GTG GCA TCC ACG AAA CTA-TAMRA	
Cα: probe	6FAM-GTG GCA TCC ACG AAA CTA-TAMRA	
TCR repertoire		
VB1	GCACAACAGTTCCCTGACTTGCAC	
VB2	TCATCAACCATGCAAGCCTGACCT	
VB3	GTCTCTAGAGAGAAGAAGGAGCGC	
VB4	ACATATGAGAGTGGATTTGTCATT	
VB5.1	ATACTTCAGTGAGACACAGAGAAAC	
VB6.1	AGGCCTGAGGGATCCGTCTC	
VB7	CCTGAATGCCCCAACAGCTCTC	
VB8	ATTTACTTTAACAACAACGTTCCG	
VB9	CCTAAATCTCCAGACAAAGCTCAC	
VB10	CTCCAAAAACTCATCCTGTACCTT	
VB11	TCAACAGTCTCCAGAATAAGGACG	
VB12	AAAGGAGAAGTCTCAGAT	
VB13.1	CAAGGAGAAGTCCCCAAT	
VB14	GTCTCTCGAAAAGAGAAGAGGAAT	
VB15	AGTGTCTCTCGACAGGCACAGGCT	
VB16	AAAGAGTCTAAACAGGATGAGTCC	
VB17	CAGATAGTAAATGACTTTCAG	
VB18	GATGAGTCAGGAATGCCAAAGGAA	
VB19	CAATGCCCCAAGAACGCACCCTGC	
VB20	AGCTCTGAGGTGCCCCAGAATCTC	
3'CB	TTCTGATGGCTCAAACAC	

Table 2 Immunologic functions and clinical manifestations of patients with profound T-cell immunodeficiency on diagnosis

	P1	P2	P3	P4	P5	P6	P7	Normal range
Sex/age at diagnosis	M/3M	M/4M	F/7M	M/17M	M/5M	M/5M	F/4M	(n = 7[c])
Genetic defects	Unidentified	Unidentified	Unidentified	Unidentified	IL2RG (Trp74Gly)	IL2RG (Arg226Lys)	RAG2 (heterozygous)[b]	
Lymphocyte (/mm3)	604	5700	295	114	297	7506	474	2500–5600
Immunoglobulin (mg/dL, IU/ml)								
IgG	124	231	146	1210[a]	245	99	83	294–1069
IgA	23	<6	<6	168	30	<6	<6	16–84
IgM	12	<4	<4	273	31	21	<4	41–149
IgE	<19	<19	<19	<19	<19	<19	<19	
Lymphocyte subsets (%)								
CD4	23	21	3	34	2	1	1	35–64
CD8	15	19	2	23	3	20	1	12–28
CD19	18	54	12	32	76	74	2	6–41
NK	47	15	54	8	2	2	92	3–18
CD4+ memory	16	16	2	11	2	0	0	2–22
CD19+ memory	2	2	15	0	2	0	0	1.4–6.6
Activated lymphocyte	3	7	2	39	16	31	19	4–26
Lymphocyte proliferation [c.p.m. for amitogens (PHA, ConA and PWM) and antigens (*Candida* and BCG)]								
PHA 10 μg/mL	3124	4235	2457	7498	4264	6547	3573	83,042–201,534
ConA 10 μg/mL	NA	2159	1897	3197	2716	4465	3198	41,326–85,507
PWM 0.1 μg/mL	NA	NA	NA	6381	1507	3123	2493	23,179–42,397
Candida 10 μg/mL	2348	1283	598	5465	1294	191	346	8457–24,269
BCG 0.25 μg/mL	NA	NA	477	2523	349	196	453	4267–14,738
Infections	Chronic diarrhea Pneumonia	CMV encephalitis Epilepsy Pneumonia	Salmonella sepsis Bronchiectasis Influenza pneumonia	BCGitis Candidiasis Pneumonia Bronchiectasis ORSA cellulites Chronic diarrhea	BCGitis *Pneumocystis jiroveci* pneumonia Bronchiectasis *E. coli* sepsis *Pseudomonas* sepsis	BCGitis *Pneumocystis jiroveci* pneumonia	Candidiasis *Pneumocystis jiroveci* pneumonia Bronchiectasis	
HSCT (source)	No	No	No	Yes (cord blood)	Yes (cord blood)	Yes (cord blood)	Yes (bone marrow)	
Prognosis (mortality cause)	Mortality at 5 mo (respiratory failure)	Mortality at 6 mo (respiratory failure)	Mortality at 8 mo (respiratory failure)	Alive	Alive	Alive	Mortality at 8 mo (pneumothorax)	

[a] After regular monthly IVIG infusion.
[b] Paternal allele with missense mutation Ser 205 Tyr and maternal allele with deletion mutation 1366 T, frameshift, stop at the 484.
[c] Normal range was obtained from 7 age-matched healthy controls receiving regular vaccination.

reverse transcriptase (Invitrogen. Each cDNA sample was amplified using a V beta-specific primer with a C-3' primer (Table 1) at a final concentration of 0.3 uM in each reaction. In a 1:3 dilution of fluorescent PCR products, 3 uL were combined with an equal amount of formamide dye and heat denatured at 90°C for 3 minutes. Applied Biosystems GeneScan Software (Foster City, CA, USA) was used for the collection and analysis of the fluorescent-labeled PCR products. A fluorescent size marker was employed to determine the length of each band.

For evaluation of fluorescent spectra-types, histograms were generated from data collected either by an ABI/DNA Sequencer or a Molecular Dynamics FluorImager. Using a labeled TCR BC region primer and TCR BV-specific primers, both band number and intensity were examined. The overall intensity of the bands was a function of the cycle number and the starting concentration of cDNA. The peaks' patterns were defined by lightly changing the previous definitions.[21] A CDR3 fragment profile with 5–9 peaks/vβ families and a Gaussian (bell shaped) distribution was called the "polyclonal" pattern. Oligoclonal profile resulted in a pattern of no more than four peaks per vβ family, while the presence of a peak with an area >50% of the total area for that family was defined as restricted or skewed.

Results

Patient characteristics

In the period 2005–2008, there were seven enrolled patients, including two females, with profound T cell immunodeficiency and <30% of the normal lymphocyte proliferation to the mitogen PHA[12,18] who presented as interstitial cytomegalovirus or *Pneumocystis jiroveci* pneumonia (P2, P5, P6, and P7), oral candidiasis (P4 and P7), or failure to thrive and/or chronic diarrhea (P1 and P4) (Table 2). Decreased IgG level was all below the normal range except in P4, who had normal immunoglobulins but obvious lymphopenia and hepatosplenomegaly.

Two missense mutations of IL2RG (Try74Gly and Arg 226Lys, X-linked) and one complex mutation of RAG2 (missense mutation Ser 205 Tyr and deletion mutation 1366 T, frameshift, stop at the 484; autosomal recessive) were identified. Three patients (P1, P2, and P3) developed severe and refractory pneumonia after their first admission and died of respiratory failure before transplantation. Four patients had successful HSCT but P7 developed Grade III graft-versus-host disease (GVHD) and died of pneumothorax secondary to multiple bronchopleural fistulas.

T-cell receptor excision circles (TRECs) evaluation

Two evaluation systems of absolute and relative quantity of TRECs were utilized. For absolute quantity, a standard T&A cloning plasmid with one PCR product of TRECs was constructed and transfected into XL-1 blue DH-5α component cells. Direct sequencing demonstrated that the inserted DNA had no mutation and each insert was present in a single copy. Small aliquots of standard dilutions containing 10^6 to 10^2 copies of this vector were stored at −80°C. Seven age-matched healthy children were enrolled as controls. The detectable TRECs ranged from 3.03×10^3 to 1.44×10^5 fewer than controls (2.48×10^5 to 8.53×10^6) based on 10^6 PBMCs (Table 3). For relative quantity, consistent Cα chain was used as an internal control for each individual. Similar patterns of lower expression ratio were detected (6.07×10^{-6} to 2.43×10^{-5} in patients versus 1.92×10^{-4} to 1.17×10^{-2} in controls). Normalized TRECs were obtained in three patients after 6 months post-transplantation.

TCR (CDR3) size spectra-typing analysis

Healthy controls had polyclonal patterns with Gaussian (bell shaped) distribution in Vβ TCR repertoire (Fig. 2A). All seven

Table 3 T-cell receptor excision circles (TRECs) in patients with profound T-cell immunodeficiency

	P1	P2	P3	P4	P5	P6	P7	Normal range ($n = 7$)
At diagnosis	3M	4M	7M	17M	5M	5M	4M	
Genetic defects	Unidentified	Unidentified	Unidentified	Unidentified	IL2RG	IL2RG	RAG2	
GvHD[a]				Grade II	Grade I	Grade I	Grade III	
TRECs copy number/10^6 PBMCs								
Before transplantation	3.03×10^3	1.44×10^5	4.84×10^4	2.92×10^4	5.24×10^4	6.88×10^4	6.01×10^4	2.48×10^5 −8.53×10^6
Post-transplant 6 mo	NA	NA	NA	8.25×10^6	8.04×10^6	9.85×10^5	NA	
TRECs / Cα								
Pre-transplant	6.07×10^{-6}	7.94×10^{-5}	3.41×10^{-5}	5.53×10^{-5}	2.43×10^{-5}	3.12×10^{-5}	4.00×10^{-5}	1.92×10^{-4}
Post-transplant 6 mo	NA	NA	NA	1.28×10^{-4}	1.64×10^{-2}	5.64×10^{-3}	NA	−1.17×10^{-2}

[a] Prophylactic GvHD contained cyclosporine and methylprednisolone and pre-condition medications, including anti-thymocyte globulin (ATG) 15 mg/kg twice daily, i.v. for 4 consecutive days (Day -4 to Day -1).
NA = not available.

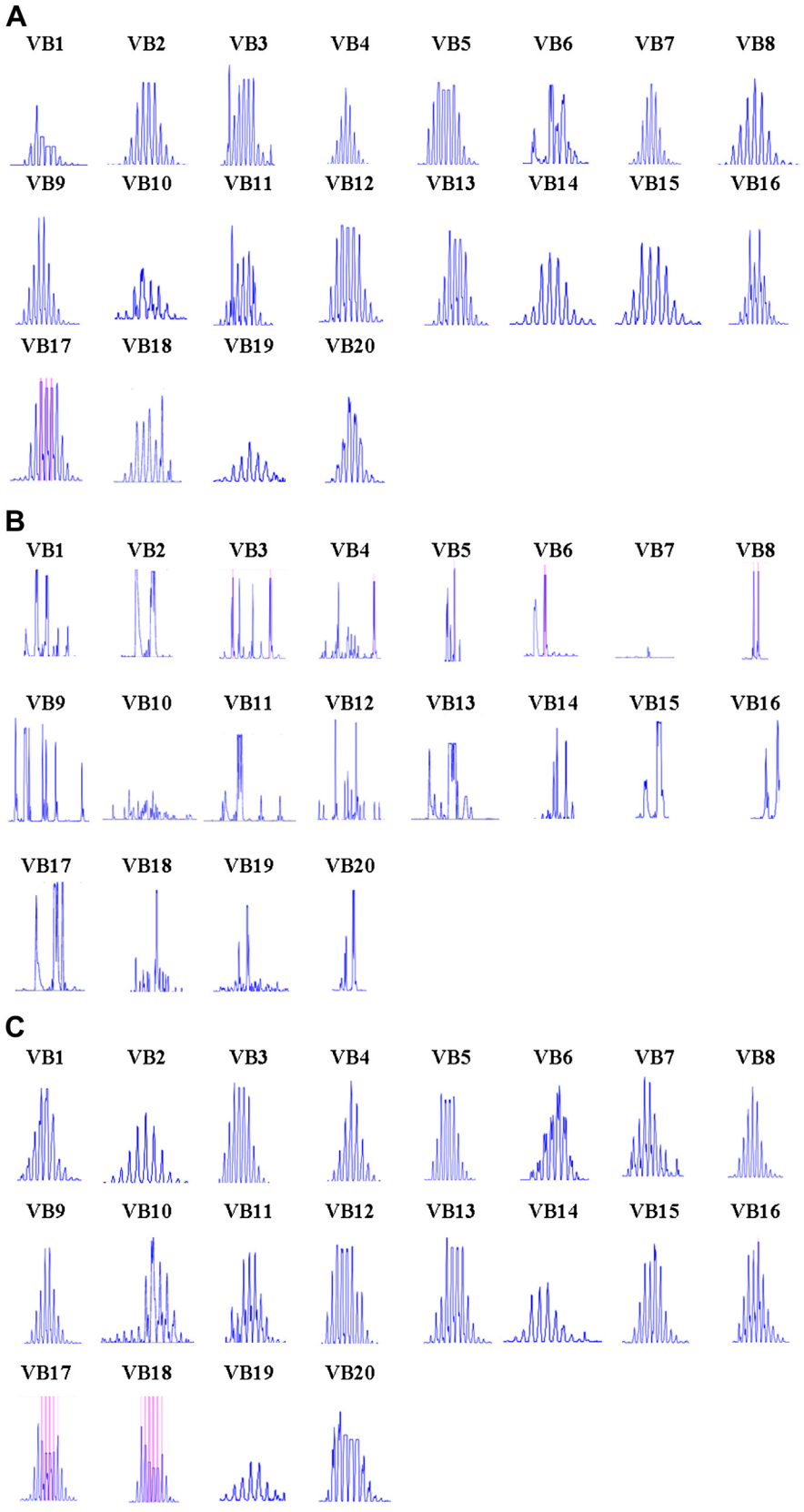

Figure 2. (A) Polyclonal TCR repertoire was detected in healthy controls; (B) oligo- and skewed TCR repertoire was present in patients with profound T-cell immunodeficiency, represented by patient P5; (C) these were reconstructed after umbilical cord stem cell transplantation.

patients had oligo- or/and restricted TCR repertoire (a representative demonstration in Fig. 2B). After successful transplantation, donor progenitor hematopoietic stem cells developed into effective T cells with polyclonal TCR repertoire patterns (Fig. 2C).

Discussion

TRECs were first reported in 1988 by Douek et al[16] to study changes in the frequency of recent thymic emigrants with age and in the case of HIV infection. T cell receptor gene rearrangements occur in maturing thymocytes and TRECs are subsequently detected in thymocytes and in mature T cells.[16,22–24] For profound T-cell defective function determined using neonatal Guthrie cards, TRECs evaluation is useful in recognizing patients with DiGeorge syndrome, idiopathic T-cell lymphopenia, SCID with IL2RG, JAK3, ADA, LIG4 and Rac mutation, and secondary disorders of lymphocyte extravasation, including chylothorax, chyloperitoneum and gastroschisis in Wisconsin, California, Massachusetts, and Japan[2,25–27]

Instead of Newborn screening in Guthrie cards (NBS) with whole blood, lymphocytes were directly utilized to count recent thymic emigrants. Leaky (in P1, P2, and P4) or maternal (in P6) T cells mainly presented as residual transplacental memory cells, with obviously decreased TRECs. Patients with TRECs below the normal range had higher infectious susceptibility to opportunistic pathogens (i.e., BCGitis, CMV, *Pneumocystis jiroveci* pneumonia, and Candida), compatible to T cell defects. However, the relationship of the TRECs value to the severity and frequency of infection was not inversely correlated, which might be ascribed to environmental hygiene and surrounding pathogens.

"TCR repertoires" are rearranged to form highly diverse hyper-variable regions of TCR during the process of V(D)J recombination and are responsible for the antigenic specificity of the individual TCR.[15] Polyclonal distribution is detected in infant healthy controls at 3 months of age, showing capability for a broader reactivity to multiple antigens. In contrast, patients of the same age, but with severe defective T cells, only reveal oligo- or/and restricted TCR repertoires caused by disturbed V(D)J recombination and hyper-somatic mutation. In line with TRECs, patients with defective T cell functions who have oligo- or restricted TCR repertoires have increasing opportunistic infections with increasing age. Oligo- or restricted patterns of a TCR repertoire seem to be related to the expression of residual trans-placental maternal T memory or leaky cells (in P1, P2, P4, and P6).[28–30]

Successful umbilical cord blood stem cell transplantation reconstructed T cell immunity after 6 months in this series. Immuno-reconstitution after HSCT reportedly depends on two different pathways.[31–33] The first wave consists of thymic-independent graft-derived mature donor T cells to the periphery, followed by antigen or cytokine expansion. The resulting circulating T cells have limited T-cell repertoire diversity and are unable to respond to a broad range of pathogens.[34,35] The second wave is a thymic-dependent pathway that involves the selection of graft-derived precursor cells in the host's thymus and/or possibly in the periphery.[36–40] This process accounts for a more durable reconstitution of the T-cell compartment, with a more diverse T-cell repertoire. The first wave is mainly from mature memory cells and may cause GVHD. In patients with GVHD and infectious complications, increased effective mature T-cell proliferation may dilute the recent thymic emigrants and consequently decrease the TRECs value.[17] Umbilical cord blood stem cell transplantation, with no or fewer donor's maternal transplant memory cells, attenuates the severity of GVHD in the first wave (P4, P5 and P6 vs. P7) and rapidly promotes the second wave for TCR repertoire diversity in the thymus and periphery at 6 months post-transplantation.

Pre-natal genetic diagnosis for PIDs with family history and identified genetic mutation allows for early intervention and prevention of irreversible sequelae. To those patients with profound defective T cells, with *de novo* mutation and unidentified molecular basis, lower TRECs and restricted TCR repertoire compatible with severe impaired PHA-stimulated lymphocyte proliferation and phenotypic severity in the period of infancy are authentic ways for early recognition and treatment, even HSCT under infection-free conditions. However, the frequency and severity of infections are not inversely correlated to lower TRECs value and restricted TCR repertoire diversity in this series.

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