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TREC and KREC profiling as a representative of thymus and bone marrow output in patients with various inborn errors of immunity

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Summary

Primary immune deficiency (PID) disorders are clinically and molecularly heterogeneous diseases. T cell receptor excision circles (TRECs) and κ (kappa)-deleting excision circles (KRECs) are markers of T and B cell development, respectively. They are useful tools to assess T and B cell function and immune reconstitution and have been used for newborn screening for severe combined immunodeficiency disease (SCID) and agammaglobulinemia, respectively. Their profiles in several genetically confirmed PIDs are still lacking. The objective of this study was to determine TREC and KREC genomic profiling among various molecularly confirmed PIDs. We used real-time–quantitative polymerase chain reaction (RT–qPCR)-based triplex analysis of TRECs, KRECs and β-actin (ACTB) in whole blood genomic DNA isolated from 108 patients with molecularly confirmed PIDs. All agammaglobulinemia patients had low KREC counts. All SCIDs and Omenn syndrome patients secondary to mutations in *RAG1*, *RAG2*, *DCLRE1C* and *NHEJ1* had low TREC and KREC counts. *JAK3*-deficient patients had normal KREC and the TREC count was influenced by the type of mutation. Early-onset ADA patients had low TREC and KREC counts. Four patients with zeta-chain-associated protein kinase 70 (ZAP70) had low TREC. All purine nucleoside phosphorylase (PNP) patients had low TREC. Combined immunodeficiency (CID) patients secondary to *AK2*, *PTPRC*, *CD247*, *DCLREC1* and *STAT1* had normal TREC and KREC counts. Most patients with ataxia–telangiectasia (AT) patients had low TREC and KREC, while most *DOCK8*-deficient patients had low TRECs only. Two of five patients with Wiskott–Aldrich syndrome (WAS) had low TREC counts as well as one patient each with bare lymphocyte syndrome (BLS) and chronic granulomatous disease. All patients with Griscelli disease, Chediak–Higashi syndrome, hyper-immunoglobulin (Ig)M syndrome and *IFNGR2* had normal TREC and KREC counts. These data suggest that, in addition to classical SCID and agammaglobulinemia, TREC/KREC assay may identify ZAP70 patients and secondary target PIDs, including dedicator of cytokinesis 8 (DOCK8) deficiency, AT and some individuals with WAS and BLS.

Keywords: agammaglobulinemia, ataxia telangiectasia, chronic granulomatous disease, Hyper IgE, severe combined immunodeficiency

Abbreviations: CNV, copy number variation; CGD, chronic granulomatous disease; DBS, dried blood spot; DOCK8, dedicator of cytokinesis 8; HIES, hyper-IgE syndrome; KREC, kappa-deleting recombination excision circles; NBS, newborn screening; PID, primary immunodeficiency disease; qPCR, quantitative polymerase chain reaction; SCID, severe combined immunodeficiency disease; SNV, single nucleotide variant; STAT3, signal transducer and activator of transcription 3; TREC, T cell receptor excision circle; WAS, Wiskott-Aldrich syndrome.

Introduction

Primary immunodeficiency diseases (PIDs) are a phenotypically and molecularly heterogeneous group of diseases with more than 300 known genetic defects that affect the development or the function of the immune system [1,2]. Patients affected with these genetic defects present with varying clinical presentations that range from mild to life-threatening infections, dysregulated immunity, inflammation and malignancy [1–3]. PIDs are classified based on the affected immunological system and clinical presentations [2]. These include defects in antibody production by B cells (humoral immune deficiencies) and/or T cell functions (cellular immunity), impaired antigen presentation, defects in natural killer (NK) cell cytotoxicity, Toll-like receptor (TLR) activation, phagocytosis, macrophage activation and complement.

During T and B cell development, premature T and B cells undergo the process of recombination of receptor gene segments. This occurs at the T cell (TCR) and B cell receptor (BCR) loci to generate the diversity of T and B cell repertoires, respectively, that are required to develop effective immunity against a wide range of antigens, such as bacteria, viruses and others [4,5]. These recombination processes result in the formation of extrachromosomal non-replicating excision circles. Quantitative measurements of these excision circles can be used clinically to assess the degree of T and/or B cell defects. Multiple types of TRECs are generated during the process of TCR rearrangement, and the TCR delta deletion TREC (delta rec psi-J-alpha signal joint TREC) appears to be the most accurate TREC for measuring thymic output. Conversely, BCR rearrangement results in the formation of κ (kappa)-deleting excision circles (KRECs). Each (B/T) lymphocyte is believed to produce a single copy of TREC or KREC which have been used to develop newborn screening (NBS) methods for T or B cell lymphopenia, such as severe combined immunodeficiency disease (SCID) and X-linked agammaglobulinemia (XLA), respectively [6–9]. TRECs are generated during the latest stages of T cell development just before they exit to the periphery. Hence, their levels in the peripheral circulation represent thymic activity [10–13]. Also, the longevity of naive T cells in the peripheral circulation, as well as TRECs intracellular degradation and dilution by peripheral T cell division, represent limitations that should be kept in mind. Conversely, KRECs are formed in the early stages of development up to mature B lymphocytes. Hence, KREC numbers in the periphery represent fresh and functional B cells [14].The utility of TREC and KREC in NBS aims to provide early diagnosis of severe forms of PID which have a significant impact on patient morbidity and mortality [14].

TREC measurements have not only been used for NBS: this method was also used as a tool to assess T cell immune reconstitution after thymic or hematopoietic stem cell transplantation (HSC) transplantation [15–18]. Thymic output has been studied using TREC measurements, with the effect of aging showing an inverse correlation where TREC numbers decreased by more than 95% between the ages of 25 and 60 years [19]. Also, TREC measurements have been used in the studies of T cell generation in neonates [20]. T cell homeostasis was also evaluated in patients with rheumatoid arthritis [21], HIV and in response to highly active anti-retroviral therapy (HAART) [22]. In such studies, genomic DNA (gDNA) extracted from whole blood was used.

The combined triplex assay of the TREC, KREC and β-actin (ACTB) screening approach is useful for the detection of severe forms of PID that present with a low number of TREC, KREC or both [23,24]. These assays have been validated by screening large numbers of newborn babies. Babies with low TREC or KREC numbers were then recalled for second-tier testing [25]. Conversely, using a duplex (TREC, ACTB) assay, we recently conducted a SCID NBS pilot study that showed a high incidence of SCID in the Saudi newborn population (1 : 2906 live births) [26].

In this study, we sought to assess TREC and KREC profiling using genomic DNA extracted from whole blood samples from a large and diverse group of patients with molecularly confirmed diagnoses of PIDs.

Materials and methods

This study was approved by the institutional review board at King Faisal Specialist Hospital and Research Center (KFSHRC), RAC no. 2130-027. The study was also supported by the Kingdom of Saudi Arabia National Science, Technology and Innovation Plan's Strategic Technologies Grant KACST: 13-BIO 755-20 under the King Abdulaziz City for Science and Technology (Riyadh, Kingdom of Saudi Arabia).

Sample collection

All patients identified and reported in this study received comprehensive clinical and laboratory evaluations as part of their routine clinical care at the Pediatric Immunology and Allergy Clinics at King Faisal Hospital and Research Center. Previously consented, archived extracted DNA samples from this collection were used for this study. Written informed consent to obtain blood samples or to use the stored DNA was obtained from each patient or his/her parents if available; otherwise, the sample was anonymized. Data of interest collected from the patients' medical records were secured as governed by the institutional policies on patient confidentiality and privacy.

DNA extraction

Genomic DNA of children known to have various PIDs (and their families, when available) was isolated from peripheral blood samples obtained by venipuncture as described previously using standard protocols. DNA was diluted to a working concentration of 10 ng/μl [27].

Sequence analysis and copy number analysis of PID genes

Bi-directional Sanger sequencing was performed in all gDNA samples. Sequencing primers were designed to amplify the region of interest, which was sequenced as previously described [28]. Sequence data were aligned against the reference GenBank sequences and examined for variation. For novel mutation validation, anonymized 250 DNA samples derived from normal Saudi blood donors were sequenced to rule out potential novel Saudi normal single nucleotide polymorphisms (SNPs). The Affymetrix high-definition scan SNP-microarray platform (Affymetrix, Santa Clara, CA, USA) was used to evaluate patients with dedicator of cytokinesis 8 (DOCK8) deficiency for possible copy number abnormalities.

Real-time–quantitative triplex polymerase chain reaction (RT–qPCR)

RT–qPCR of gDNA of individuals with known diagnoses of primary immune deficiency was used to quantify TRECs, KRECs and ACTB, which served as an internal control for determining the efficiency of the RT–qPCR reaction. Reagents for this assay (SCREEN-ID neonatal screening kit) were purchased from Mabtech Diagnostics (Nacka Strand, Sweden) (https://www.mabtech.com/), and the assay was performed as per the manufacturer's recommendations which have been previously described [25,26]. Briefly, the RT–qPCR reactions were performed in a final volume of 20 μl containing 100 ng (10 ng/μl) gDNA, 1× TaqMan Gene Expression Master Mix, 20 μM TREC primers, 25 μM KREC primers, 7·5 μM ACTB primers, 15 μM 6-carboxyfluorescein (6FAM)-labeled minor groove-binding (MGB) TREC- and NED-labeled MGB ACTB probes, 17·5 μM VIC-labeled MGB KREC probe and 0·8 μl of 10 mg/ml bovine serum albumin (BSA). The 96-well plate reactions were carried out on the ABI QuantStudio 7 real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR conditions were: initial cycle at 50°C for 2 min and a heating cycle at 95°C for 10 min, followed by 45 cycles of 30 s at 95°C and 30 s at 60°C. An individual cycle threshold for TREC, KREC or ACTB was fixed for automated data collection and analysis of the amplification during the exponential phase. Calibration curves were generated by 10-fold serial dilution using TREC-KREC-ACTB. All analyzed RT–qPCR assays met the quality requirements of similar slopes and R^2 values > 0·97. While amplification of ACTB was used to assess PCR efficiency, this was disregarded if TREC and KREC copy numbers were above the cut-off level.

Ethical approval

This study was submitted to the Institutional Review Board of King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia, and was approved by the Research Advisory Committee through established procedures.

Results

Patient demographics and T/B lymphocyte subset flow cytometry analysis

This large group of patients represents a wide array of inborn errors of immunity. In 100 patients, the median age at sample collection was 39 ± 72 months (0·25–372) and prehematopoietic stem cell transplantation lymphocyte subset analysis results were available on 90 subjects (Supporting information, Table S1). The median count for $CD3^+$ cells was 1672·5 ± 1932 (reference: 1600–2700), $CD4^+$ cells 706 ± 1322 (reference: 1000–1700), $CD8^+$ cells 480 ± 843·9 (reference: 600–1000), CD19 cells 275 ± 806 (reference: 400–800), NK $CD56^+$ and 16^+ cells 220 ± 556·9 (reference: 200–400). Lymphocyte subpopulations counts were low in 21 ($CD3^+$), 58 ($CD4^+$), 50 ($CD8^+$), 45 ($CD19^+$) and 65 (NK $CD56^+$, 16^+) samples. However, naive and memory T cell counts were not available for many samples. TREC and KREC counts were low in 50 and 34 samples, respectively, while 29 samples were low for both markers, as shown in Supporting information, Table S1.

PID genetic mutations detected by Sanger sequencing and SNP microarray analyses

In this large cohort of 108 patients with a wide spectrum of PIDs (Table 1), missense mutations were the most common (n = 44) followed by stop–gain mutations (n = 24), frameshift mutations (n = 19) and splicing mutations (n = 13), which were all identified via Sanger sequencing. Four of the frameshift mutations were identified in four of the six patients with ataxia-telangiectasia (AT). Large intragenic deletions detectable by SNP-microarray analysis were confirmed in eight patients, seven of which involved *DOCK8*, and one deletion spanned exons 1–3 of *DCLRE1C* (ARTEMIS). In 11 patients, we detected eight unique and novel mutations (Table 1), six of which involved *DOCK8* (including five multi-exon deletions). While *STAT3* and *BTK* mutations are heterozygous and hemizygous (X-linked), respectively, all remaining mutations reported here are homozygous. Clinically and biochemically, all patients with novel mutations had typical presentation except for cases 5,

Table 1. Combined TREC/KREC counts and DNA mutation analysis in 108 patients with various inborn errors of immunity

Sample Name	Clinical phenotype	Gene	gDNA variant	Protein change	Reference	TRECs/µl	KRECs/µl
08R-01806	Agammaglobulinemia	*BTK*	NM_000061:exon8:c.C763T	p.R255X	[45]	1494	1·3
12R-01584		*BTK*				894	1·2
08R-01805		*BTK*				861	1·7
11R-00514		*BTK*	NM_000061:exon20:c.1909-9T>C	n.a.	[46]	1·2	631
11R-00404	CID	*AK2*	NM_001625:exon6:c.C545A	p.A182D	[46]	24	19
09R-00412		*AK2*				64	250
09R-00413		*AK2*				235	1932
11R-01877		*PTPRC*	NM_002838:exon32:c.T3545C	p.L1182S		273	656
12R-03326		*CD247 (CD3Z)*	NM_198053:exon2:c.58^{+}8C>T	n.a.		318	219
11R-00457		*DCLRE1C*	NM_022487:exon10:c.C614G	p.S205C		47	154
13R-01205		*ZAP70*	NM_001079:exon12:c.G1606A	p.G536S		0·8	8
13R-01204		*ZAP70*				12	23
09R-00001		*STAT1*	NM_007315: c.1220T>G	L407R		15	26
06R-00778	SCID	*RAG1*	NM_000448:exon2:c.T1201C	p.S401P	[47]	0·1	0·7
12R-02223		*RAG1*	NM_000448:exon2:c.555delG	p.R186fsX15	[46]	0	0
11R-00776		*RAG1*	NM_000448:exon2:c.G2210A	p.R737H	[48]	0	0
13R-00820		*RAG1*	NM_000448:exon2:c.C1180T	p.R394W	[49]	0	0·1
12R-02253		*RAG1*	NM_000448:exon2:c.G2041T	p.E681X	[46]	0	0
06R-00454		*JAK3*	NM_000215:exon 8: c.1012 delG	A338LfsX14	This report	0	350
08R-01741		*JAK3*	NM_000215:exon7:c.C913T	p.Q305X	[46]	0	23
11R-00138		*JAK3*	NM_000215:exon3:c.G308A	p.R103H		67	270
11R-00199		*RAG2*	NM_000536:exon2:c.374_375delCA	p.T125RfsX10		0	0
08R-00802		*RAG2*	NM_000536:exon2:c.G686C	p.R229P	[50]	0	0
09R-00477		*RAG2*	NM_000536:exon2:c.T547C	p.F183L	[46]	0	0
09R-00114		*RAG2*	NM_000536:exon2:c.442C>T	p.R148X	[47]	0	0
10R-00041		*RAG2*	NM_000536:exon2:c.1403_1406delATCT	p.H468RfxX16	[46]	0	0
12R-03041		*ADA*	NM_000022: c.385G>A	p.V129M	[51]	3	2
07R-01680		*ADA*	NM_000022:exon10:c.956_960delAAGAG	p.E319GfsX3	[52]	56	65
06R-00782		*ADA*	NM_000022:exon 10:c.956_960delAAGAG	p. E319GfsX3		0	0
07R-00376		*DCLRE1C*	(ARTEMIS) deletion exon 1 – 3	n.a.	[53]	0	0
08R-00625		*DCLRE1C*	NM_001033855:exon6:c.G403A	p.G135R	[46]	0	0
07R-01595		*ZAP70*	NM_001079.3: exon 12:c.1606G>A	p.G536S		12	6
10R-00846		*ZAP70*				319	1242
09R-00066		*ZAP70*	NM_001079:exon5:c.569G>C	p.R190T		5	632
08R-00795		*PNP*	NM_000270:exon5:c.487T>C	p.S163P	[54]	0	82
11R-02131		*PNP*				0	3
09R-00329		*PNP*	Splice: NM_000270:exon2:c.12-1G>C	n.a.	[46]	0	75
10R-00194	SCID with microcephaly, growth retardation and sensitivity to ionizing radiation	*NHEJ1*	NM_024782:exon4:c.390^{+}1G>C			0·4	0
10R-00190		*NHEJ1*				2	6
11R-00581	MSMD	*IFNGR2*	NM_005534:exon5:c.C705A	p.Y235X	[55]	508	1159

Table 1. (Continued)

Sample Name	Clinical phenotype	Gene	gDNA variant	Protein change	Reference	TRECs/μl	KRECs/μl
08R-00113	Omenn syndrome	*RAG1*	NM_000448:exon2:c.555delG	R186fsX15	[46]	0	0
11R-00236		*RAG2*	NM_000536:exon2:c.1332C>G	p.I444M	[56]	0	0
10R-00808	AT	*ATM*	NM_000051: c.C8732A	p.T2911N	This report	1·7	4·4
08R-01809		*ATM*	NM_000051:exon61:c.8833_8834delCT	L2945VfsX10	[57]	5·5	4·5
11R-01857		*ATM*	NM_000051:exon63:c.9066delA	p.G3023AfsX10	[46]	2·4	3·9
07R-01996		*ATM*	NM_000051:exon8:c.903_904insT	p.A302CfsX3		1	1
08R-1863		*ATM*	ATM:NM_000051:exon5:c.381delA	p.V128X	[58]	0·1	0·3
08R-01896		*ATM*	NM_000051:exon63:c.9066delA	p.G3023AfsX10	[46]	34	3
08R-01634	HIES	*DOCK8*	NM_203447:exon7:c.827^{+}6T>C	n.a.	[28]	19	146
12R-00998		*DOCK8*	NM_001190458:exon7:c.647delT	p.L216RfsX24	This report	2·2	171
12R-03319		*DOCK8*	DOCK8 deletion of exons 6 to 8	n.a.	This report	12	81
11R-02472		*DOCK8*		n.a.	This report	8·2	479
12R-03038		*DOCK8*		n.a.	This report	0	0
09R-00002		*DOCK8*	NM_203447:exon44:c.T5625G	p.Y1875X	[28]	24	390
10R-00007		*DOCK8*	NM_203447:exon40:c.C5132A	p.S1711X		3	192
06R-01268		*DOCK8*	NM_203447:exon44:c.T5625G	p.Y1875X		26	1277
07R-02055		*DOCK8*				23	980
11R-00115		*DOCK8*	DOCK8 deletion up to exon 19	n.a.		0·8	20
11R-00061		*DOCK8*	NM_203447:c.4626^{+}5G>A		[46]	20	339
11R-00422		*DOCK8*	NM_203447:exon40:c.C5132A	p.S1711X	[28]	11	128
11R-00761		*DOCK8*	NM_203447: c.4626^{+}5 G>A	n.a.	[46]	0·1	839
11R-00753		*DOCK8*	DOCK8 deletion of exons 1–13		This report	2	67
13R-00808		*DOCK8*	NM_203447:exon44:c.T5625G	p.Y1875X	[28]	42	2617
08R-00244		*DOCK8*	NM_203447:exon40:c.C5132A	p.S1711X		0	123
11R-00140		*DOCK8*	NM_203447:exon44:c.T5625G	p.Y1875X		7	354
07R-00097		*DOCK8*	Whole DOCK8 deletion up to intron 1 of KANK1	n.a.		0·3	0·7
10R-00180		*DOCK8*	Deletion up to exon 19		This report	7	418
11R-02144		*DOCK8*	NM_203447:exon44:c.T5625G	p.Y1875X	[28]	19	90
09R-00179		*STAT3*	NM_003150:exon20:c.G1868T (hetero)	p.W623L	[46]	139	162
11R-00298		*STAT3*	NM_003150:exon13:c.G1145A (hetero)	p.R382Q	[59]	50	60
07R-00795		*STAT3*	NM_003150:exon13:c.1144C>T (hetero)	p.R382W		107	64
09R-00184		*STAT3*				153	31
09R-00182		*STAT3*				675	748
11R-01442	WAS	*WAS*	NM_000377:exon4:c.G397A	p.E133K	[60]	14	110
12R-02926		*WAS*	M_000377: c.11dupG	M6NfsX32	[61]	31	80
11R-00333		*WAS*	NM_000377:exon2:c.G257A	p.R86H	[62]	119	28
11R-02389		*WAS*				0	88
12R-02269		*WAS*	NM_000377:exon1:c.G91A	p.E31K	[63]	144	611
11R-02714	BLS	*RFXANK*	NM_003721:c.C477A	p.S159R	This report	29	333
12R-02302		*RFXANK*	NM_003721:exon6:c.A362T	p.D121V	[64]	20	172
12R-02646		*RFXANK*				20	386
13R-00818		*RFXANK*	NM_134440:exon4:c.271^{+}1G>C	n.a.	[46]	1·2	2·4

Table 1. (Continued)

Sample Name	Clinical phenotype	Gene	gDNA variant	Protein change	Reference	TRECs/μl	KRECs/μl
09R-00132		*RFXANK*	NM_003721:exon3:c.8_11dupTTAC	Q5YfsX22		84	86
10R-00303		*CIITA*	NM-000246: c.G2551C	p.A851P	This report	133	912
11R-00156	CGD	*CYBB*	NM_000397:exon6:c.G618A	p.W206X	[65]	104	322
08R-00975		*CYBB*	NM_000397:exon5:c.C388T	p.R130X	[66]	0	581
11R-00186		*CYBB*	NM_000397:exon13:c.1618delG	p.E540KfsX7	[67]	323	363
07R-01787		*CYBB*	NM_000397:exon10:c.1217T>A	p.L406X	[46]	247	646
08R-01338		*NCF2*	NM_000433:exon2:c.C229T	p.R77X	[68]	213	481
08R-00833	GS	*MLPH*	NM_001042467:exon2:c.G104A	p.R35Q	[46]	49	65
07R-00416		*MLPH*				28	70
10R-00003		*MLPH*	NM_001042467: exon8: c.987delC	p.S330fsX15		159	290
10R-00558		*RAB27A*	NM_004580:c.148_149delAGinsC	p.R50QfsX35	[69]	541	372
11R-00810A		*RAB27A*	NM_004580:exon5:c.A400C	p.K134Q	[46]	90	161
07R-00817		*RAB27A*	NM_004580:exon6:c.598C>T	p.R200X	[70]	151	239
07R-00962		*RAB27A*				888	631
11R-02385		*RAB27A*	NM_004580:exon2:c.53_54delCT	p.S18WfsX15	[71]	122	87
12R-02807	CHS	*LYST*	NM_000081:exon23:c.G6694T	p.G2232X	[46]	45	77
13R-01455		*LYST*	NM_000081:exon37:c.9044^{+}1G>T	n.a.		659	712
11R-00696	HIGM	*CD40*	NM_001250:exon3:c.256^{+}2T>C		[72]	240	323
12R-02921		*CD40*				84	18
07R-00087		*CD40*				705	1551
11R-00167		*CD40LG*	NM_000074:exon5:c.719_720delAT	p.N240SfsX3	[46]	1417	674
11R-02335		*CD40LG*	NM_000074:exon5:c.A506G	p.Y169C	[73]	115	1175
10R-00971		*AICDA*	NM_020661:exon3:c.G169A	p.V57M	[46]	53	218
10R-00970		*AICDA*				16	63
10R-00972		*AICDA*				137	340
07R-00662		*AICDA*	NM_020661:exon2:c.152A>T	p.N51I		109	234

All variants (mutations) in this table are homozygous except for *STAT3* (heterozygous) and *BTK* (X-linked hemizygous, ♣).

TREC = T cell receptor excision circle; n.a. = not applicable; KREC = kappa deleting recombinant excision circle; PNP = purine nucleoside phosphorylase; CID = combined immunodeficiency; SCID = severe combined immunodeficiency; MSMD = Mendelian susceptibility to mycobacterial disease; AT = ataxia telangiectasia; HIES = hyper-immunoglobulin (Ig)E syndrome; WAS = Wiskott-Aldrich syndrome; BLS = bare lymphocyte syndrome; DOCK8 = dedicator of cytokinesis 8; CGD = chronic granulomatous disease; GS = Griscelli syndrome; CHS = Chediak–Higashi syndrome; HIGM = hyper-IgM.

6 and 7, who had atypical AK2 deficiency which was recently reported to cause hypogammaglobulinemia and limited T cell dysfunction [29], and cases 11 and 12 with zeta-chain-associated protein kinase 70 (ZAP70) deficiency who presented with CID phenotype without CD8 deficiency.

Triplex (TREC, KREC, ACTB) assay

Using a cut-off of ≤ 15 TRECs/μl and < 10 KRECs/μl, all agammaglobulinemia patients [4] secondary to *BTK* defects had low KREC and normal TREC counts. All SCIDs and Omenn syndrome patients secondary to *RAG1* [6], *RAG2* [6], *DCLRE1C* [2] and *NHEJ1* [2] had low TREC and KREC counts. All SCIDs secondary to *JAK3* deficiency had normal KRECs and two of three had low TRECs. Two of the three ADA-deficient patients had low TRECs and KRECs. Interestingly, two patients of three with ZAP70 deficiency had low TRECs and low KREC in one patient. All purine nucleoside phosphorylase (PNPP)-deficient patients [3] had low TRECs, and one of them had low KREC. CID patients secondary to AK2 [3], protein tyrosine phosphatase receptor type C (PTPRC) [1], CD247 [1], DNA cross-linking repair protein 1 (DCLREC1) [1] and signal transducer and activator of transcription 1 (STAT-1) [1] had normal TRECs and KRECs, while the CID patients with atypical ZAP70 (no CD8 deficiency) had a low TREC count. Five of six AT patients had low TREC and all had low KREC counts. Thirteen of 20 DOCK8-deficient patients had low TRECs and two had low KRECs. Two of five patients with Wiskott–Aldrich syndrome (WAS) had low TRECs. One of six patients with bare lymphocyte syndrome (BLS) had low TRECs and KRECs. One of the five chronic granulomatous disease patients had a low TREC count. All patients with Griscelli disease [8], Chediak–Higashi syndrome [2], hyper-immunoglobulin (Ig)M syndrome [9] and interferon gamma-receptor receptor 2 (IFN-γR2) [1] had normal TREC and KREC counts.

Discussion

TRECs and KRECs are considered markers of new lymphocyte output. They are used in research studies, but not routinely used in the routine clinical evaluation of patients with PIDs. TREC levels are extremely low or undetectable in all patients with typical SCID, and have been used effectively for routine NBS for SCID [7,30]. KRECs can be used to screen for agammaglobulinemia [6,8]. Currently, there is no universally validated NBS method for other PIDs.

TREC levels have been extensively assayed in children with PIDs in whom the decreased number and function of T and/or B cells result in severe impairment of immunity. Limited KREC studies were performed for various types of PIDs. Roifman *et al*. studied TRECs among 51 patients with T cell dysfunction. All classical SCIDs patients had low TREC numbers while partial ADA, interleukin (IL)-2γR (p. R222C mutation), CD40L, forkhead box protein 3 (FoxP3) and IL-10R had normal TREC results [31]. Using the same multiplex assay reported here, Borte *et al*. applied this approach to study 49 original stored Guthrie cards from patients diagnosed with SCID (n = 18), XLA (n = 4), AT (n = 4), Nijmegen breakage syndrome (n = 2), common variable immunodeficiency (CVID) (n = 4), IgA deficiency (n = 15) and X-linked hyper-IgM syndrome (HIGM) (n = 2) and demonstrated its ability to identify patients with XLA, AT, NBS and WAS [23]. Separately, in two of three young children with DOCK8-related HIES, we found low dried blood spot (DBS) TREC copy numbers [32]. Subsequently, a few additional reports demonstrated low TREC counts in DBS samples from patients with various secondary (non-SCID) target disorders such as AT [33], Nijmegen breakage syndrome [34,35], cartilage hair hypoplasia (CHH) [36], WAS [36,37] and DNA ligase 4 (LIG4) deficiency [38]. Table 2 summarizes the primary immunodeficiency diseases in which TREC and KREC numbers have been evaluated both in the literature as well as in our current study.

In this report, we confirm that a subset of patients with AT, WAS and the majority of DOCK8-deficient individuals had low TREC and/or KREC counts (Table 1, Fig. 1), thus potentially allowing early NBS for these disorders. It is interesting to note that one of our three cases with ADA deficiency had normal TREC counts and therefore would have been missed on NBS. Either late-onset or partial ADA deficiency may explain such phenomena, as a significant subset (15–20%) of ADA-deficient patients exhibit a 'delayed clinical onset' and present with less severe, but slowly progressive, combined immune deficiency later in life [39]. It was believed that some PID patients with residual T cells, such as ZAP70 deficiency, will be missed by TREC-based NBS. ZAP70 deficiency, a TCR signaling defect, is characterized by normal/elevated number of circulating lymphocytes, absent $CD8^+$ T cells, abundant $CD4^+$ T cells, normal number and activity of natural killer cells and normal number with reduced function of B cells. In this cohort, four of five cases with ZAP70 deficiency had low TREC counts, a finding that was previously reported by Roifman *et al*. [31], thus making some patients with this disorder potentially amenable to TREC-based detection. In contrast, a recent publication reported that the TREC count was normal at birth and undetectable at age of 9 months in a patient with ZAP70 defect

Table 2. Primary immunodeficiency diseases in which T cell receptor excision circles (TREC) and kappa deleting recombinant excision circles (KREC) have been evaluated in the literature and this study

Disease	Genetic defect	TREC	KREC	References
Severe combined immunodeficiency disease	*ADA*	Low	Not done (n.d.)	[31,74,75]
	Partial *ADA*	Normal (N)	n.d.	[31]
	ADA	Low	Low	This report
	Partial *ADA*	N	N	This report
	AK2	Low	Low	[23], this report
	AK2 (p.A182D mutation*)*	N	N	This report
	CD3G	Low	n.d.	[31]
	CD247 (CD3Z)	N	N	This report
	DCLRE1C	Low	Low	This report
	IL2RG	Low	N	[7,23,31,75]
	IL2RG (p.R222C mutation*)*	N	N	[31]
	JAK3	Low	N	[7,23,75], this report
	JAK3 (p.R103H mutation*)*	N	N	This report
	LIG4	Low	n.d.	[31,75]
	PNP	Low	n.d.	[74]
		Low	Low	This report
	PTPRC	N	N	This report
	NHEJ1	Low	Low	This report
	RAG1	Low	Low	[23,31,75], this report
	RAG2	Low	Low	[31,75], this report
	RMRP	Low	n.d.	[31]
	ZAP70	Low	n.d.	[31]
		Low or N	Low or N	This report
Agammaglobulinemia	*BTK*	N	Low	[8,23], this report
Ataxia-telangiectasia	*ATM*	Borderline	Low	[23]
		Low (5 of 6)	Low	This report
Hyper-immunoglobulin (Ig)E syndromes	*DOCK8*	Low	n.d.	[32]
		Low (13 of 20)	N in 18 and low in 2 of 20	This report
	STAT3	N	N	This report
Wiskott–Aldrich syndrome	*WAS*	Low	Low	[76]
		Low (2 of 6)	Low (1 of 6)	This report
Bare lymphocyte syndrome	Not reported	Low	n.d.	[31]
	RFX5	Low	n.d.	[41]
	RFXANK	N (1 of 5 low)	N (1 of 5 low)	This report
	CIITA	N	N	This report
Hyper-IgM syndrome	*CD40L*	N	N	[23,31,76], this report
	CD40	N	N	This report
	AICDA	N	N	This report
Griscelli syndrome	*RAB27*	N	N	This report
	MLPH	N	N	This report
Chediak–Higashi syndrome	*LYST*	N	N	This report
Nijmegen breakage syndrome	*NBN*	N	Low	[23,77]
Immune dysregulation	*FOXP3*	N	n.d.	[31]
	IL2RA	Low	n.d.	[31]
	IL10RA	N	n.d.	[31]
Mendelian susceptibility to mycobacterial diseases	*IFNGR2*	N	N	This report
	STAT1	Borderline	N	This report
Chronic granulomatous disease	*CYBB*	Low	n.d.	[43]
	CYBB	N (low in 1 of 4)	N	This report
	NCF2	N	N	This report

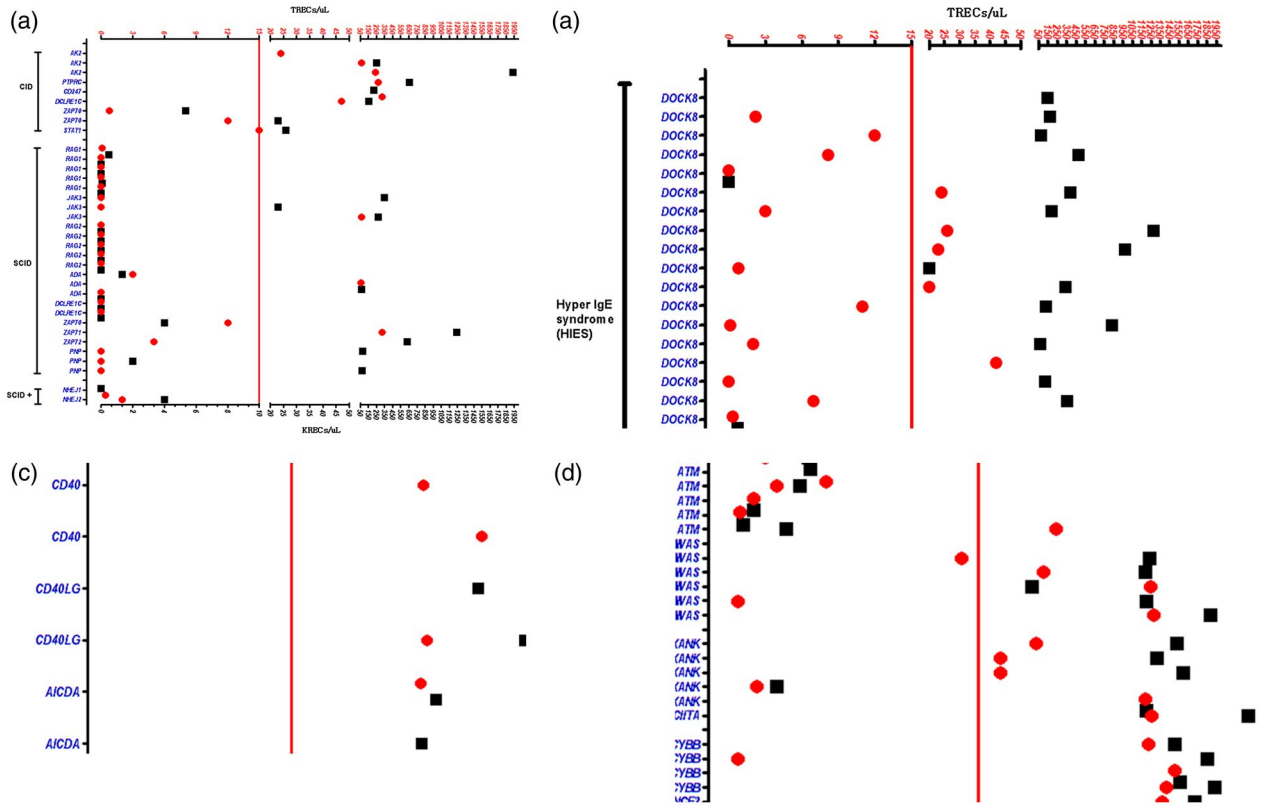

Fig. 1. T cell receptor excision circle (TREC) and κ (kappa)-deleting excision circle (KREC) counts in a cohort of 108 patients with various primary immune deficiencies. Primary immune deficiency (PID) categories and related genes are displayed on the *y*-axis and TRECs/KRECs on the *x*-axis. Vertical red lines within each group represent TREC (red circles) and KREC (black squares) count cut-offs at 15 and 10, respectively. BLS = bare lymphocyte syndrome; CGD = chronic granulomatous disease; CHS = cartilage hair hypoplasia syndrome; CID = combined immunodeficiency; HIES = hyper-immunoglobulin (Ig)E syndrome; HIGM = hyper-IgM syndrome; SCID = severe combined immunodeficiency.

[40], reflecting possible variations that could be observed at different ages.

Major histocompatibility complex (MHC) class II deficiency, also known as bare lymphocyte syndrome (BLS) type II, is a primary immunodeficiency disorder that results from the lack of expression of MHC class II molecules on the surface of immune cells rather than an intrinsic defect in T cell development. It is another disease that is expected to be missed by TREC based newborn screening. Recently, an MHC II-deficient patient was identified through SCID NBS [41]. Interestingly, one of our six patients had low TREC and KREC counts. The possible mechanism for this observation is not clear, but might be related to T cell depletion secondary to chronic unresolved infections. In this report, we showed for the first time, to our knowledge, that patients with SCID secondary to CD3Z (CD247) and PTPRC (CD45) deficiency have a normal TREC and KREC, confirming the notion that these rare types of SCID will unlikely to be identified by the current SCID NBS. The roles of the different CD3 chains in T cell differentiation are not well known. Mutations in those chains impair T cell development to varying degrees. Deficiency of CD3δ or CD3ε causes severe T cell lymphopenia, while CD3γ or CD247 (CD3Z) causes mild T cell lymphopenia, and this probably explains the normal TREC level in our patient with CD3Z defect [42].

We noticed unexpectedly that one of the five patients with chronic granulomatous disease (CGD), a phagocyte defect, had a low TREC count, an observation that was previously observed in some patients with CGD, which is thought to be due to T cell depletion (through unknown mechanisms) [43,44]. This finding supports our observation, and calls for additional comprehensive T cell evaluation and monitoring in these patients.

Genomic DNA extracted from whole blood samples of individuals with fully characterized genetic mutations is the best approach to determine which PID can be detected by NBS using TREC and KREC quantification, followed by validation on DBS. In this study, we explored the genomic TREC and KREC profiles of 108 patients with diverse molecularly confirmed PIDs as representing the largest series of patients ever studied, and showed

that various PID disorders are associated with low TREC and/or KREC copy numbers, not limited to SCID and agammaglobulinemia. Such findings will possibly increase the list of secondary targets for NBS. Additionally, as the majority of our patients' DNA samples were collected beyond the neonatal period, our results suggest the potential utility of this triplex assay beyond the newborn period to evaluate the immune system during work-up of various immunodeficiency diseases in addition to the assessment of immune reconstitution post-HSCT. As these assays were performed well beyond the newborn period, these results should be validated by assays in the newborn period.

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Disclosures

The authors declare no conflicts of interest.

Author contributions

M. D. designed the study, analyzed data and prepared the manuscript. H. A.-M. and A. H. participated in the data analysis and manuscript preparation. G. A.-D. and A. J. performed the triplex (TREC, KREC, ACTB) screening assay. F. E. helped with the data analysis. A. M. A., B. A., R. A., H. A.-D. and I. A. provided the patients' information. All authors approved the final version for submission.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Flowcytometry profiles of T/B lymphocytes and corresponding TREC/KREC counts in patients with primary immune deficiencies. Highlighted cells in the table indicate low counts as defined by the reference ranges: TREC < 15, KREC < 10 and reference ranges for T/B cell subsets: CD3 1,600-2,700; Helper T CD4 1,000-1,700; CD8 600-1,000; CD19 400-800; NK CD56 &16 200-400; CD4/CD8 Ratio 1.3-2.0. NA not available.