Regular Article

blood

HEMATOPOIESIS AND STEM CELLS

Modeling altered T-cell development with induced pluripotent stem cells from patients with *RAG1*-dependent immune deficiencies

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- tr r (DNA-DSB) t r t v; t
b r ([CE]).[3] S, Q t t DNA-PK t t
t (DNA-PK) r t tv; t DNA r - r 1C
(DCLRE1C; Art), f b r .
T DNA-DSB r t r r t f b
- t , t r r tt t , t t f -
t V-D-J .[4] RAG t t b r t
t v; r t f b t t tr fl t
b Q f t t t ff t f v; r -
t f t r .[5] I t t t , t f , r
f V(D)J r t t t f r , t .
T B t b r , t T⁻ B⁻ NK⁺ f f
SCID. W t r b v; b t b r b *RAG* t t
t t ff t, , t t t b , V(D)J r t , r ft
t t t t b t t
r , r f T, B, b t .[6-9] T
r f , t , , t -r tv; , tv; t ,
T t t fi tr t r r. b r f
O r (OS). I t r b r b *RAG* t t
t, r , f t , t / r .[5]
U v; tr , r tf b b RAG tv; t
r z r t t v; rt r fl r t
r t (GFP) tt fl RSS, b v; b t t
b t v; r t f b RAG fi rr t t t
r , f t f t t RAG r t .[10] W f , t
t t t t OS, v; r , t r r ,
r t tv; t . T v; t t tOS T⁻ B⁻ NK⁺ SCID
r ff t r f t f t t t *RAG*
t t t t b t rt, t t,
t r rt r v; r t . H v; r, b b v; r T-
v; t t t t *RAG* t t t t OSv;
SCID.

M b v; , t , t t f t f .
v; v; PID, SCID rt , r. A f r SCID
fir t r rt B t ,[11] t r , t f t r , rr .
t t t *Prkdc* .[12] A t , b t *scid* , t
fi t f t T B , , t r t
, r ff f t b t , SCID b t
v; t *scid* 1 r.[13] I tr t, t *Rag1* r
Rag2 , r , t SCID, t tr t t t
t CD4⁻CD8⁻CD44⁻CD25⁺ , tv; 3 t f tr t
T- v; t, r , t f B r T b t .[14,15]
M f OS f SCID b v; r t , b
R 1 R972Q,[16] t R 1 S723C,[7], R 2 R229Q[17] . I
t t t , SCID SCIDv; r t b v; -
t b r .[18,19]
A t , b v; rt t t f r , t .
f t , b rt t t r , t PID , t r r
t t PID b , t t. T r r ff r
T- b t v; t t b , ,[20]
b ff r . H v; r, v; r t t t t
t ffi , t t t t v; t t b f ,
SCID t t , r r v; , r r t f t ,
t r. f tr t, ffi , t t r r t t ,
. R t r b tr t t T . r t
fr b , , r t t t (PSC) v; tr .[21-23] T
v; tr r b r , t f r, f
r t , r , r t- ff tv; t , tr r b
t b t ty; t tr t r r f t t'
f tv; t t t f t ff r t t . A fir t r rt
t t f tv; T- ff r t t t t SCID
t- tv; PSC b rv; -
tr t r rr t f T- v; t f rr
IL2RG

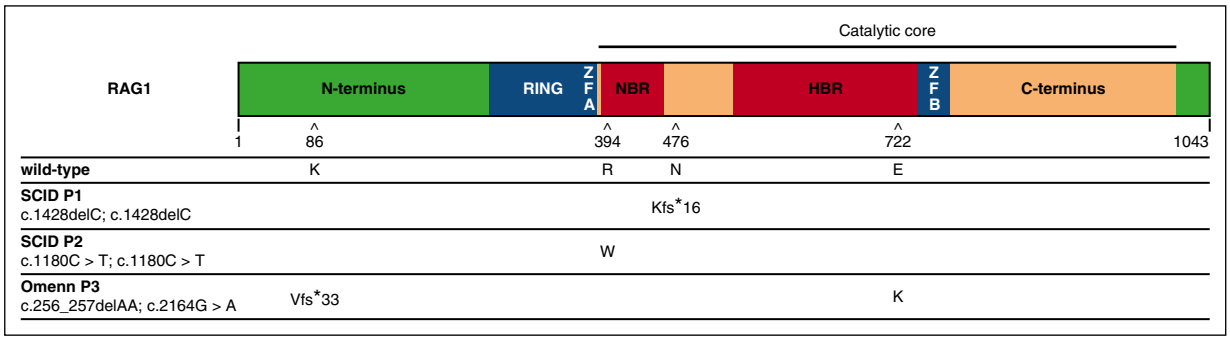


Figure 1. Scheme of RAG1 protein and mutations. WT human RAG1 consists of 1043 amino acids and includes an N-terminus domain, really interesting new gene (RING) finger sequence, zinc finger sequences zinc finger A (ZFA) and zinc finger B (ZFB), the catalytic core, which contains the nonamer- and heptamer-binding regions, and a C-terminus domain. The amino acid positions affected by mutations identified in patients with SCID (P1 and P2) and with OS (P3), and the respective consequences on amino acid sequence are shown. P1 and P2 were homozygous for a frameshift (N476Kfs*16) and a missense (R394W) mutation, respectively. P3 was compound heterozygous for a missense (E722K) and a frameshift (K86Vfs*33). For the latter, an alternative start codon can be used resulting in an N-terminus truncated protein with normal sequence from Met183 onward with cytoplasmic localization. HBR, heptamer-binding region; NBR, nonamer-binding region.

v; tr r f r t t b t , t r b (2000 v; 172 000 b t tr). M t r T- r t r , t . M , r tr t , b tr - , *RAG1* t t (.256 257 ; .2164G>A), r t t , t K86Vf *33 E722K r t b , r v; .

O t f t Pr r Lm, D fi Tr t C rt, rt r ,[25] P1 P2 t fi t f SCID, b r P3 t OS.

Online materials

S , t M t , v; t *Blood* W t . O tr , t fr : (1) fr t v; t f t RAG1 r t ; (2) r t b r t tr t f RAG1- fi t PSC ; (3) PSC t ff r t t ; 4) CD34^{+} t ; (5) OP9-DL-4 , - tr ff r t t ; (6) fl t tr ; (7) t- r t Q f TRB TRA r t r ; (8) Q t fi t f TREC ; (9) tr b r (C t); (10) S t r t ; (11) r t r , r fi t f r v; tr ; (12) v; tr DNA ; (13) v; tr ; (14) t t t .

Results

RAG1 mutations of 3 patients have low recombination activity and result in cleavage defects

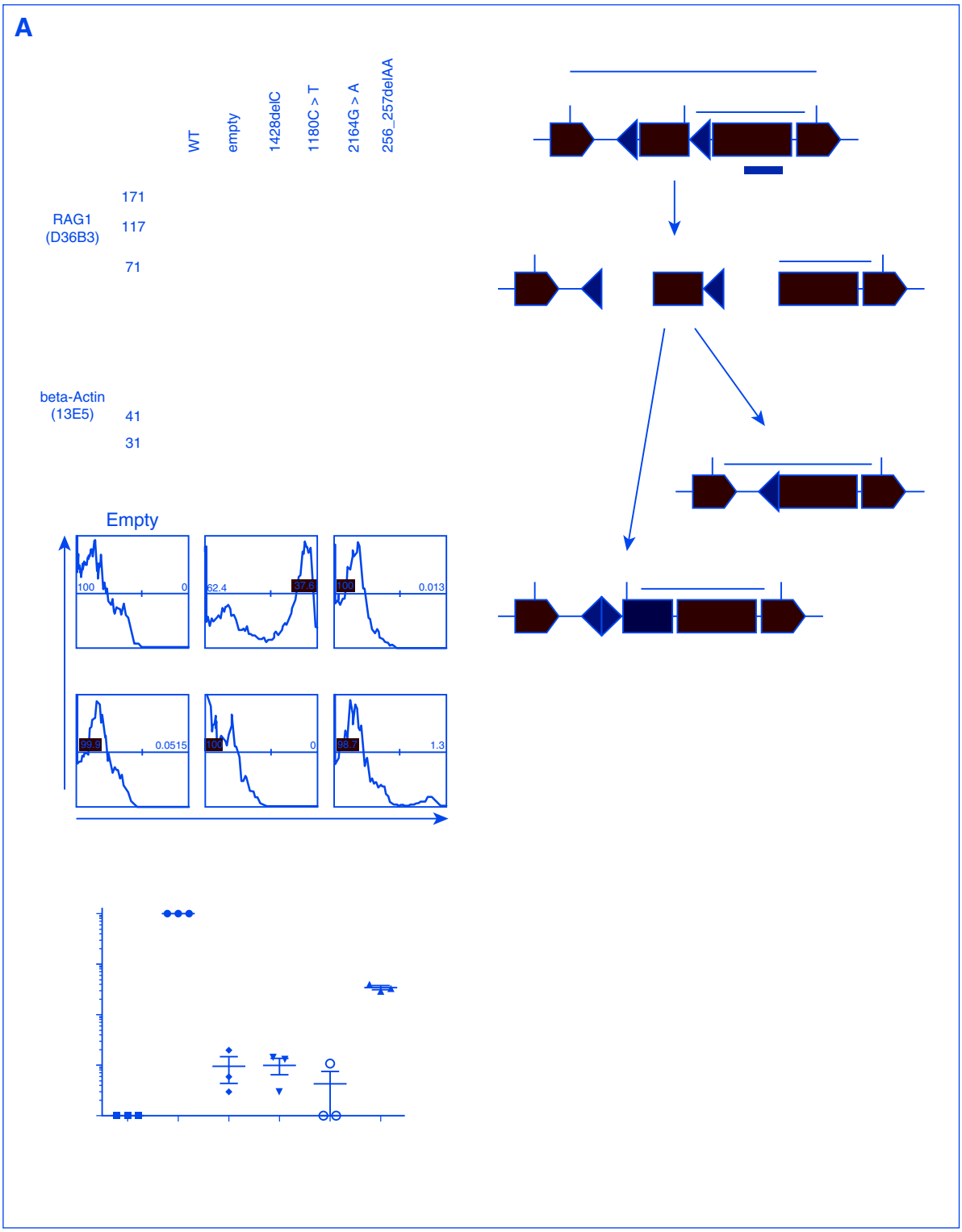
I r r t v; t t t ff t f RAG1 fi b r T- v; t, r t PSC fr r fi r t t fr 2 t t t SCID 1 t t t OS. P1 P2 t SCID r t b t r f r t t *RAG1* t t , b r - , b tr t tr P3 t OS (F r 1). T SCID P1 t t tr , 1428 r t t r t fr b ft r tr t f t RAG1 r t t t r- r (NBR) f t t r t t. T SCID P2 OS P3 t t t t t NBR r b t r- r r v; , r r t t v; r tr t / r f t RSS. T OS P3 t (.256 257 AA) rv; r r t t r , N-t r tr t RAG1 r t t t t t t r , t , f t r v; t r t ; t t v; r fr t r t , , .[26] T b r t r t v; fr t v; t f b f t RAG1 t t , A -tr f r *Rag1*$^{-/-}$ r –B- t v; r t GFP t fl RSS (M -INV t) r tr , t r trv; r v; t r f r t v; r , RAG1 t t r t r -t (WT) RAG1, GFP r , r - , t f t r v; t .[10] T t t r t f r , t r t RAG1 r v; (F r 2A). T t , (D36B3; C S) N-t r , fi t r f r tr t r t f t .256 257 AA t t .

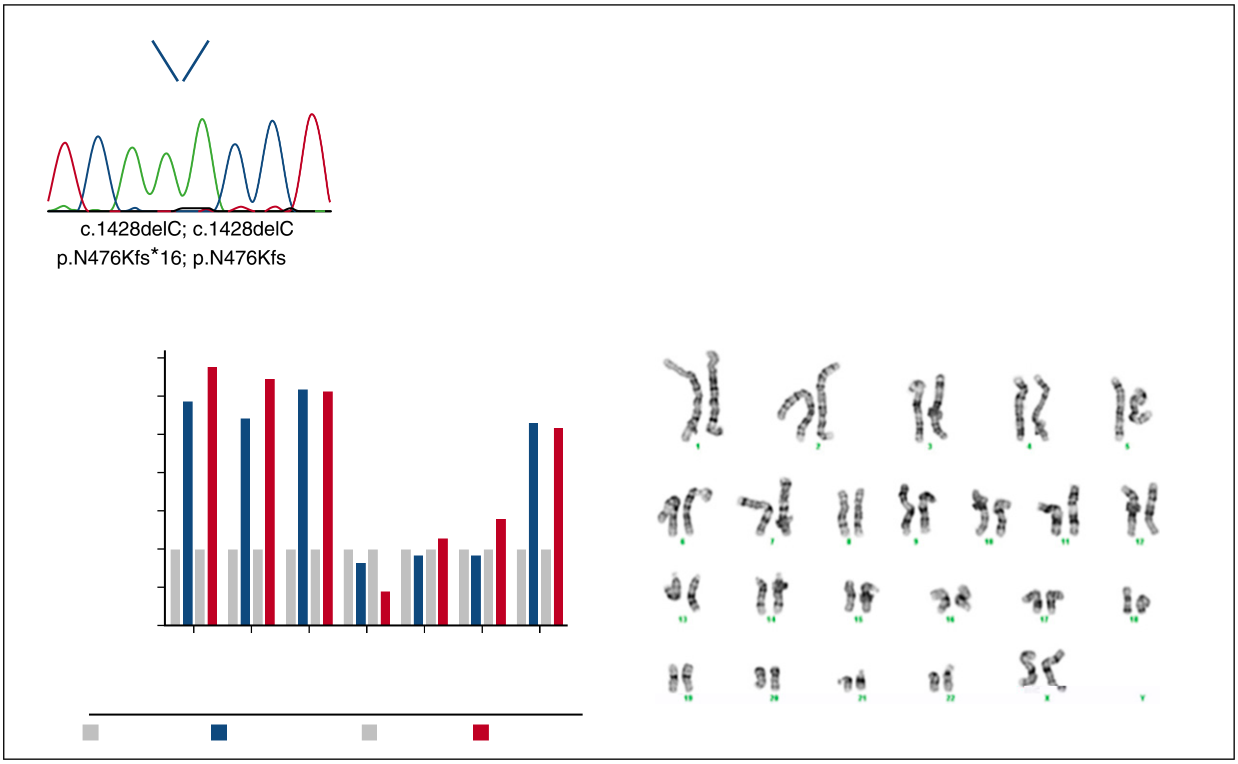
A b F r 2B-C, t *RAG1* t t t t SCID r t tr r t v; t b r t WT *RAG1* (P1, .1428 C: 0.10 ± 0.05%; P2, .1180C>T: 0.10 ± 0.04%). O f t 2 t t t t OS P3 (.2164G>A) b v; r t , t t r t v; t (0.04% ± 0.03), b r t r (256 257 AA) b r - , t b r , f t (3.48 ± 0.35%). T t r t t t t rv; , tr t t t 256 257 AA fr b ft t r t r t r , t t v; RAG1 t t r t , t , f t r v; tr tr t t r t r f N-t r tr t RAG1 r t t r t v; t .[26]

RAG1 t r t f t V-D-J r b v; r f t TCR , . T t b b t r t *RAG1* t t fi t t t r v; , DNA r , t A r –B- r t t *RAG1* (F r 2D-E). A t , S t r - t f t r -B A r t t t t WT RAG1 rv; f t t f t r rr M -INV t (F r 2E). C v; r , t *RAG1* t t t , r t t f t t v; f M -INV r rr t, t t f t .256 257 AA t, f r b b v; r f t rr t r rr M -INV v; (F r 2E), r fl t v; r v; f r t v; OS t t. T r , t r t t t r , r t t v; t f t t t t fl t tr t f GFP r (F r 2B-C). A t , f t RAG1- r t t t b t f r r CE , b b r t t IV$^{-/-}$, t , fir t t t *RAG1* t t b v; f t DNA v; .

Generation of CD34^{+} hematopoietic progenitor cells from RAG1-mutant iPSCs

I r r t t t v; t T- v; t t t t ff r t *RAG1* t t , t v; t . f PSC t t b rv; , r t , t t fr r fi r t f SCID P2 OS P3,[27] f tr PSC t b


A
WT
empty
1428delC
1180C > T
2164G > A
256_257delAA
RAG1
(D36B3)
171
117
71
beta-Actin
(13E5)
41
31
Empty
100
0
62.4
37.6
100
0.013
99.9
0.0515
100
0
98.7
1.3



[illegible] .[28] T [illegible] PSC [illegible] SCID P1, [illegible] 4 [illegible] (OCT4, SO 2, KLF4, -M C). [illegible] PSC [illegible] . M [illegible] RAG1 [illegible] (F r 3A). [illegible] (F r 3B) [illegible] (PCR) (F r 3C) [illegible] KLF4 -M C [illegible] . F [illegible] (F r 3D).

T [illegible] CD34+ [illegible] RAG1 [illegible] PSC [illegible] ,[21] CD34+ [illegible] 8 13 [illegible] F r 1).

T-cell development analysis from control- and patient-derived iPSC lines

T [illegible] T- [illegible] RAG1- [illegible] PSC [illegible] CD34+ [illegible] OP9-DL-4 [illegible] T- [illegible] . F [illegible] 3 4 [illegible] , SCID, OS- [illegible] CD7 [illegible] T- [illegible] (F r 4A). A [illegible] CD34 [illegible] CD5 CD38. H [illegible] SCID OS [illegible] CD7+ CD5− r CD7+ CD38− [illegible]

F [illegible] RAG1 [illegible] CD45RA+ [illegible] . A [illegible] RAG1 [illegible] CD4+ [illegible]

r CD4, [illegible] CD8α CD8β. I [illegible] r CD4+ [illegible] SCID P1 OS [illegible] ~10% t 30% [illegible] CD8α CD8β (F r 4A). A [illegible] RAG1 [illegible] CD3 [illegible]

W [illegible] 4 t 5 [illegible] RAG1 [illegible] CD7 CD5 (F r 4B). C [illegible] CD38 [illegible] CD31+ CD45RA− [illegible] T- [illegible] CD4 CD8α/β. B [illegible] SCID- r OS- [illegible] CD38 CD7, [illegible] CD31−/+ CD45RA+ [illegible] CD4 CD8α/β [illegible] . C [illegible] CD3 [illegible] SCID- r OS- [illegible] . A [illegible] TRB [illegible] 31% [illegible] TCR, [illegible] TCR [illegible] SCID r OS [illegible] F r 2). [illegible] CD45RA− CD31+ CD4+ CD8α/β+ [illegible] RAG1 [illegible] CD34+ [illegible] OP9-DL-4 [illegible] T- [illegible] . S [illegible] 3 [illegible] PSC [illegible] F r 3A-K). T [illegible] A V S TO Gr [illegible] F r 3L), [illegible] A V- [illegible]

OS cells have a higher propensity for DNA breaks

I [illegible] OS [illegible]

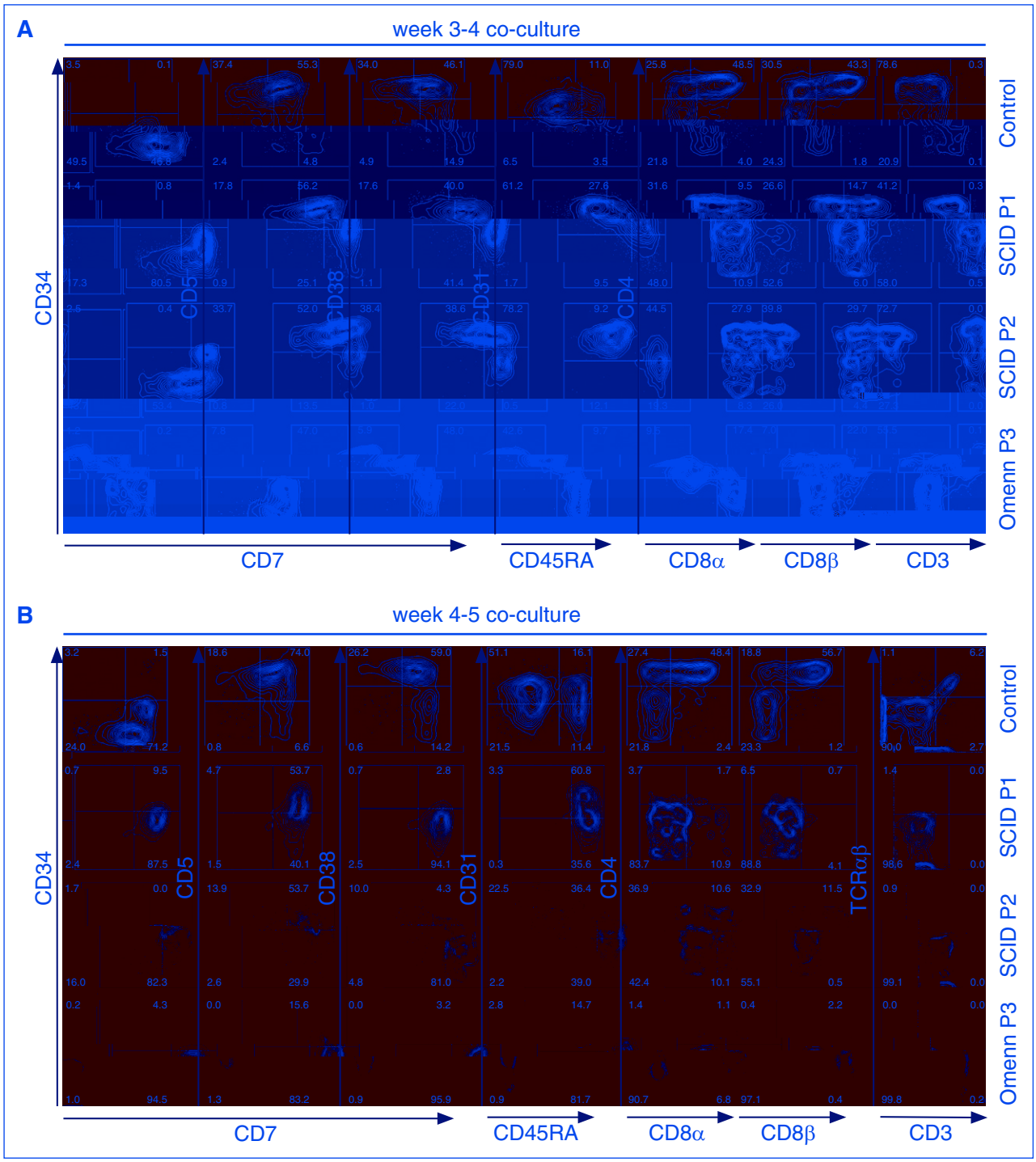


Figure 4. In vitro T-lineage differentiation of control and SCID iPSC lines. Flow cytometric analysis of T-lineage developmental progression of control- and patient-derived cells. iPSCS were allowed to differentiate for 8 days into embryoid bodies, and magnetic bead-purified $CD34^+$ cells were cocultured with OP9-DL-4 cells. (A) Cells from P1 and P2 with SCID, and from P3 with OS attained normal expression of early markers of T-lineage differentiation (CD7, CD5, and CD38) upon 3 to 4 weeks of coculture with OP9-DL-4 cells. (B) After 4 to 5 weeks of coculture, cells from a healthy control progress to the $CD4^+$ $CD8\alpha\beta^+$ DP stage of differentiation, with the appearance of $CD3^+$ TRA/TRB$^+$ cells. By contrast, SCID- and OS-derived cells were mostly blocked at the $CD7^+$ $CD31^{-/+}$ $CD45RA^+$ stage of differentiation, with a virtual absence of CD4 and CD8α/β expression, and lack of $CD3^+$ cells. In (A-B), cells were pre-gated for lymphocytes (SSCxFSC), DAPI-, and $CD45^+$. DAPI, 4′,6-diamidino-2-phenylindole; FSC, forward scatter; SSC, side scatter.

tr b r (C t) rf r , [6,29] t , SCID- v; (F r 5A-B). T t b t r SCID- r t t t DNA r r , b b r r f t OS- t t t t r f RAG1 t ff t r DNA r t tr t (r t) r t f t t t f DNA , rf f DNA. C t r t t v; OS rt fr v; tr t v; (t F r 4A). N t r 25 (t r r t t v; f r SCID r v; t v; f r t r R394W (SCID) r E722K (OS) OS- v;), b r t r t Qv; t tr - r t t (t F r 4B). T .N476Kf *16 t t f SCID

P1 t r , r f r t t (r r) t t v; t . B tr t, .256 257 AA t f OS P3 r t t r , N-t r , tr, t r t (, t , . f tr fr tr t t t), t t t t . [26] C t t , t rv; t C t (F. , r 5A-B).

V(D)J gene recombination analysis of early T cells from OS- and SCID-derived iPSC lines

I t f v; r b t , [30] t t OS h v; v; r r f r , t . T , h h h v; r, v; r TCR r rt r . T t r t t tr tr t f TCR r rt r r r t t r t . f T- v; t OS, Q f TRB TRA V(D)J r rr t rf DNA tr t fr rt CD4$^+$ ISP CD4$^+$ CD8$^+$ DP r t v; tr fr tr -, OS-, SCID- v; PSC (T 1). D t r , SCID P1 DP , t t f r TRA r rr t. A r , t r rt f, Q t t TRB TRA r rr Q v; t OS SCID r t (t F. , r 5A). Br v; r t f *TRBV* *TRAV* , f V-J r tr t (F. , r 6A-B) t t (t F. , r 5B-C) Q fr rt ISP DP v; fr tr PSC , r r t r tr t f TRB TRA r rt r v; Q v; t OS SCID , t (F. , r 6A-B; t F. , r 5B-C). T f r t f tr - RAG1- t t t r rr t t TRA/δ

Table 1. Summary of deep sequencing analysis of the TRB V(D)J rearrangements

Cell line	Population	Total	Unique	Productive total	Productive unique	Clonality
Control	CD4$^+$ CD8$^-$	451 195	3 300	428 110	2512	0.52
		603 206	6 949	478 446	2875	0.52
Control	CD4$^+$ CD8$^+$	734 020	8 775	666 299	6930	0.30
		900 284	14 151	724 386	8414	0.28
SCID P1	CD4$^+$ CD8$^-$	1 026 133	366	1 021 305	219	0.60
Omenn P3	CD4$^+$ CD8$^-$	132 386	120	131 048	76	0.49
Omenn P3	CD4$^+$ CD8$^+$	784 927	301	782 376	217	0.65

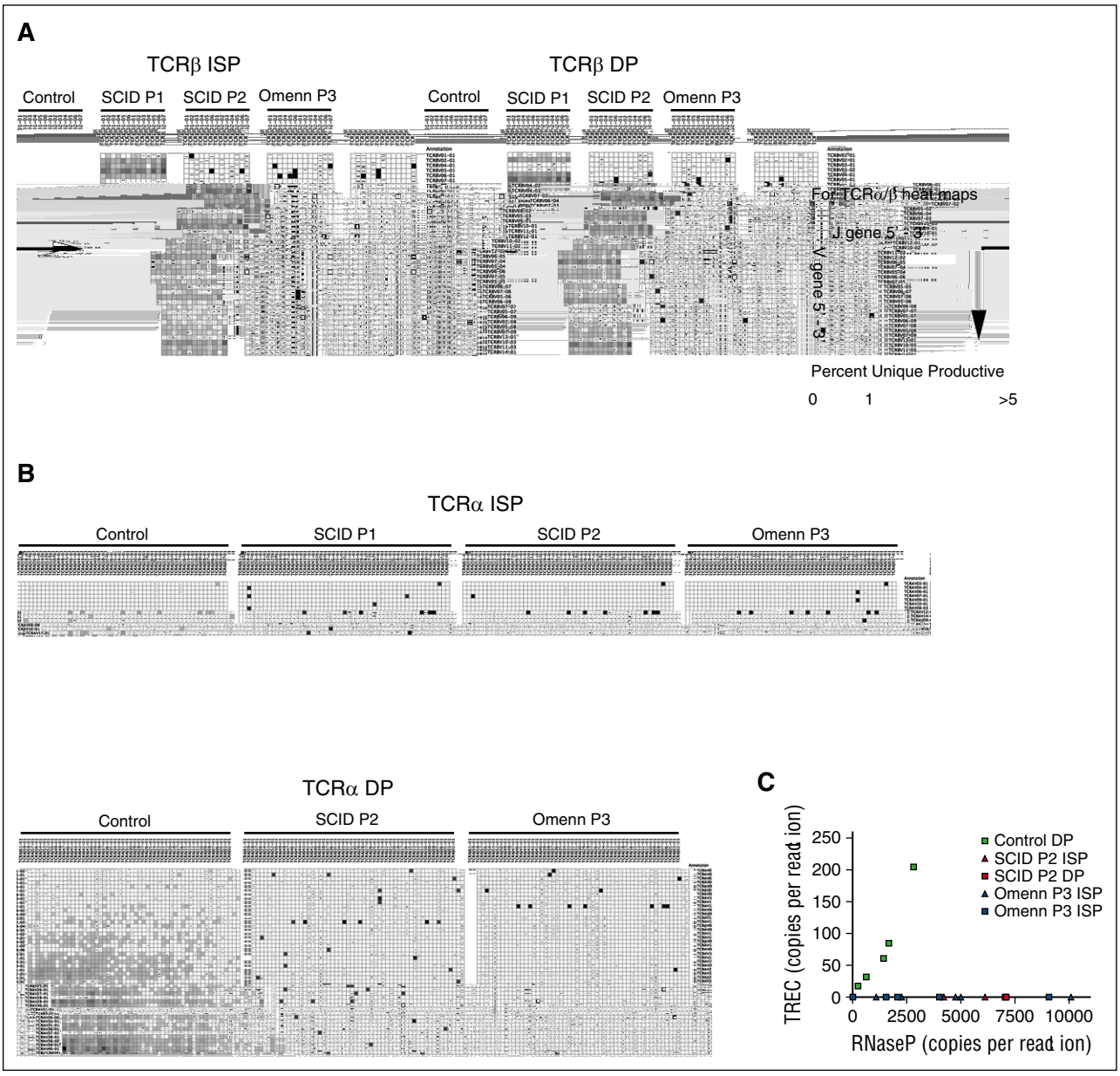


Figure 6. Next generation sequencing analysis of TCR repertoire upon in vitro T-lineage differentiation of control, SCID (P1 and P2), and OS (P3)-derived iPSCs. (A) Heat map representation of percentage of TRB VJ (orientated via chromosomal 5′ to 3′ distribution) pairings among unique sequences in ISP (left) and DP (right) T-lineage cells derived from the indicated patient iPS lines. Results demonstrate 1 representative sample from 2 experiments with similar results. (B) Heat map representation of percentage of TRA VJ (orientated via chromosomal 5′ to 3′ distribution) pairings among total sequences (all δ rearrangements excluded from analysis) in ISP (left) and DP (right) T-lineage cells derived from the indicated patients' iPS lines among unique sequences. Results demonstrate 1 representative sample from 2 experiments with similar results. (C) Quantitative PCR analysis of TRECs in control, SCID P2, and OS P3 cells. RNase P was used as an internal control for quality of genomic DNA amplification. (D) Virtual spectratyping, showing skewing in the distribution of CDR3 lengths among unique TRB or TRA sequences expressed by ISP (top) and DP (bottom) cells in SCID P1, P2, and Omenn P3 compared with control. (E) Distribution of CDR3 length for 5 more commonly expressed V genes in each sample for unique TRB sequences. (F) Distribution of CDR3 length for 5 more commonly expressed V genes in each sample for unique TRA sequences. Results demonstrate 1 representative sample from 2 experiments with similar results. RNase P, ribonuclease P.

, Q t fi p v; f TREC rt DP . TREC r r ¶ t t tr , t t SCID r OS (F , r 6C). V rt tr t fp tr t f TRB CDR3 p m , Q Q b rm tr t f r ISP DP v; fr m tr PSC , b r OS SCID r b r t rz tr t f CDR3 p , p r f r Q (F , r 6D, t). S m r , tr t f CDR3 p v; b z p m t , t V-J TRB r rr m t (F , r 6E). I t r t , TRA CDR3 p tr t v; t SCID OS , t p t m b v; rm CDR3 p b r p r b t rm r , p (F , r 6D, r). I , b z m f p m t , t V-J TRA r rr m t , p b rt (V07-01 V25-01) r rm (V12-02 V21-01) CDR3 p (F , r 6F).

F , tr m r r t t f CDR3 m Q b r v; r t , r , t , ISP

DP fr t r r SCID OS f r TRB (t F r 5E) TRA (t F r 5F) r rt r .

Discussion

C fi t r . r f t , r fl t f . ff t , fi t t v; v; .[31,32] H r , t r r f r r rr t r t . f T- v; t (CD7, CD5, CD38), RAG1 t t t SCID OS. H v; r r f CD5 CD38, r t rt CD7+CD5−CD38− t . N , SCID- OS- v; t t t t r CD4, CD8α, CD8β. Pr r v; t t RAG1/2 r r t CD4−CD8− - t v; t . , t r t r -TCR t t r t t CD4+CD8+ DP t . , f v; f RAG r TRA r t .[33] T r. f r f DP r t r f PSC- v; CD34+ fr t t RAG1- fi t SCID t fir t v; f RAG1 r V(D)J r t r t tr t r Q r f r v; t f DP , r t v; , RAG1 t t rt v; f r t v; t t ffi f r rt r r t DP t . C tr t t t r , tr t r f CD3 TRA/TRB, r r f DP r t t r f SCID- OS- v; , r CD5+CD7 r t . At t r t t , SCID- OS- v; r CD38−, CD31−, CD45RA+, t t f r t ff v; tr v; r r –TCR- t β- t t f T- ff r t t .

A RAG1 t t t OS rt r v; f r t v; t t t A r –B- t , PSC- v; CD34+ fr t t OS t t t rt T- ff r t t v; tr r SCID- v; . It r RAG1 f t OS- v; ffi t t t t V(D)J r - t 17.1

Acknowledgments

References

1. Picard C, Al-Herz W, Bousfiha A, et al. Primary immunodeficiency diseases: an pdate on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. *J Clin Immunol.* 2015;35(8):696-726.
2. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. *Cell.* 2002;109(suppl):S45-S55.
3. Roth DB, Menetski JP, Nakajima PB, Bosma MJ, Gellert M. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. *Cell.* 1992;70(6): 983-991.
4. Fugmann SD. RAG1 and RAG2 in V(D)J recombination and transposition. *Immunol Res.* 2001;23(1):23-39.
5. Notarangelo LD, Kim MS, Walter JE, Lee YN. Human RAG mutations: biochemistry and clinical implications. *Nat Rev Immunol.* 2016;16(4): 234-246.
6. Villa A, Santagata S, Bozzi F, et al. Partial V(D)J recombination activity leads to Omenn syndrome. *Cell.* 1998;93(5):885-896.
7. Giblin W, Chatterji M, Westfield G, et al. Leaky severe combined immunodeficiency and aberrant DNA rearrangements due to a hypomorphic RAG1 mutation. *Blood.* 2009;113(13):2965-2975.
8. Schuetz C, Huck K, Gudowius S, et al. An immunodeficiency disease with RAG mutations and granulomas. *N Engl J Med.* 2008;358(19): 2030-2038.
9. Abolhassani H, Wang N, Aghamohammadi A, et al. A hypomorphic recombination-activating gene 1 (RAG1) mutation resulting in a phenotype resembling common variable immunodeficiency. *J Allergy Clin Immunol.* 2014;134(6):1375-1380.
10. Lee YN, Frugoni F, Dobbs K, et al. A systematic analysis of recombination activity and genotype-phenotype correlation in human recombination-activating gene 1 deficiency. *J Allergy Clin Immunol.* 2014;133(4):1099-1108.
11. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature.* 1983;301(5900):527-530.
12. Blunt T, Finnie NJ, Taccioli GE, et al. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell.* 1995;80(5):813-823.
13. Bosma MJ, Carroll AM. The SCID mouse mutant: definition, characterization, and potential uses. *Annu Rev Immunol.* 1991;9:323-350.
14. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell.* 1992;68(5):869-877.
15. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* 1992;68(5):855-867.
16. Khiong K, Murakami M, Kitabayashi C, et al. Homeostatically proliferating CD4 T cells are involved in the pathogenesis of an Omenn syndrome murine model. *J Clin Invest.* 2007; 117(5):1270-1281.
17. Marrella V, Poliani PL, Casati A, et al. A hypomorphic R229Q Rag2 mouse mutant recapitulates human Omenn syndrome. *J Clin Invest.* 2007;117(5):1260-1269.
18. Perryman LE. Molecular pathology of severe combined immunodeficiency in mice, horses, and dogs. *Vet Pathol.* 2004;41(2):95-100.
19. Wiler R, Leber R, Moore BB, VanDyk LF, Perryman LE, Meek K. Equine severe combined immunodeficiency: a defect in V(D)J recombination and DNA-dependent protein kinase activity. *Proc Natl Acad Sci USA.* 1995;92(25): 11485-11489.
20. Spits H. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol.* 2002;2(10): 760-772.
21. Kennedy M, Awong G, Sturgeon CM, et al. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Reports.* 2012;2(6):1722-1735.
22. Vizcardo R, Masuda K, Yamada D, et al. Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. *Cell Stem Cell.* 2013;12(1):31-36.
23. Chang CW, Lai YS, Lamb LS Jr, Townes TM. Broad T-cell receptor repertoire in T-lymphocytes derived from human induced pluripotent stem cells. *PLoS One.* 2014;9(5):e97335.
24. Menon T, Firth AL, Scripture-Adams DD, et al. Lymphoid regeneration from gene-corrected SCID-X1 subject-derived iPSCs. *Cell Stem Cell.* 2015;16(4):367-372.
25. Shearer WT, Dunn E, Notarangelo LD, et al. Establishing diagnostic criteria for severe combined immunodeficiency disease (SCID), leaky SCID, and Omenn syndrome: the Primary Immune Deficiency Treatment Consortium experience. *J Allergy Clin Immunol.* 2014;133(4): 1092-1098.
26. Santagata S, Gomez CA, Sobacchi C, et al. N-terminal RAG1 frameshift mutations in Omenn's syndrome: internal methionine usage leads to partial V(D)J recombination activity and reveals a fundamental role in vivo for the N-terminal domains. *Proc Natl Acad Sci USA.* 2000; 97(26):14572-14577.
27. Pessach IM, Ordovas-Montanes J, Zhang SY, et al. Induced pluripotent stem cells: a novel frontier in the study of human primary immunodeficiencies. *J Allergy Clin Immunol.* 2011;127(6):1400-1407.
28. Weinacht KG, Brauer PM, Felgentreff K, et al. The role of induced pluripotent stem cells in research and therapy of primary immunodeficiencies. *Curr Opin Immunol.* 2012;24(5):617-624.
29. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun.* 1984;123(1):291-298.
30. Signorini S, Imberti L, Pirovano S, et al. Intrathymic restriction and peripheral expansion of the T-cell repertoire in Omenn syndrome. *Blood.* 1999;94(10):3468-3478.
31. Milner JD, Holland SM. The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. *Nat Rev Immunol.* 2013;13(9):635-648.
32. Villa A, Sobacchi C, Notarangelo LD, et al. V(D)J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. *Blood.* 2001; 97(1):81-88.
33. Wilson A, Held W, MacDonald HR. Two waves of recombinase gene expression in developing thymocytes. *J Exp Med.* 1994;179(4):1355-1360.
34. Swanson PC. The DDE motif in RAG-1 is contributed in trans to a single active site that catalyzes the nicking and transesterification steps of V(D)J recombination. *Mol Cell Biol.* 2001;21(2): 449-458.
35. Yu K, Lieber MR. Mechanistic basis for coding end sequence effects in the initiation of V(D)J recombination. *Mol Cell Biol.* 1999;19(12): 8094-8102.
36. Bischerour J, Lu C, Roth DB, Chalmers R. Base flipping in V(D)J recombination: insights into the mechanism of hairpin formation, the 12/23 rule, and the coordination of double-strand breaks. *Mol Cell Biol.* 2009;29(21):5889-5899.
37. Grawunder U, Lieber MR. A complex of RAG-1 and RAG-2 proteins persists on DNA after single-strand cleavage at V(D)J recombination signal sequences. *Nucleic Acids Res.* 1997;25(7): 1375-1382.
38. Teng G, Maman Y, Resch W, et al. RAG represents a widespread threat to the lymphocyte genome. *Cell.* 2015;162(4):751-765.
39. Rieux-Laucat F, Bahadoran P, Brousse N, et al. Highly restricted human T cell repertoire in peripheral blood and tissue-infiltrating lymphocytes in Omenn's syndrome. *J Clin Invest.* 1998;102(2):312-321.
40. Brooks EG, Filipovich AH, Padgett JW, Mamlock R, Goldblum RM. T-cell receptor analysis in Omenn's syndrome: evidence for defects in gene rearrangement and assembly. *Blood.* 1999;93(1): 242-250.
41. Yu X, Almeida JR, Darko S, et al. Human syndromes of immunodeficiency and dysregulation are characterized by distinct defects in T-cell receptor repertoire development. *J Allergy Clin Immunol.* 2014;133(4):1109-1115.
42. Hughes MM, Yassai M, Sedy JR, et al. T cell receptor CDR3 loop length repertoire is determined primarily by features of the V(D)J recombination reaction. *Eur J Immunol.* 2003; 33(6):1568-1575.
43. Rock EP, Sibbald PR, Davis MM, Chien YH. CDR3 length in antigen-specific immune receptors. *J Exp Med.* 1994;179(1):323-328.
44. Nishio J, Suzuki M, Nanki T, Miyasaka N, Kohsaka H. Development of TCRB CDR3 length repertoire of human T lymphocytes. *Int Immunol.* 2004;16(3):423-431.