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Research paper

Long-term robustness of a T-cell system emerging from somatic rescue of a genetic block in T-cell development

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ABSTRACT

Backgound: The potential of a single progenitor cell to establish and maintain long-term protective T-cell immunity in humans is unknown. For genetic disorders disabling T-cell immunity, somatic reversion was shown to support limited T-cell development attenuating the clinical phenotype. However, the cases reported so far deteriorated over time leaving unanswered the important question of long-term activity of revertant precursors and the robustness of the resulting T-cell system.
Methods: We applied TCRβ-CDR3 sequencing and mass cytometry on serial samples of a now 18 year-old SCIDX1 patient with somatic reversion to analyse the longitudinal diversification and stability of a T-cell system emerging from somatic gene rescue.
Findings: We detected close to 10^5 individual CDR3β sequences in the patient. Blood samples of equal size contained about 10-fold fewer unique CDR3β sequences compared to healthy donors, indicating a surprisingly broad repertoire. Despite dramatic expansions and contractions of individual clonotypes representing up to 30% of the repertoire, stable diversity indices revealed that these transient clonal distortions did not cause long-term repertoire imbalance. Phenotypically, the T-cell system did not show evidence for progressive exhaustion. Combined with immunoglobulin substitution, the limited T-cell system in this patient supported an unremarkable clinical course over 18 years.
Interpretation: Genetic correction in the appropriate cell type, in our patient most likely in a T-cell biased self-renewing hematopoietic progenitor, can yield a diverse T-cell system that provides long-term repertoire stability, does not show evidence for progressive exhaustion and is capable of providing protective and regulated T-cell immunity for at least two decades.
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Reseach in context

Evidence before this study

The potential of a limited number of progenitor cells to establish and maintain long-term protective T-cell immunity in humans has so far been estimated based on data from 3 different model situations.

1. In patients with genetic disorders disabling T-cell immunity, somatic reversion was shown to support limited T-cell development attenuating the clinical phenotype. However, the best studied case reported so far deteriorated over time leaving unanswered the important question of long-term activity of revertant precursors and the robustness of the resulting T-cell system.
2. Gene therapy trials in primary immunodeficiencies have shown long-term reconstitution of a polyclonal T-cell repertoire following treatment with gene-corrected HSC. The most detailed study reported normal repertoire diversity in SCID-X1 patients up to 15 years after gene therapy. However, the number of different integration sites detected in purified T cells was in the range of about 1,000, implying that they were descendants of a large number and not a few HSPC clones.
3. Studies transplanting barcoded human purified HSCs into NSG mice have shown that only few of the transferred HSPC clones seeded the thymus and that less than 10 HSPC could provide a polyclonal repertoire. However, in this murine experimental model, diversity was assessed only at a single time point by low resolution CDR3 spectratyping.

Added value of this study

The unique constellation in our patient provides several advantages over the model systems analysed so far to estimate the potential of a single progenitor cell to establish and maintain long-term protective T-cell immunity:

1. Analysis of an SCIDX1 patient with revertant T cells, but no NK cells allowed pinpointing the genetic reversion to a T-cell committed precursor.
2. Repeated analysis until 18 years of age with evidence for long-term phenotypic stability and longitudinal repertoire robustness indicated reversion in a self-renewing precursor.
3. High resolution NGS CDR3β repertoire analysis revealed a surprisingly diverse repertoire and allowed detailed analysis of repertoire structure and comparison to healthy individuals and patients undergoing gene therapy
4. The age of the patient enabled a clinical observation period of almost 2 decades, providing an opportunity to judge the long-term protective and regulatory function of the resulting T-cell system

Implications of all the available evidence

Genetic correction of the appropriate cell type, in our patient most likely in a T-cell biased self-renewing hematopoietic progenitor, can yield a diverse T-cell system that provides long-term repertoire stability, does not show evidence for progressive exhaustion and is capable of providing protective and regulated T-cell immunity for at least two decades.

1. Introduction

T lymphocytes are part of the multi-lineage hematopoietic system emerging from hematopoietic stem cells (HSCs). The central role of T cells in adaptive immunity is highlighted by genetic defects that abrogate T-cell development, which causes severe combined immunodeficiency [1]. SCID is lethal within the first two years of life, unless patients undergo hematopoietic stem cell transplantation (HSCT). Hypomorphic mutations in genes whose loss of function is associated with SCID, such as incomplete RAG deficiency [2,3] often exhibit residual T-cell immunity and hence present with a less severe clinical phenotype [4]. This group of incomplete defects of T-cell development also includes patients with thymic dysgenesis on the basis of DiGeorge anomalies [5].

The ability of T lymphocytes to provide protection against diverse pathogens relies on their structurally diverse T cell antigen receptor [6,7]. The generation of TCR diversity during thymic maturation of T-cell committed hematopoietic stem and progenitor cells (HSPC) results in an estimated TCR diversity between 10^6 and 10^8 in human blood [8–10]. This repertoire forms the basis of T-cell mediated protective immunity. The sequence of the TCR CDR3 regions that contribute to the surface interaction with peptide/MHC complexes essentially functions as a unique barcode for a particular clone of T cells, the expansion and contraction of which are driven by homeostatic cues and the presence of antigen. Sequence analyses of the CDR3 regions of the β-chains of the TCR heterodimer (CDR3β) are frequently used to estimate the diversity of the TCR repertoire and to follow the fate of expanded clones, which in some instances have been tracked for up to 20 years in humans [11].

An important biological question is how much a single progenitor cell can contribute to long-term protective T-cell immunity in humans. This problem has been addressed in 3 different model situations. First, Bousso et al. ingeniously exploited the observation of a SCIDX1 patient with somatic genetic rescue of the T-cell compartment (P1) to characterize a human T-cell system evolving from a single revertant progenitor [12,13]. CDR3β spectratyping and sequencing of a small number of CDR3β regions suggested the presence of at least 1000 different TCR sequences. However, lack of evidence for continuous thymic output indicated reversion in a short-lived T-cell precursor lacking self-renewal capacity. Progressive functional exhaustion of the limited T-cell compartment was considered to explain the eventual clinical deterioration leading to HSCT at age 6 years [14]. This case therefore left unanswered the important question of long-term activity of revertant precursors and the robustness of the resulting T-cell system. Second, gene therapy trials in primary immunodeficiencies have reported normal repertoire diversity in SCID-X1 patients up to 15 years after gene therapy. However, the number of different integration sites detected in purified T cells was in the range of about 1,000, implying that they were descendants of a large number of HSPC clones [15]. Third, studies transplanting barcoded human purified HSCs into NSG mice have shown that only few of the transferred HSPC clones seeded the thymus and that less than 10 HSPC could provide a polyclonal repertoire. However, resulting TCR diversity was assessed only at a single time point by low resolution CDR3β spectratyping [16] and the implications of this murine experimental model for human T-cell development may be limited.

Here, we report the longitudinal study of another SCIDX1 patient with somatic gene reversion (P2)(14). Using CDR3β sequencing, we document that the T-cell diversity in P2 far exceeded that of P1. Unexpectedly, despite transient expansions of individual clones, we observed long-term repertoire stability over 8 years without phenotypic evidence for increasing T-cell exhaustion. Importantly, the limited T-cell system provided protective and regulated immunity for now 18 years. These findings can best be explained by reversion in a long-lived or self-renewing committed T-cell progenitor and continued low-level thymic output over years. They support

emerging concepts of long-term self-renewal and proliferation capacities of stem and progenitor cells with biased lineage output.

2. Material and methods

2.1. Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Stephan Ehl (stephan.ehl@uniklinik-freiburg.de).This study did not generate new unique reagents.

2.2. Patients and healthy donors

Informed consent for the performed studies was obtained from all participants. The study was conducted according to the Declaration of Helsinki and approved by Ethics Committees of the University of Freiburg (No. 230/11-120179)

2.3. Flow cytometry, cell sorting and proliferation

Immunophenotyping was performed using peripheral blood derived mononuclear cells (PBMCs). Single cell suspensions were stained at 4°C for 20min with the antibodies summarized in table S2. For analysis of T−cell proliferation, PBMCs were labeled with CFSE (Molecular Probes) for 15 min at 37°C. Cells were seeded into a 96−well flat−bottom plate and left untreated or stimulated with plate bound CD3 antibody 300 ng mL−1 (OKT3, eBioscience) and soluble CD28 antibody 1 μg mL−1 (CD28.2, BD Biosciences) or PHA (Remel) at a final concentration of 2.5 μg mL−1. Cells were harvested after 4 days, stained for CD8 and CD4 and analysed by flow cytometry. For intracellular staining, cells were fixed and permeabilized with IntraPrep Permeabilization Reagent (Beckman Coulter) according to manufacturer's instructions. DAPI (Invitrogen) or Fixable Viability dye eFluor780 (eBioscience) were used for dead cell exclusion. Data were acquired with a Gallios or Navios Flow cytometer (Beckman Coulter). Data was analysed using FlowJo10.4 software (Tree Star). Cell populations were sorted with MoFlo Astrios (Beckman Coulter).

2.4. Genetic analysis

Cell population specific genomic analyses of IL2RG exon 3 was performed by exon amplification and subsequent Sanger sequencing with the ABI Prism® Terminator v1.1 Sequencing Kit (ABI). Sequences were separated on an ABI Prism® 3100 Genetic Analyzer (ABI). Area under the peak values were obtained using the Mutation Surveyor® Software (Softgenetics). The Sanger sequencing results were confirmed by single molecule sequencing of genomic amplicons of the complete IL2RG locus by nanopore sequencing (Oxford Nanopore Technologies).

2.5. TREC quantification

The ddPCR workflow was performed with the QX100TM system (Bio-Rad) according to the manufacturers instructions. 800ng genomic DNA per sample isolated from PBMCs was digested with HindIII (NEB) for 2.5h at 37°C and heated to 80°C for 20min. Primers and probes used for detection of TRECs are listed in Table S3, Rpp30 was used as reference gene as previously described [17]. Primers were used at 0.9μM and probes at 0.25μM final concentration. 300ng of genomic DNA were used to setup the reaction mixture in 2x ddPCR Supermix for probes no dUTP (Bio-Rad). 20μl reaction mixture and 70μl droplet generation oil (Bio-Rad) were loaded into a DG8 cartridge (Bio-Rad) and placed in the automated droplet generator (Bio-Rad). Droplets were manually transferred with a multichannel pipette into a 96well PCR plate and sealed with a Plate sealer (Bio-Rad). PCR amplification was performed with the following conditions: 95°C for 10 minutes, followed by 40 cycles of 30sec at 94°C and 1min at 58°C and a final step at 98°C for 10 minutes. Plates were directly read in the QX100 Droplet Reader (Bio-Rad). Data was analysed using QuantaSoft software version 1.7.4 (Bio-Rad).

2.6. Staining and CyTOF acquisition

Sample preparation was performed as described [18] with antibodies listed in Table S4. A Helios mass cytometry instrument (Fluidigm) was used for sample acquisition. Data analysis was performed using the Cytobank platform and FlowJo10.4 software (Tree Star). UMAP v2.1 was used as a plugin for FlowJov10.4, detailed parameters of UMAP analysis are provided in Table S5. The data was obtained according to the "Guidelines for the use of flow cytometry and cell sorting in immunological studies".

2.7. CDR3β sequencing and analysis

Genomic DNA from peripheral blood mononuclear cells was isolated using the QiAamp DNA Blood Mini Kit (Qiagen). TCRβ chain CDR3 regions were sequenced using the Immunoseq™ Assay (Adaptive Biotechnologies). CDR3 regions were amplified in a bias-controlled multiplex PCR and sequenced using the Illumina platform (Illumina). CDR3β sequences were aligned to a reference genome, collapsed and filtered for identification and quantification as previously described [19]. VDJ gene definitions are based on the International ImMunoGeneTics system [20]. Productive unique and total sequences were analysed with the ImmunoSEQ Analyzer and with R software (version 3.4.2). Mutual Information (MI), a measure of dependence between 2 variables, was calculated for V and J gene segment usage of unique and total clonotypes:

$$MI(X;Y) = H(X) + H(Y) - H(X,Y),$$

where Shannon entropy (H) is defined as:

$$H(X) = -\sum p(x) log2(p(x)).$$

Clonality was calculated as normalized reciprocal of Shannons entropy [21] and slopes of power law distributions were calculated as previously described [22]. Differentially abundant clonotypes between 2 samples were identified as previously described [23,24]. To assess CDR3β overlap between samples we calculated Baroni Urbani and Buser Overlap index [25,26]:

$$BUB(t1,t2) = \frac{n12 + \sqrt{n12d12}}{n1 + n2 - n12 + \sqrt{n12d12}}$$

To assess similarity in clonotype distribution, Morisita Horn Index was calculated using EstimateS software version 9.1.0 [27].

2.8. Statistical analysis

Linear regression analysis and F-tests were calculated using GraphPad Prism 8.

2.9. Data availability

CDR3β sequencing data used in this study will be available for download and analysis at ImmuneACCESS®, an open-access, shareable repository (Adaptive Biotechnologies).

3. Results

3.1. Somatic gene rescue reconstituting T cells, but not NK cells is sufficient to provide long-term protective immunity in a SCIDX1 patient

At 6 years of age, patient P2 [14] had been found to carry a deleterious germline IL2RG variant, c.452T>C; p.Leu151Pro, in all investigated cell types with the exception of $\alpha\beta$ and $\gamma\delta$ T cells. Immunoglobulin substitution was initiated and he has been free of any significant infection or manifestation of immune dysregulation since then. Stability of the genetic mosaicism was confirmed in a blood sample obtained at 18 years of age. While TCR$\alpha\beta$+ and TCR$\gamma\delta$+ T cells purified by flow cytometry carried the IL2RG wild-type sequence, CD19+ B cells and granulocytes exhibited the mutant allele (Fig. 1a). NK cells were at the detection limit by flow cytometry and could therefore not be purified for genetic analysis (Fig. S1a/b). The genetic studies indicate that the mosaicism in P2 was stable over time, best explained by somatic gene reversion in one or several progenitor cells that provided long-term reconstitution of $\alpha\beta$ and $\gamma\delta$ T cells, but not of NK cells (Fig. 1b).

3.2. Stable T-cell populations over time despite very low thymic output

Between the age of 6 and 18 years, P2 had low, but stable counts of CD4+ (91-313 cells/μl) and CD8+ T cells (144-614 cells/μl) (Fig. 1c, d). TCR$\gamma\delta$+ T cells were found in normal numbers and exhibited the expected age-related decline (Fig. 1e). The distribution of naïve (CD27+CD45RA+), central memory (CM; CD27+CD45RA-), effector memory (EM; CD27-CD45RA-) and effector memory RA (TEMRA; CD27-CD45RA+) cells was skewed towards CM cells, with very few naïve cells among CD4 and CD8 T cells. Over time, this constellation was stable among CD4, but more variable among CD8 T cells (Fig. 1f, g). Recent thymic emigrants, characterized by CD45RA+CD31+ surface expression [28], were detectable, albeit more than 100-fold below reference values, compatible with persistent low-level thymic output (Fig. 1h). T-cell receptor excision circles (TRECs) represent an established indicator of ongoing thymic output [29]. However, digital droplet PCR failed to reliably detect TRECs in T cells from P2 (Fig. 1i), indicating that TREC levels were at or below the detection limit of our assay and reduced at least 100-fold compared to age-matched donors. In an age-matched patient with incomplete DiGeorge syndrome (DGS) and a similar degree of chronic T-cell lymphopenia, TRECs could also not be detected by our method (Fig. 1i), attesting to its limited sensitivity. Collectively, the low percentage of naïve T cells associated with the long-term numeric stability of T cells suggests a minimal, but persistent thymic output.

3.3. Stable T-cell differentiation over time without evidence for increasing exhaustion

The proliferation capacity of P2 CD4+ and CD8+ T cells was consistently normal in the period between 6 and 18 years of age (Fig. S1c). To look for more subtle evidence of cellular exhaustion of the limited T-cell system over time, we performed a longitudinal analysis of T-cell exhaustion markers, including the inhibitory receptors PD1, LAG3, TIGIT and TIM3, as well as the ectoenzyme CD38, the chemokine receptor CXCR5, and the co-regulatory receptor CTLA4 [30]. We were able to study six patient samples collected over 8 years and compared them to samples taken from 6 healthy individuals (HD) in a similar age range; for consistency, the samples were analysed in parallel in one mass cytometry by time of flight (CyTOF) experiment. Gated on CD3+ T cells, UMAP-based dimensionality reduction [31] delineated distinct populations of CD4+ T cells, CD8+ T cells and TCR$\gamma\delta$+ T cells. The FlowSOM algorithm for unsupervised clustering identified four clusters each of CD4+ T cells (clusters 5-7 and cluster 9) and CD8+ T cells (clusters 0-2 and cluster 4), as well as one cluster of TCR$\gamma\delta$+ T cells (cluster 3) and one cluster of CD4-CD8- double negative T cells (cluster 8) (Fig. 2a). Clusters 2 and 9 showed the most pronounced expression of markers associated with T-cell exhaustion, including PD1, TIGIT, CTLA4 and CD38 (Fig. 2b). The frequencies of clusters 2 and 9 among CD3+ T cells of P2 were within the range observed in HDs, with the exception of a single time point for cluster 2 (Fig. 2c). In addition, cluster 2 and 9 did not show an continuous increase in expression of exhaustion related markers (Fig. 2d). Collectively, while P2s T-cell compartment showed an altered differentiation pattern including an increased proportion of memory phenotype cells and cells expressing the senescence marker CD57 (TabS1) already at the first time point, longitudinal studies did not detect a further increase in exhaustion-like or senescent T cells in P2.

3.4. Somatic gene rescue provides a limited T-cell repertoire in a SCIDX1 patient

To evaluate the TCR repertoire generated in the context of somatic gene rescue, we first performed flow cytometry using a panel of 18 Vβ−family specific antibodies at a single time point. As a reference, we included 2 patients with incomplete block in T-cell development, an age-matched patient with incomplete DiGeorge syndrome (DGS), and an infant with RAG1 deficiency presenting with Omenn syndrome (OS) (Fig. 3a). The lymphocyte subsets of these patients are summarized in Table S1. While the Vβ-usage among P2 CD4+ T cells was largely within reference values, we detected large perturbations in the CD8+ repertoire, similar to the situation in the DGS patient. As expected [32], perturbations of Vβ-usage were much more pronounced in the OS patient, where several Vβ families were not detectable in both CD4+ and CD8+ T cells (Fig. 3b,c).

For more detailed repertoire analysis, we performed targeted sequencing of the CDR3 regions of the TRB genes. We corrected for potential bias caused by different sample sizes by downsampling all samples to the lowest number of CDR3β sequences obtained in a single sample (29519). First, we analysed usage of V- and J-segments. While in HD, 108 of the 676 possible VJ combinations were not detectable, this number was higher in P2 and DGS (148 and 170), accompanied by moderate expansions of certain VJ combinations. In the OS patient, the majority of VJ combinations were not detectable; instead, some combinations were dramatically expanded in line with the inefficiency of RAG-mediated recombination (Fig. 3d). To further analyse VJ usage, we calculated the mutual information, a measure of dependence between two variables, for V- and J-segment usage for each donor. Mutual information based on unique sequences (reflecting the primary thymic repertoire) was low for HD, P2 and DGS, but ~9 fold higher for OS compared to age matched HD, again highlighting the defective V(D)J recombination in OS (Fig. 3e). However, when the frequencies of clonotypes were additionally considered, mutual information was 11 and 8-fold higher for P2 and DGS, respectively, compared to adult HD, and thus closer to OS (~21-fold higher than an age-matched control) (Fig. 3f). Thus, while VJ usage is clearly biased in P2 and DGS, this is likely caused by selective expansion of peripheral T cell clones in the respective lymphopenic environments, and not associated with altered V(D)J recombination during thymocyte development.

Next, we quantified the number of unique CDR3β sequences. In P2, across all 10 samples (7 different time points, 3 measured in duplicates) a total of 87562 individual CDR3β sequences were detected. After downsampling, a roughly 3-fold reduced number of unique CDR3β sequences was observed in both P2 and DGS relative to HD. The OS sample contained no more than 624 different clonotypes (Fig. 3g). This suggested a large contribution of expanded clonotypes to the T-cell populations. In HD, the 500 most frequent clonotypes represent only about 11% of all CDR3β sequences; by contrast, the 10 most frequent clonotypes in OS furnish 50% of all CDR3β sequences. The distributions of P2 and DGS occupy an intermediate

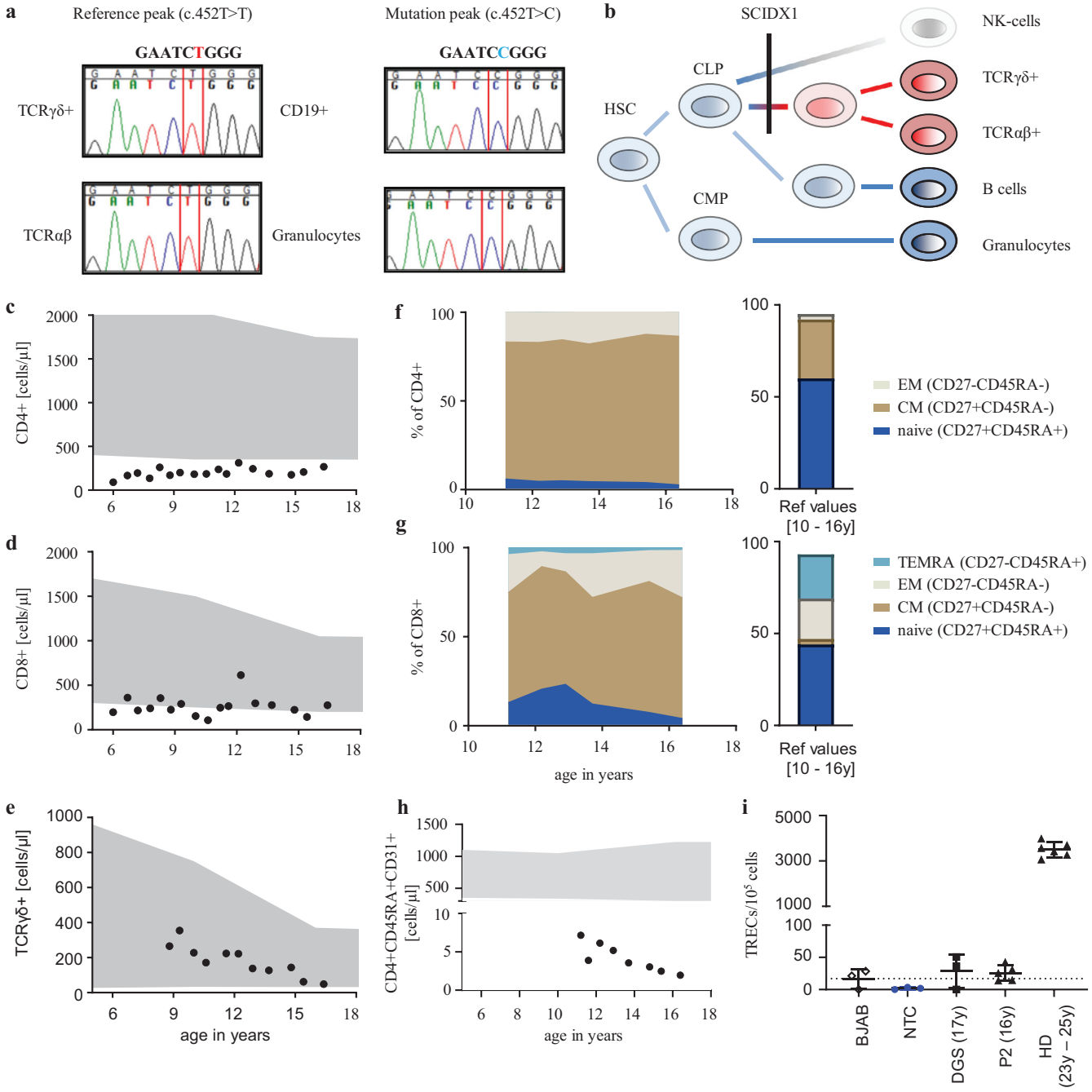

Fig. 1. Somatic gene rescue provides a longitudinally stable T-cell system in a SCIDX1 patient.

(a) Sanger sequencing of sorted lymphocyte subsets. CD3+TCR$\gamma\delta$+, CD3+TCR$\alpha\beta$+ T cells and CD19+ B cells were isolated by FACS. The pellet of Ficoll density centrifugation was the source of the granulocyte fraction. These results were validated by nanopore sequencing (data not shown). (b) Scheme of the hematopoietic system in P2. SCIDX1 patients are characterized by a complete block in NK and T-cell development with normal development of B cells. In P2, somatic genetic rescue allowed development of both $\alpha\beta$ and $\gamma\delta$ T cells while not sufficiently reconstituting NK cells. Peripheral blood counts for CD4+ (c), CD8+ (d) and TCR$\gamma\delta$+ (e) T cells and differentiation pattern of CD4+ (f) and CD8+ (g) T cells based on surface expression of CD27 and CD45RA. EM=effector memory, CM=central memory, TEMRA=effector memory T cells re-expressing CD45RA. Right graphs show published reference values [62]. (h) Peripheral blood counts for CD4+CD45RA+CD31+ Recent Thymic Emigrant T cells. (i) Number of T cell receptor excision circles (TRECs) per 100.000 T cells as determined by digital droplet PCR. NTC = no template control. BJAB = Burkitt lymphoma B cell line (negative control), DGS = patient with severe DiGeorge Syndrome. Dotted line represents mean background measurement in the TREC-negative BJAB cell line. For BJAB and DGS three independent measurements of the same sample are shown. For P2, samples from three time points are shown, for one of which three independent replicates were performed. For HD (23-25y) 2 replicates each of 3 donors are shown.

position, where the 100 most frequent clonotypes cover approximately 50% of all CDR3β sequences (Fig. 3h). Notably, in P2 and DGS, around 25%-40% of CDR3β sequences were contributed by rare clonotypes, i.e., clonotypes with ranks beyond 500, whereas such rare clonotypes did not exist in OS, illustrating the much larger overall diversity in P2 and DGS. We characterized the diversity of the CDR3β sequence repertoires by two additional parameters. First, we calculated the clonality score (Fig. 3i), which is defined as normalized reciprocal of Shannon's entropy [21]; clonality scores scale from 0 to 1, and approach 1 when the repertoire is dominated by few expanded clonotypes. Second, we calculated the slope of the respective power law distributions (Fig. 3j-l) following the procedure described by DeWolf et al [22]; the slopes increase with increasing diversity. Both measures confirmed the intermediate constellation P2 and DGS, a

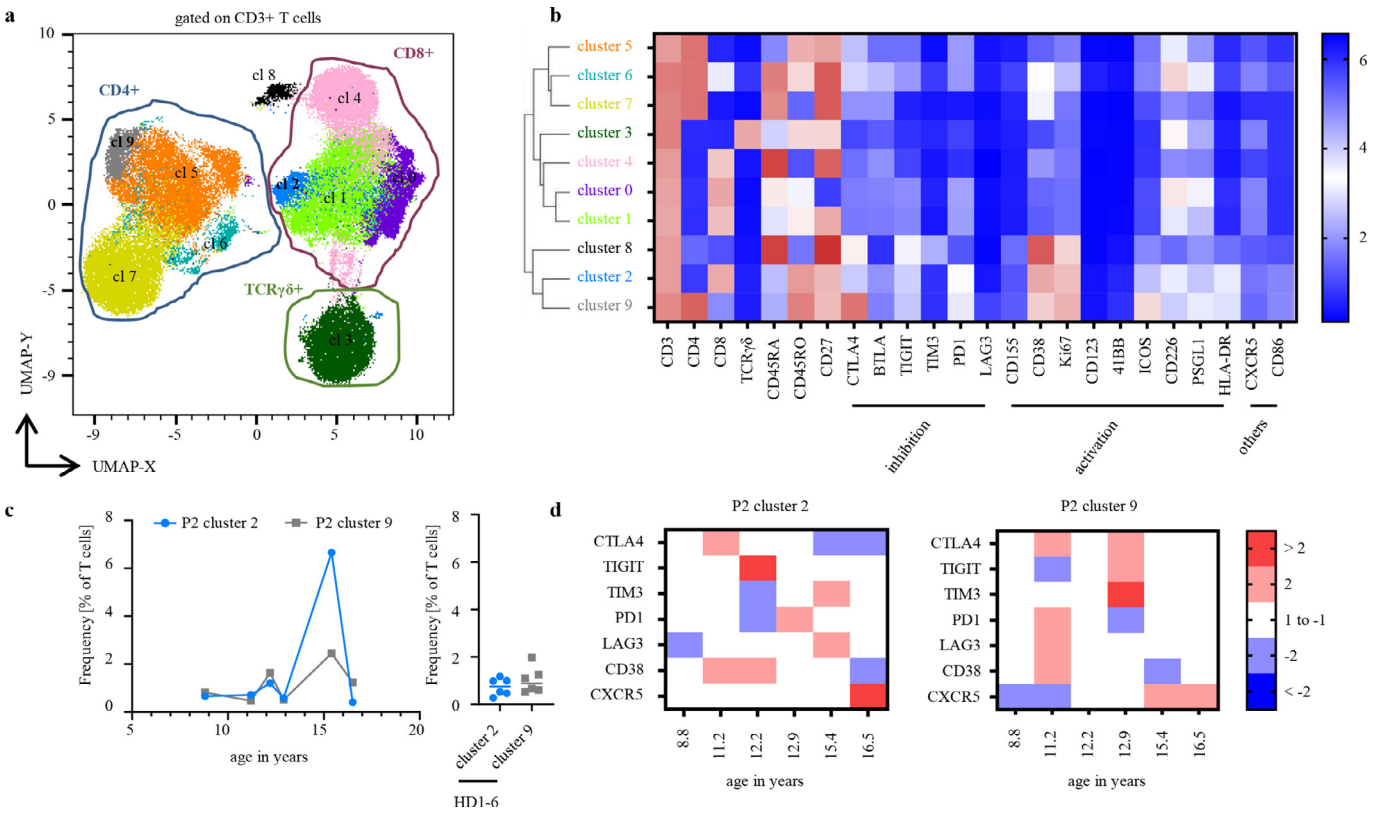

Fig. 2. No evidence for continuous exhaustion of the T-cell pool over 7 years.

(a) PBMCs analysed by mass cytometry and projected after multi-dimensional reduction with UMAP. Cells were gated on CD3+ T cells. 6 longitudinal samples of P2 and samples of 6 HD obtained at single time points are included in the analysis. CD4+ T cells, CD8+ T cells and TCR$\gamma\delta$+ T cells are circled. Clustering analysis was performed using FlowSOM and overlaid in respective colour. (b) Dendrogram and expression levels of indicated markers on clusters identified by FlowSOM. Arcsinhyp normalized mean intensities are displayed. (c) Abundance of cluster 2 and cluster 9 in longitudinal samples of P2 and individual samples of HDs. (d) Longitudinal expression of selected markers upregulated on exhausted T cells for cluster 2 and cluster 9 of P2. Rows are scaled to have a mean of 0 and SD of 1.

reduced diversity compared to HD, but much higher diversity than OS.

3.5. *Increased dynamics of top clones across an overall stable T-cell repertoire*

From a clinical point of view, a key question concerns the stability of the limited T-cell system in P2. To this end, we analysed longitudinal samples from one HD, P2 and DGS. To describe the dynamics of repertoire diversity, we used the numbers of unique CDR3β sequences (Fig. 4a), the clonality scores (Fig. 4b), and the slopes of power law distributions (Fig. 4c). In line with previous reports [11,33–35], in HD all three measures remained stable within the 3 years analysed. In P2, the number of unique CDR3β sequences also remained stable with the exception of a reduction at the age of 12.9 years (Δ3 years in Fig. 4a-c), which was associated with an increase in the clonality score and a decreased slope in the power law distribution. Notably, in DGS, we detected an increase in diversity as evidenced by an increased number of unique CDR3β sequences, a decrease in the clonality score, and a higher slope in the power law distribution; we attribute this phenomenon to the recovery from severe illness involving cardiac surgery complicated by infection that was present at initial sampling. In HD, Vβ-family usage among CD4+ and CD8+ T cells as well as the frequency of the top 100 clones remained stable over 3 years (Fig. 4d-f). While Vβ-family usage among P2 CD4+ T cells remained stable for over 5 years (Fig. 4g), 2 Vβ-families among CD8+ T cells showed dramatic expansion and contraction over time (Fig. 4h, red and blue lines). Analysis of the 100 most frequent clonotypes identified by CDR3β sequencing revealed that these changes were mainly caused by two distinct clonotypes with the corresponding V-segment usage (Fig. 4i). In DGS, the most drastically expanded clonotypes contracted over 4 years, while previously undetectable clonotypes expanded with time (Fig. 4j-l). Thus, the limited T-cell repertoires of P2 and DGS are characterized by a much more dynamic behaviour of individual clones when compared to HD. In contrast to the situation of DGS, in P2 no clear clinical events such as infections or vaccinations could be associated with these alterations.

3.6. *Increased overall TCR population dynamics is determined by top clones*

To further explore the longitudinal dynamic of the limited T-cell repertoire, we calculated the number of significantly expanded and contracted clonotypes as previously described [23,24]. In HD, only 5 significantly expanding and 6 significantly contracting clonotypes were detected during the three years of observation. For P2, the picture was very different; 166 expansions and 201 contractions of clonotypes were detectable within 8 years (Fig. 5a,b). Taking all five 3-year intervals available in the dataset into consideration, the T-cell repertoire of P2 was marked by 90±25.3 (mean, SD) expansions and 83±46.7 contractions of clonotypes per interval. For HD, biological replicates and samples taken 3 years apart demonstrated a low but stable degree of overlap of the analysed T-cell populations when applying the Baroni-Urbani and Buser Overlap Index [25](Fig. 5c). The degree of overlap was much higher for P2, where we found a moderate linear decline over time, highlighting the more dynamic repertoire evolution (Fig. 5c). The similarity of the overall CDR3β population structure between biological replicates and longitudinal samples can be characterized by the Morisita Horn (MH) similarity Index [36](Fig. 5d). MH Index is scaled from 0 to 1, with greater

a

Patient	Mutation	CD4 [cells/µl]	naive CD4+ [%]	CD8 [cells/µl]
Reference (10-16y)	-	400 - 2100	37 - 97	300 - 1300
P2 (13y)	SCIDX1 reversion	186	5.6	266
DGS (13y)	22q11del	74	2.8	47
Reference (1w-2m)	-	1000 - 4800	59 - 100	200 - 2700
OS (1mo)	RAG1 comp het	10082	0.1	53

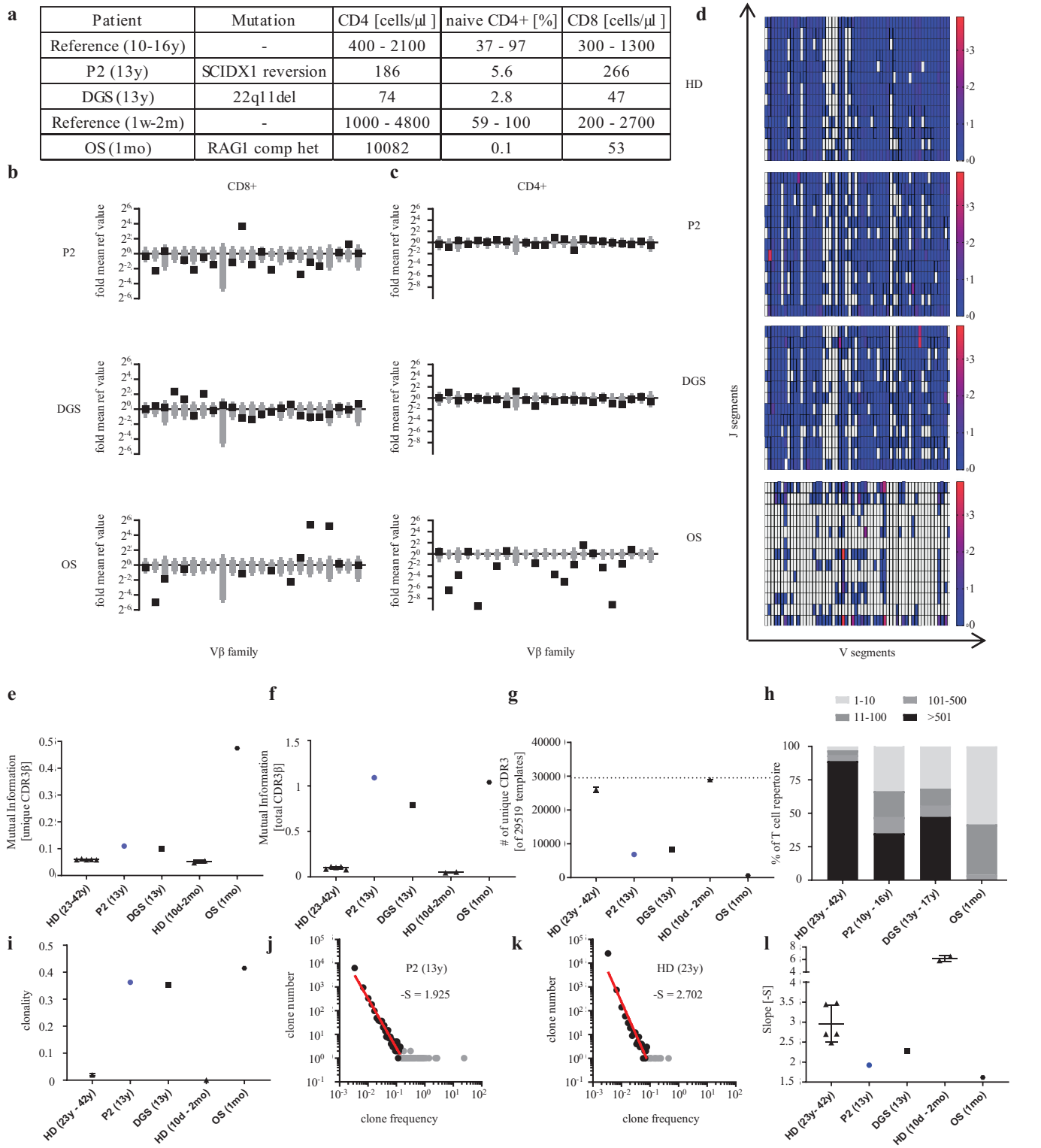

Fig. 3. Somatic gene rescue provides a surprisingly broad, but reduced T-cell repertoire.

(a) Abundance of lymphocyte subsets in peripheral blood of CID patients analysed for TCR$\alpha\beta$ repertoire diversity. Reference are values taken from [62]. Vβ-usage analysed by flow cytometry for CD8+ (b) and CD4+ (c) T cells. Data are depicted as fold change over mean reference values. Grey bars indicate standard deviation of reference values. (d) Analysis of V and J segment usage by CDR3β sequencing. For analysis of CDR3β sequencing data, all samples were downsampled to contain sequences from equal amounts of TCR$\alpha\beta$+ T cells. Colour code represents square root normalized frequency among productive CDR3β rearrangements. Mutual information was calculated based on unique (e) and total (f) sequences for each donor. (g) Number of unique CDR3β regions. Dashed line represents total number of templates after downsampling. Frequency of top 10, top 100, top 500 clonotypes among productive rearrangements (h) and clonality score (i) were calculated for each donor. (j/k) Representative power law slopes for P2 and 1 HD. Expanded clonotypes (grey) were excluded for calculation of the slope as described [22]. Red line indicates linear regression for clone number plotted against clone frequency of non-expanded (black) clonotypes. (l) Slopes of power law distribution were calculated for all donors. In e-l, age at analysis is given in parentheses. DGS = DiGeorge syndrome. OS = Omenn Syndrome.

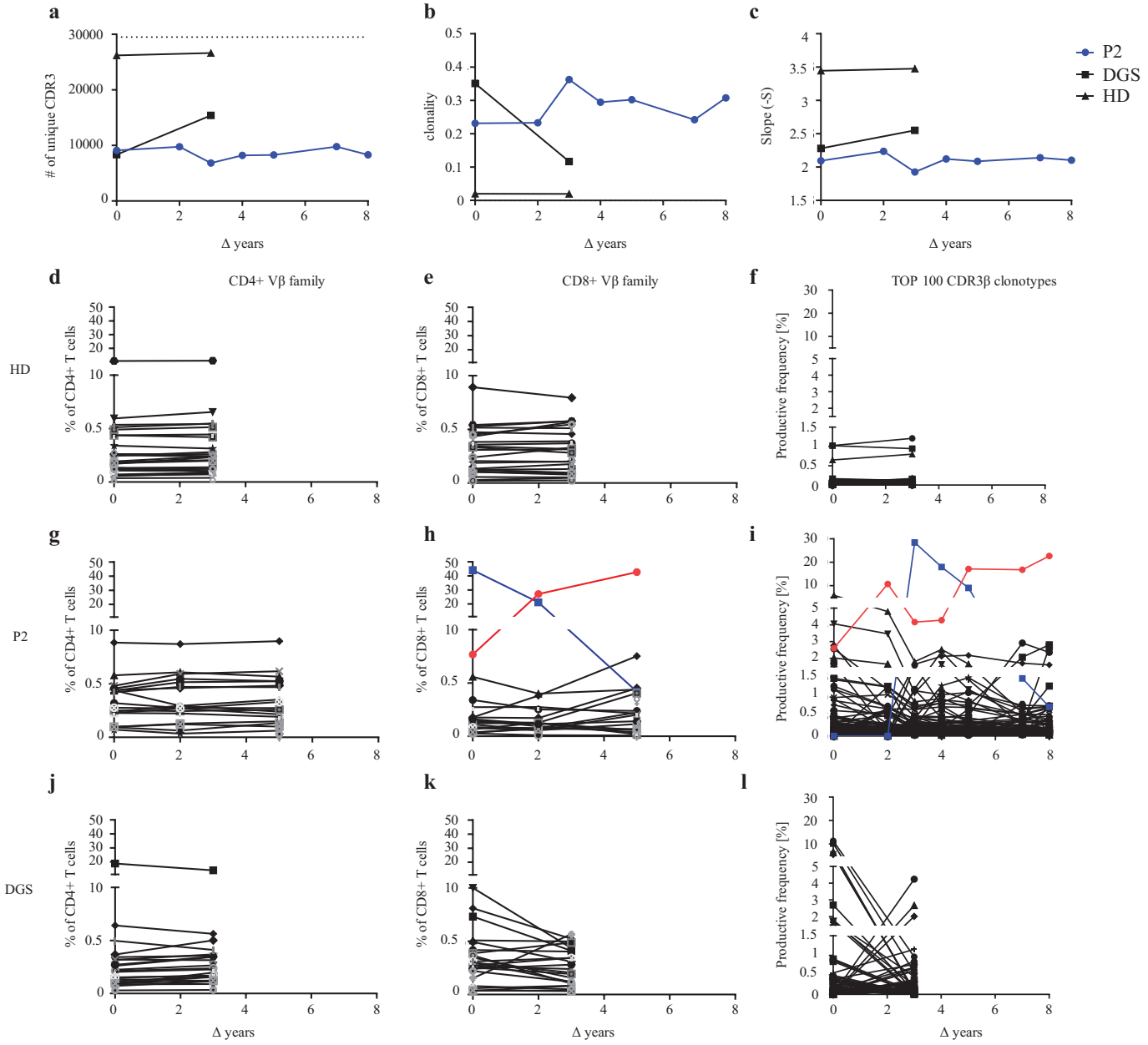

Fig. 4. Increased dynamics of top clones across an overall stable T-cell repertoire.

Longitudinal samples from one healthy donor (HD), P2 and DGS were analysed by flow cytometry and CDR3β sequencing. The number of unique CDR3β regions (a), clonality score (b) and slopes of power law distributions (c) were analysed in longitudinal samples. Longitudinal analysis of Vβ-family usage by flow cytometry in CD4+ (d/g/j) and CD8+ (e/h/k) T cells and productive frequency of the top 100 CDR3β clonotypes by sequencing are depicted (f/i/l). For P2, the most drastically expanded CD8+ T cell clones with matching Vβ-family usage are highlighted in blue and red respectively.

values indicating higher similarity. A high degree of similarity was detected between biological replicates (delta years = 0) in all donors; this was seen in HD, where less than 3% of sequences were shared, and in P2, where the overlap was about 20%. In HD, the similarity between CDR3β repertoires remained stable over 3 years. When calculating MH based on all clonotypes of P2, a linear correlation between MH index and time was observed (Fig. 5f; R^2=0.2713; p=0.0091 (F-test)). However, when the 2 most abundant clonotypes, highlighted in red and blue in Fig. 4i, were removed from analysis at each time point, correlation of MH with time increased (Fig. 5e/g; R^2= 0.8680; $p < 0.0001$ (F-test)). This again highlights the more dynamic evolution of the repertoire of P2 and the large impact of few dominant clonotypes on the population structure within a more limited T-cell repertoire.

4. Discussion

Is it possible that somatic rescue of genetically determined human T-cell deficiency supports the establishment and maintenance of a stable and robust human T-cell system that provides long-term protective immunity? Here, we have addressed this important biological and clinical question in a longitudinal study of a SCIDX1 patient with evidence of genetic reversion. Our data provide the following insights: [1] The observation of the long-term stability of the TCR repertoire in the absence of NK cell regeneration identifies a T-cell biased self-renewing hematopoietic progenitor as the site of genetic reversion. [2] The diversity of the resulting TCR repertoire was at least 100-fold higher than previously observed in a similar case of somatic rescue. [3] The gross architecture of the TCR repertoire appeared

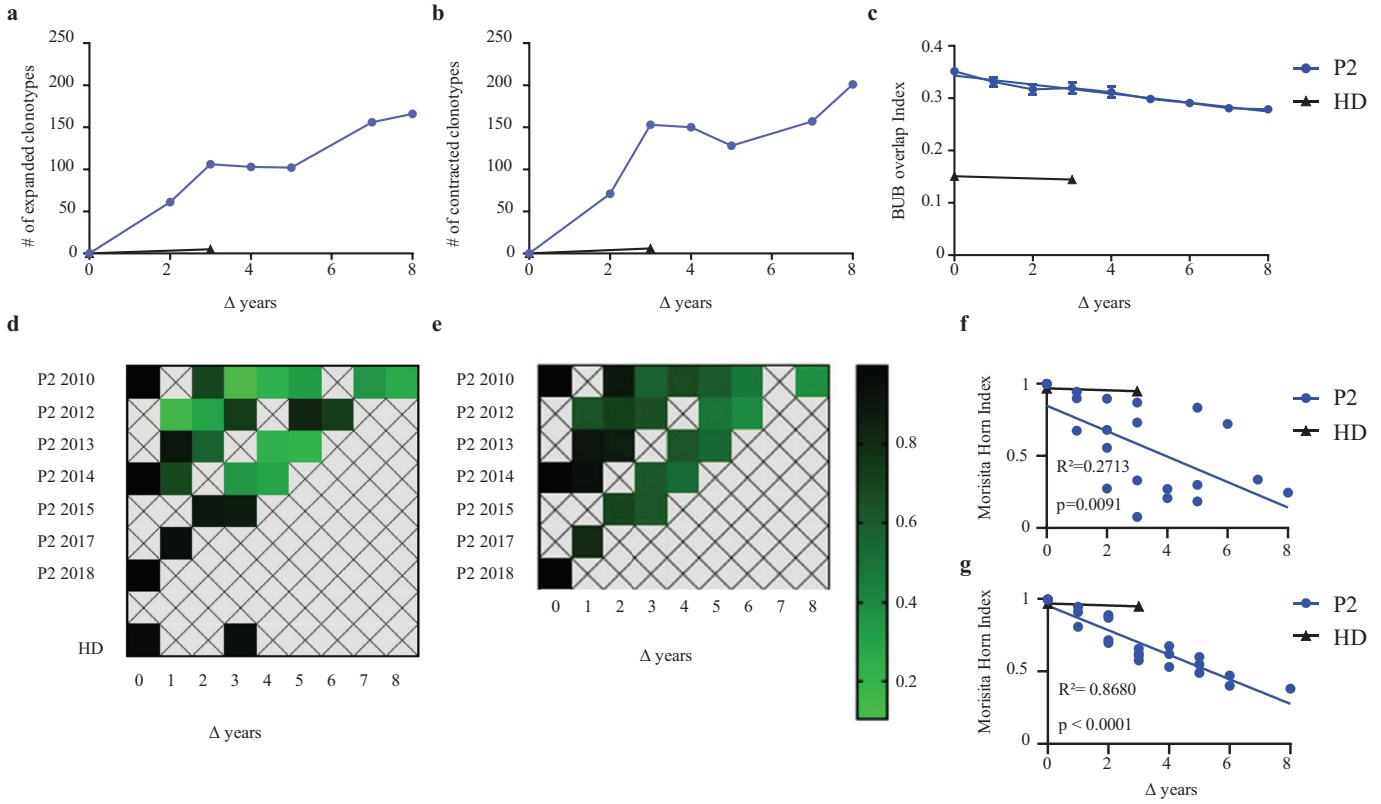

Fig. 5. Increased overall CDR3β population dynamics is determined top clones.

The numbers of significantly expanding (a) and contracting (b) clonotypes between longitudinal samples were calculated as previously described [23,24] (c) BUB overlap index was calculated on longitudinal samples for 1 HD and P2. Morisita Horn (MH) Index was calculated considering all clonotypes (d) and excluding the top 2 clonotypes for P2 (e; highlighted in red and blue in Fig. 4l). MH index visualized against time between blood draws including all productive rearrangements (f) and excluding the top2 clonotypes for P2 (g). Solid blue lines show linear regression for P2 (F: R^2=0.2713; p=0.0091 (F-test); G: R^2= 0.8680; p < 0.0001 (F-test)).

stable and robust over at least 8 years, despite dramatic expansions and contractions of individual TCR clonotypes. [4] A T-cell system emanating from progenitors after the reversion of the genetic lesion is capable of providing protective and regulated T-cell immunity for at least 18 years.

P2 [14] showed stable genetic mosaicism, expressing the *IL2RG* wild type allele in all $\alpha\beta$ and $\gamma\delta$ T cells and the loss-of-function L151P allele in other cell lineages investigated. Two explanations are possible. [1] The patient may represent a germline mosaic, such that a certain fraction of HSPC is genetically wildtype for the IL2RG gene. However, reverted cells were not detected in cell types that do not gain a developmental advantage from the genetic correction [37–39] including a buccal swab, granulocytes or B cells. Hence, if a germline mosaic indeed existed in P2, it must be below our detection limit of about 5%. Moreover, we observed that NK cells, strictly depending on IL2RG signaling for proper development but with the potential for homeostatic expansion in lymphopenic hosts [40,41], were insufficiently reconstituted. This suggests that few if any HSPCs constitutively carry the wildtype *IL2RG* sequence [2]. Hence, our observations are compatible with the interpretation of a somatic reversion of the *IL2RG* mutation.

Statistically, genetic reversion in more than one progenitor cell is unlikely. First, around 50,000-200,000 HSCs actively contribute to steady state hematopoiesis [42]. Assuming that about 5 to 20% of these are T-cell biased HSPC, ten cell divisions of each these 10,000 HSPCs in the first years of life would yield a pool of ~10^7 cells. Assuming a mutation rate per mitosis of 0.02 per genome [43], the pool of HSPCs would acquire 2×10^5 mutations. Given a haploid genome size of 3×10^9 base pairs, the probability of a second reversion would be $2 \times 10^5/6 \times 10^9 = 3.3 \times 10^{-5}$. Second, whole genome sequencing of in vitro expanded single HSPC yielded estimates of ~14 acquired mutations per year [44]. Again, considering 1×10^4 relevant precursors, the probability for reversion of a specific nucleotide would be $14 \times 1 \times 10^4/6 \times 10^9 = 2.3 \times 10^{-5}$ per year. Third, from a clinical point of view, somatic reversion of pathogenic germ-line mutations is a rare observation. Assuming that 1% of X-SCID patients acquire a reversion, the probability for a second reversion would be 1×10^{-4}. Even assuming that 10% of patients acquire a reversion, the probability of a second hit would be only 1% (and 1×10^{-3} for a third hit, 1×10^{-4} for a fourth hit). Taken together, the genetic stability of HSC and the scarcity of reported patients with somatic gene rescue favor the interpretation of a reversion in a single cell as the most likely explanation for the genetic mosaicism in our patient.

What is the nature of the revertant progenitor? In mice, thymic progenitors maintain NK cell potential until the DN2a stage [45] whereas β-selection and exclusion of TCR$\gamma\delta$ fate occurs in the DN3 stage. Importantly, thymic NK cells have not rearranged the TCRγ locus [46]. The absence of NK cells therefore indicates that the reversion must have occurred in a cell that is already committed to the T-cell lineage, yet in the hematopoietic tree is situated proximal to the bifurcation of $\alpha\beta$ and $\gamma\delta$ T cells. The classical model of hematopoiesis assumes that self-renewal capacity is restricted to multipotent stem cells at the tip of the hematopoietic hierarchy [47]. However, increasing evidence challenges this view by demonstrating heterogeneity among stem cells and self-renewing capacity among hematopoietic stem progenitor cells (HSPCs) with restricted lineage potential [48–50]. For instance, it has been shown in mice that in the absence of progenitor import from bone marrow, thymic T-cell progenitors can acquire self-renewing capacity and become long-lived [51,52]. Moreover, by tracking viral integration sites after transplantation of

gene corrected stem cells, Six et al [53] provided evidence that HSPC with self-renewal capacity and T-cell restricted output also exist in humans. In the case of P2, the presence of recent thymic emigrants and the long-term stability of numbers and clonotypes of the peripheral T-cell repertoire strongly suggest that the revertant progenitor cell is still active after 18 years. Hence, our observations provide additional support for the existence of such a T-cell committed self-renewing HSPC in humans.

We identified a surprising repertoire diversity of 87562 unique CDR3β sequences in P2 across 10 samples. On average, 36.4%±2.5 (mean,SD) of the CDR3β sequences were unique and not detected in any other sample. This lack of saturation indicates that the total number of CDR3β sequences of P2 exceeds the observed number of sequences. The absolute CD3+TCR$\alpha\beta$+ counts/ml of blood in P2 were 3 to 4-fold below those of healthy donors and the number of unique CDR3β sequences detected among a similar number of CD3+TCR$\alpha\beta$+ T cells was reduced ~3-fold. Thus, in a blood draw of equal size ~10-fold fewer unique CDR3β sequences were physically detected in P2 compared to HD. It is important to state, however, that the true repertoire size of P2 cannot be well estimated with these data. As recently pointed out by Mora et al.[54], this is mainly due to the fact that limited sampling (around 10^5 of a total of 10^{11} T cells in a single HD blood sample) cannot capture many small frequency clones, also referred to as the "missing species problem"[55]. The need to model the distribution of these undetected clones leads to uncertainties in repertoire size estimation ranging between 10^6 and 10^{10}[54]. Attempts to correct for this fact using various statistical methods so far failed to provide a gold-standard for extrapolation from CDR3β sequencing data to full repertoire size [8–10]. Thus, while the observed repertoire in P2 is ~100-fold higher than reported for P1 [12], the difference to the repertoire of a healthy individual remains unknown.

Gene therapy trials in primary immunodeficiencies have shown long-term reconstitution of a polycloncal T-cell repertoire following treatment with gene-corrected HSC [56–60]. Clarke et al. analysed repertoire diversity in SCID-X1 patients up to 15 years after gene therapy by NGS CDR3β sequencing. They detected 5,000-33,000 individual CDR3β sequences in these patients, which was not different from healthy controls. At first sight, the diversity found in our patient is of similar magnitude. However, the key difference to our study is the number of HSPC responsible for generation of this diverse repertoire. In the study by Clarke et al, the number of different integration sites detected in T cells was in the range of 1,000, implying that they were descendants of a large number of HSPC clones [15]. On average, this would indicate that each HSPC gave rise to several dozens of T cell clones, suggesting poor expansion of T cell progenitors before rearrangement of the TCRB locus. Other studies transplanting barcoded human purified HSCs into NSG mice have shown that only few of the transferred HSPC clones seed the thymus and that less than 10 HSPC can provide a polyclonal repertoire [16]. However, in this murine experimental model, diversity was assessed only at a single time point by low resolution CDR3β spectratyping. In our human study, we document longitudinal diversity arising from natural gene correction at high resolution, adding a novel dimension to the diversification potential of a single T-cell committed HSPC.

The similar diversity and structure of the CDR3β repertoire of P2 and a patient with severe DGS allows additional conclusions. In the case of DGS, the partial loss of thymus anlagen limits the number of thymic immigrants. By contrast, for P2, we assume that the number of thymic immigrants, rather than the size of the thymic microenvironment, is the limiting factor for T-cell production. While our results show that these two conditions can lead to the same outcome, an important implication is that the progenitors entering the thymus in P2 cannot take advantage of the unusually large niche space. This observation suggests that the pathway of differentiation of a T-cell precursor is constrained by its intrinsic properties.

For P1, Bousso et al assumed that the somatic reversion occurred in a thymic progenitor with limited life-span that provided a single wave of thymopoiesis [12]. To produce the total number of CDR3β sequences observed in P2, a single thymic progenitor would have to undergo at least 16 cell cycles prior to CDR3β rearrangement, a number that far exceeds current estimates [12]. Even further expansions would be required to account for the losses resulting from non-productive rearrangements, incompatible pairing with TCRα chains, subsequent removal of TCRs by thymic selection, and diversion into the TCR$\gamma\delta$+ T-cell lineage. Hence, we consider it unlikely that a single wave of thymopoiesis, derived from a reversion in a thymic progenitor T cell, would be sufficient to produce the large T-cell repertoire that we observed in P2 over many years. Rather, it appears more likely that somatic gene rescue in P1 and P2 has occurred in cells at different levels in the hematopoietic hierarchy. While the T-cell system observed in P1 is compatible with reversion in a short-lived committed thymic progenitor, the reversion in P2 more likely affected a T cell-committed hematopoietic progenitor in the bone marrow with self-renewing capacity.

A main focus of our study was the longitudinal analysis of a restricted T-cell system. P2 was found to possess low, but stable numbers of circulating TCR$\alpha\beta$+ T cells. The very low percentage of T cells with a naïve phenotype and their increased baseline expression of inhibitory receptors and the senescence marker CD57 is likely linked to increased homeostatic proliferation in the lymphopenic environment [61]. Importantly, within the 8 years analysed, neither long-lasting alterations among T-cell subsets and their proliferative potential, nor progressively increasing expression of markers associated with T-cell exhaustion or senescence were found. Considering the uneventful clinical course, these observations argue for a phenotypically and functionally robust T-cell compartment over time.

While we observed constant clonal frequencies and stability of diversity indices in normal donors [11,33–35] a few of the most frequent clonotypes in P2 expanded and/or contracted dramatically, even though diversity metrics of the CDR3β repertoire in P2 remained largely stable over time. In our reference datasets of young healthy individuals, the 100 most frequent clonotypes make up around 5% of the repertoire, while they represented more than 60% of the CDR3β repertoire in P2. The most expanded clonotype constituted 28.5% of the entire repertoire at a single time point, while in healthy donors few clonotypes contribute more than 1% to the T-cell repertoire at any one time. No specific clinical event could be associated with the clonal expansions. Importantly, stable diversity indices indicated that these severe transient clonal distortions were tolerated by the T-cell system and did not cause long-lasting repertoire imbalance. In fact, the changes in overall repertoire structure were the result of fluctuations in the representations of the 2 most expanded clonotypes.

P1, described by Bousso et al., clinically deteriorated over time and was eventually treated with HSCT at age 6 [14]. This course suggested the progressive functional exhaustion of the T-cell compartment and questioned the ability of somatic gene correction to generate a long-term protective T-cell system. By contrast, P2 had a largely uneventful course after initiation of immunoglobulin substitution until the current age of 18 years. His T-cell system is unexpectedly diverse and surprisingly stable and robust without evidence of progressive exhaustion. Reversion in a self-renewing (P2) rather than a short-lived (P1) T-cell committed hematopoietic precursor best explains these differences. Our results imply that, in principle, if targeted to the appropriate cell type, genetic correction of few or even a single cell can provide a diverse T-cell system that is robust and protective for at least 18 years. For our patient, careful follow up is nevertheless mandatory as an ageing limited T-cell system may still at some point loose regulatory capacity or may become overwhelmed by microbial or malignant challenges.

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Declaration of Competing Interests

SF and APF are employees of Roche Ltd. The authors declare no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102961.

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