



**Abbreviations used**

CDR3: Complementarity determining region 3  
 GT: Gene therapy  
 HCT: Hematopoietic cell transplantation  
 HTS: High-throughput sequencing  
 SCID: Severe combined immunodeficiency  
 TCR: T-cell receptor  
 TRB: TCR  $\beta$

Allogeneic hematopoietic cell transplantation (HCT) or gene therapy (GT) can fully correct the T-cell deficiency of SCID.<sup>1,4,5</sup> Key features associated with favorable outcome are the speed and robustness of immune reconstitution post-HCT.<sup>5-7</sup> A diverse T-cell repertoire is an essential feature of adequate immune function after HCT for SCID. Various methods have been proposed to ascertain T-cell reconstitution, including enumeration of total and naive T cells, quantification of T-cell receptor excision circles as a measure of thymopoiesis, flow-cytometric analysis of T-cell receptor (TCR)  $\beta$  Variable (*V*) gene family expression, and spectratyping of TCR  $\beta$  (*TRB*) complementarity determining region 3 (CDR3) rearranged products. However, these assays do not adequately capture the diversity of the T-cell repertoire, and more precise methods are needed to better understand the clonotypic dynamics of immune reconstitution post-HCT. With the advent of high-throughput sequencing (HTS), it is possible to analyze in great detail the richness, diversity, and clonality of *TRB*-CDR3 products, which are responsible for peptide recognition by the T-cell antigen receptor complex.<sup>8</sup> Such analysis may provide a quantitative and qualitative measure of immune reconstitution following cellular therapies.<sup>9</sup>

We hypothesized that the degree of *TRB* diversity obtained using HTS may constitute an early biomarker predictive of long-term immune reconstitution. To test this hypothesis, we performed longitudinal HTS measurement of the *TRB* repertoire following HCT or GT for SCID and correlated the results with need for second interventions and total and naive CD4<sup>+</sup> T-cell counts. We also studied the relationship of SCID genotype, conditioning regimen, and donor type with TCR diversity post-HCT. Finally, because autoimmune manifestations (particularly autoimmune hemolytic anemia) are common in the first months after HCT,<sup>10</sup> we interrogated the *TRB* repertoire for molecular signatures of self-reactivity at various time points after HCT.

We studied 27 children with SCID who received HCT ( $n = 24$ ) or GT ( $n = 3$ ) as first line of definitive therapy between 2010 and 2017, and from whom samples were available for *TRB* repertoire analysis. All were enrolled with informed consent (as approved by the central UCSF Institutional Review Board or institutional review boards at individual enrolling sites) in Protocol 6901 ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT01186913) of the Primary Immune Deficiency Treatment Consortium, a prospective multicenter observational study evaluating treatment outcomes of patients with SCID. Table 1 lists the patients' genotypes, type of cell therapy (with second treatments if given), total and naive CD4<sup>+</sup> T-cell counts 6 months after treatment (compared to age matched reference range<sup>11</sup>), and time points of samples studied. Of 28 total HCT treatments, 10 were from 8/8 HLA-antigen (Ag) matched related or unrelated donors, whereas 18 were from 1 or more HLA-Ag mismatched donors. All patients who received allogeneic HCT developed full donor T-cell chimerism. The

composition and diversity of the *TRB* repertoire was determined pre-HCT and at 3, 6, and 12 months and then yearly posttreatment(s) up to 4 years. Treatment was considered unsuccessful when requiring a second intervention (either HCT or GT). Multiple treatments in the same subject were considered as independent events. Four patients required a second HCT; 2 others received HCT followed by GT, for a total of 33 treatments for the entire cohort. HTS of *TRB* repertoire was performed after 31 such treatments (Table 1). One patient required a boost of PBSCs after the first sample collection at 3 months due to slow T-cell reconstitution. Mean age at the first treatment was 98 days (range, 24-240 days). All patients had a minimum follow-up of 4 years.

The TCR library preparation, adapted from Zvyagin et al,<sup>12</sup> used total RNA from lysed peripheral blood (PAXgene RNA tubes; RNeasy Mini Kit, Qiagen, Inc, Germantown, Md). At least 200 ng of RNA was reverse transcribed (SMARTer PCR cDNA Synthesis Kit, Takara Bio, Inc, Mountain View, Calif) with unique molecular identifier barcodes incorporated into 5' primers for amplification to permit identification and quantification of the *TRB* repertoire. PCR products were pooled, purified (DNA Clean & Concentrator-5, ZymoResearch, Irvine, Calif), and sequenced to obtain 150 bp paired end reads (Illumina HiSeq2500). Raw sequences were filtered for productive rearrangements and analyzed for *V*, *D*, and *J* gene composition (IMGT HighV-QUEST software). The VDJ statistics file (PAST program) was used to calculate a Shannon [H] entropy index to measure repertoire diversity, and a Simpson [1-D] index of unevenness, measuring inequality in the relative representation of individual sequences in a given sample. TCR repertoire diversity was illustrated by hierarchical tree maps, using iRepertoire software, with each dot representing a unique CDR3 sequence and the size of each dot corresponding to the frequency of that sequence in the total population of sequences obtained. The differences between groups were analyzed using 2-way ANOVA (for multiple comparisons), with statistical significance indicating a 95% CI. Correlation was measured by Spearman rank correlation coefficient ( $R_s$ ). Statistical analysis was performed using Prism Software or custom script (R environment, version 3.3.2) to calculate the Cysteine index.<sup>13</sup>

## RESULTS AND DISCUSSION

Severe restriction of repertoire diversity as measured by the Shannon [H] and the Simpson [1-D] indices and clonotypic expansions were observed 3 months after cellular therapy, followed by significant improvement by 6 months ( $P < .01$ ) (Fig 1, A-C). Among all patients who received HCT, whether conditioned or nonconditioned, diversity and clonality improved more rapidly following successful as compared with unsuccessful HCT (Fig 1, D), consistent with the observation that a diverse repertoire with clone evenness is a key feature of successful immune reconstitution.<sup>8</sup> A second intervention (allogeneic HCT or GT) was needed only in patients having a 3-month H index of 5.2 or lower; none of the patients with an H index above 5.2 at 3 months required a second treatment ( $P < .0001$ ; Fig 1, D). Significant correlation ( $R_s = 0.89$ ;  $P = .001$ ) was observed at 6 months posttreatment between the H index and the total number of circulating naive CD4<sup>+</sup> T cells (Fig 1, E), a known biomarker predictive of the quality of immune reconstitution.<sup>5</sup> Analysis of the association of genotype and immune reconstitution following HCT revealed similar kinetics of development of TCR diversity in 12 patients with *IL2RG*, *IL7R*, and *JAK3* gene defects in cytokine

**TABLE I.** Patients, with cell therapies received and samples studied for TRB diversity

Patient	Sex	Genotype	Treatment type (HCT/GT)	Time points studied (months posttreatment)	Donor type*	Conditioning regimen	CD4 <sup>+</sup> T cells/mm <sup>3</sup> 6 mo posttreatment*	CD4 <sup>+</sup> naive T cells/mm <sup>3</sup> 6 mo posttreatment*
1	F	<i>RAG</i>	HCT(a)†	12, 24, 36	MMRD	None	183	0
			HCT(b)	ND	MMRD	MAC	65	6
2	F	<i>RAG</i>	HCT	12, 36, 48	URD	RIC	576	65
3	M	<i>RAG</i>	HCT	24, 36, 48	mmURD	MAC	281	132
4	M	<i>RAG</i>	HCT	12, 36, 48	MSD	None	203	107
5	F	<i>RAG</i>	HCT†	6, 24, 36, 48	MMRD	RIC	1242	384
6	F	<i>RAG</i>	HCT	6, 12, 24, 36	URD	RIC	304	119
7	M	<i>RAG</i>	HCT	6, 12, 24	URD	RIC	912	511
8	F	<i>RAG</i>	HCT	6, 12	URD	RIC	194	ND
9	F	<i>DCLRE1C</i>	HCT(a)†	12, 24, 36, 48	MMRD	None	281	3
			GT(b)	ND	GT	RIC	252	15
10	F	<i>DCLRE1C</i>	HCT(a)	3, 6, 12, 24, 36	MMRD	RIC	350	8
			HCT(b)	3, 6, 24	MMRD	RIC	427	85
11	F	<i>DCLRE1C</i>	HCT	12	MMRD	None	161	1
12	M	<i>IL2RG</i>	HCT(a)†	12, 24, 36	MMRD	None	20	11
			GT(b)	3, 6, 12	GT	RIC	187	60
13	M	<i>IL2RG</i>	HCT(a)	3	MMRD	IS	Second therapy‡	Second therapy
			HCT(b)	6, 24	mmURD	RIC	570	ND
14	M	<i>IL2RG</i>	HCT	12, 36, 48	MMRD	None	627	74
15	M	<i>IL2RG</i>	GT	3	GT	RIC	1949	779
16	M	<i>IL2RG</i>	HCT	3, 6, 48	URD	IS	>500	375
17	M	<i>IL2RG</i>	HCT	6, 12, 24, 36, 48	MMRD	IS	1417	879
18	M	<i>IL2RG</i>	HCT	3, 6, 12	MMRD	IS	920	626
19	M	<i>IL2RG</i>	GT	3, 6, 12	GT	RIC	1050	726
20	M	<i>IL2RG</i>	GT	3, 6	GT	RIC	2039	1305
21	M	<i>IL2RG</i>	HCT†	3, 12, 24, 36, 48	MMRD	None	289	87
22	F	<i>IL7R</i>	HCT	24	URD	IS	203	3
23	F	<i>IL7R</i>	HCT	6, 12, 24, 48	MMRD	None	1086	478
24	F	<i>IL7R</i>	HCT (Boost) §	3, 6, 12	MMRD	None	2291	226
25	F	<i>IL7R</i>	HCT	6, 12, 24, 36	URD	IS	217	91
26	F	<i>JAK3</i>	HCT	3, 6	MMRD	IS	1296	ND
27	M	<i>BCL11B</i>	HCT(a)	3, 6	URD	IS	Second therapy‡	Second therapy
			HCT(b)	36	URD	RIC	1120	44

Multiple cell therapies are indicated by letters following the treatment type.

F, Female; IS, immune suppression; M, male; MAC, myeloablative conditioning; MMRD, mismatched related donor; mmURD, mismatched unrelated donor; MSD, matched sibling donor; ND, not done; RIC, reduced-intensity conditioning; URD, unrelated (HLA-matched) donor.

\*Lower end of 10%-90% of healthy children aged 1-2 y 1300/mm<sup>3</sup> for CD4; 950 for CD4/CD45RA/CD62L.<sup>11</sup>

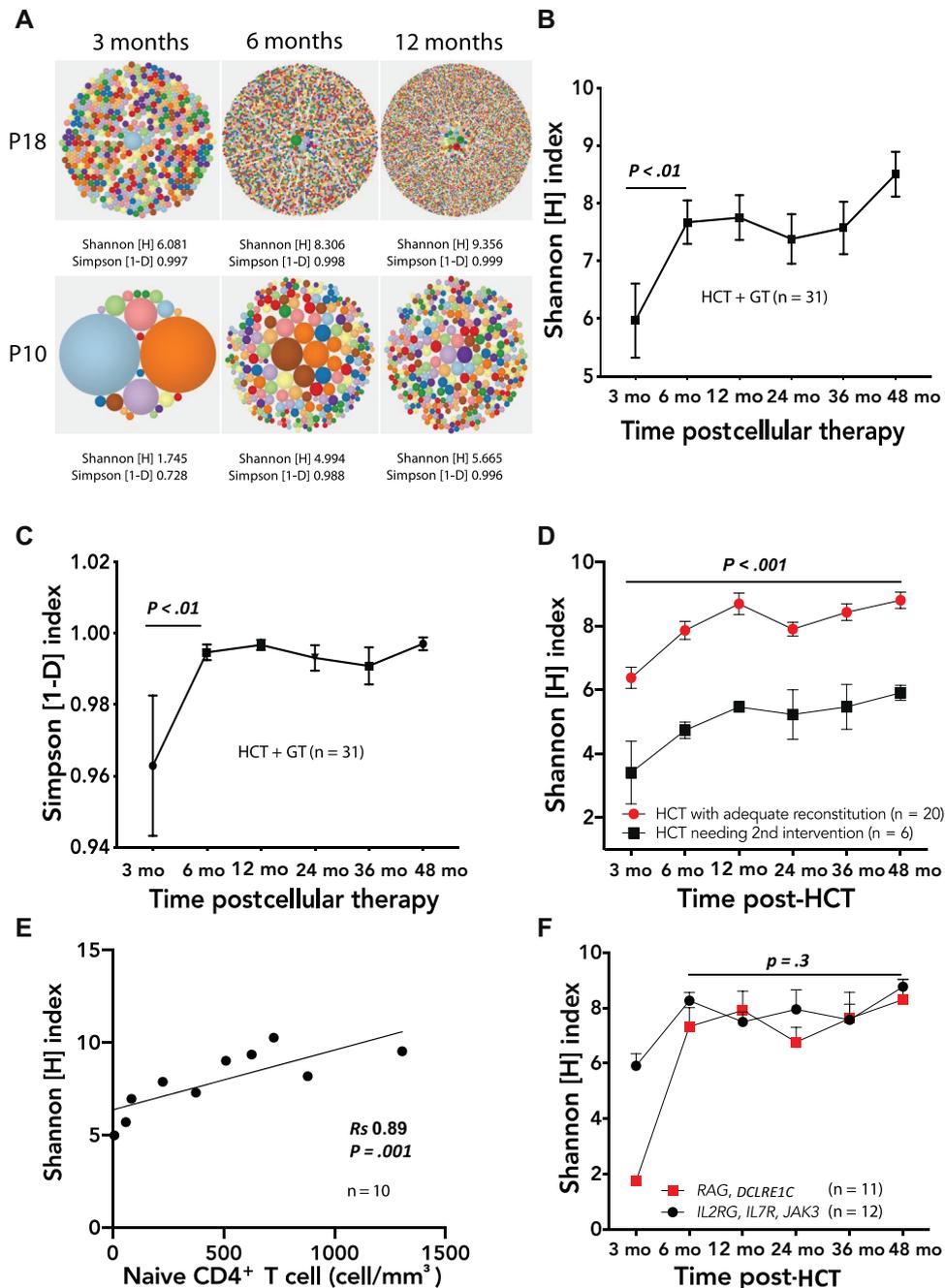
†Autoimmune complications.

‡Value not available if second treatment occurred within 6 mo of initial treatment.

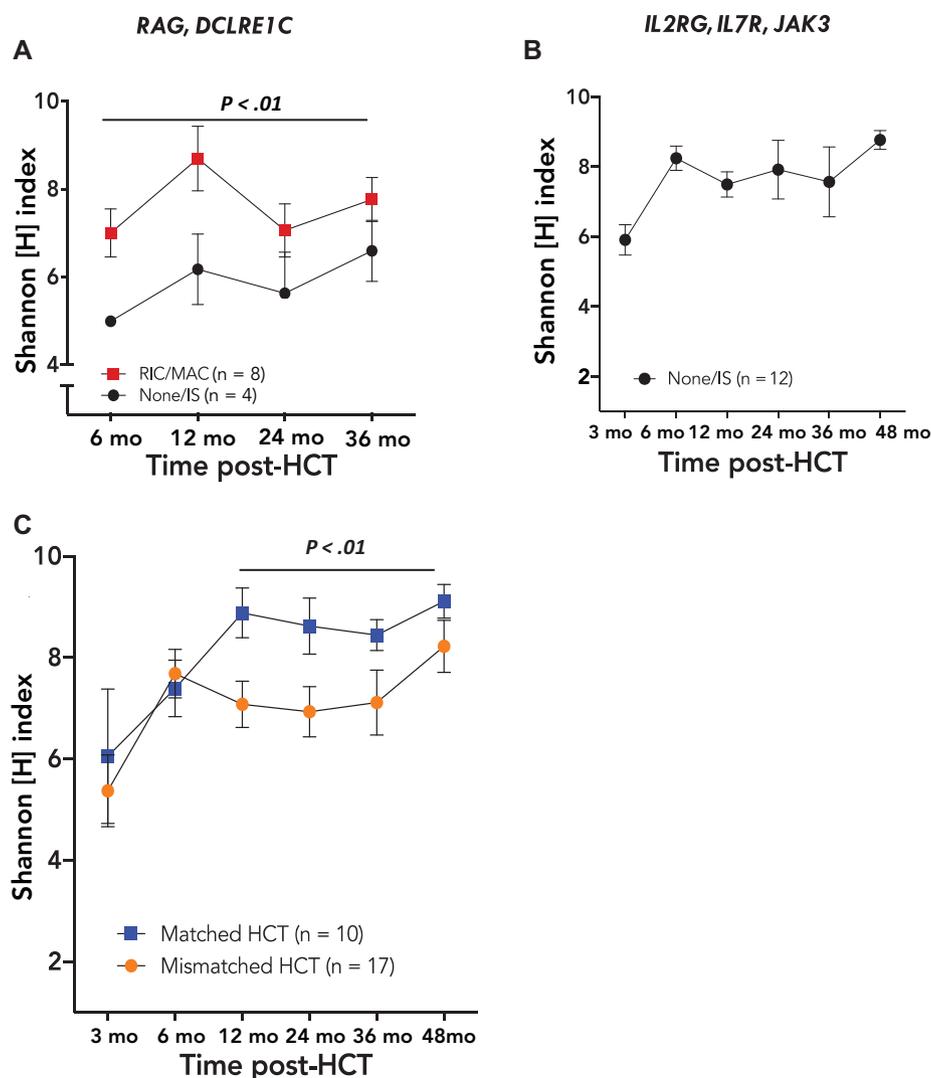
§Boost, additional cells from previous donor source administered with no conditioning between 3 and 6 mo post-HCT.

receptor signaling as compared with 11 patients with *RAG* and *DCLRE1C* defects in V(D)J recombination (Fig 1, F). In the latter group of treatments, however, HCT with no conditioning or immune suppression only (4 of 12) was associated with persistently lower diversity than HCT with myeloablative or reduced-intensity conditioning (8 of 12;  $P < .01$ ) (Fig 2, A), while in the *IL2RG/IL7R/JAK3* group (Fig 2, B) all 12 patients who received HCT with immune suppression only achieved normal diversity. Competition between autologous stem and progenitor cells versus donor-derived cells up to the double-positive stage of T-cell differentiation may have contributed to poorer immune reconstitution in the patients with *RAG* deficiency who did not receive chemotherapy, whereas the block in T-cell development occurs earlier in patients with *IL2RG*, *IL7R*, and *JAK3* defects.<sup>14</sup> Furthermore, in *RAG* and *DCLRE1C* V(D)J recombination defects, activated and hypercytotoxic natural killer cells may resist engraftment.<sup>15</sup>

Starting at 12 months post-HCT, T cells derived from a matched donor had higher diversity than those from a mismatched donor ( $P = .01$ ) (Fig 2, C). This finding may reflect more frequent use of reduced-intensity conditioning/myeloablative conditioning regimens in recipients of matched donor HCT as compared with mismatched HCT (5 of 10 vs 5 of 17) and is consistent with the superior T-cell reconstitution observed after conditioned HCT in a large cohort of patients with SCID.<sup>5</sup> In addition, a CD4<sup>+</sup> T-cell count of more than 500 cells/mm<sup>3</sup> or a naive CD4<sup>+</sup> T-cell count of more than 200 cells/mm<sup>3</sup> at 6 months post-HCT correlated with higher TRB diversity at 24 and 36 months post-HCT ( $P < .01$ ) (Fig 3, A and B), confirming previous observations that CD4<sup>+</sup> and CD45RA<sup>+</sup>CD4<sup>+</sup> T-cell counts at 6 months after HCT are predictive of sustained immune reconstitution.<sup>5</sup> Finally, a qualitative analysis showed that the TRB repertoire 3 months post-HCT in all 27 patients with SCID, including 5 who experienced autoimmune complications



**FIG 1.** **A**, Representative hierarchical tree maps showing increasing *TRB* repertoire diversity following successful HCT in patient 18 (P18, top row) and failed HCT in patient 10, treatment A (P10, bottom row). **(B)** Increasing Shannon [H] index of *TRB* diversity and **(C)** Simpson [1-D] index of clonality in all patient cellular treatments over time. The *P* values refer to differences between 3 and 6 months post-HCT. **D**, Persistently lower Shannon diversity scores following cellular treatments that ultimately needed another treatment (black squares) compared with those that were successful (red circles); differences were apparent as soon as 3 months after cellular treatments. The *P* value refers to the differences between the 2 groups considering all time points. **E**, Correlation (Spearman rank coefficient  $R_s$ ) between naive CD4<sup>+</sup> T-cell number and Shannon [H] index of diversity 6 months after a cellular treatment. **F**, Relationship between TCR diversity and genotype groupings (*IL2RG*, *IL7R*, and *JAK3*, black circles; *RAG* and *DCLRE1C*, red squares). The number of samples available at the 3-month time point was insufficient to assess whether the difference between the 2 groups was significant (only 1 sample was available for the *RAG/DCLRE1C* group at 3 months). In Fig 1, *B-D* and *F*, symbols and bars correspond to mean  $\pm$  SEM.



**FIG 2.** **A**, Relationship between conditioning and *TRB* diversity for patients with V(D)J recombination defects, with *P* value showing the difference between the 2 groups considering all time points. **B**, *TRB* diversity following unconditioned HCT for cytokine receptor defects (data were available for comparison of conditioning only from 6 months and beyond; see Table I). **C**, Relationship between donor HLA match and *TRB* diversity. Symbols and bars correspond to mean  $\pm$  SEM.

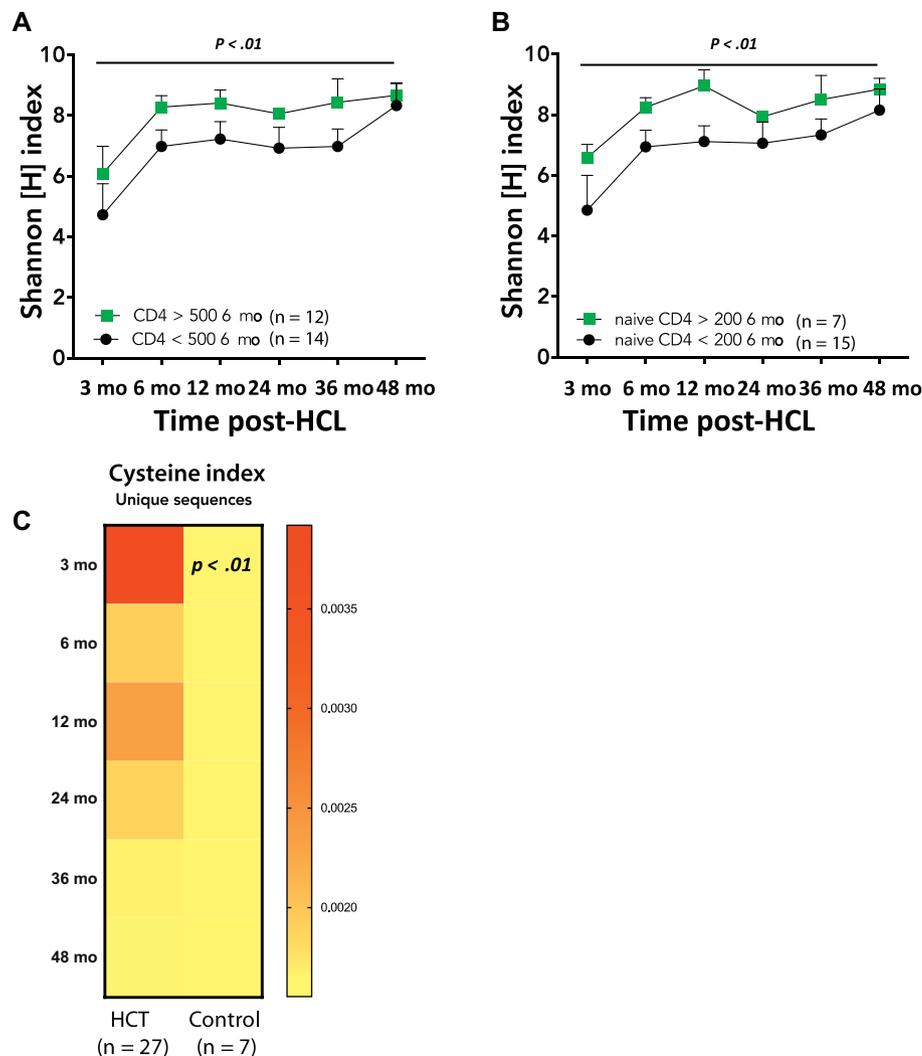
within 4 months after HCT (Table I), was enriched ( $P < .01$ ) for unique reads containing cysteine residues at the apex of the CDR3 (Fig 3, C), a biomarker of self-reactivity.<sup>13</sup> The occurrence of this molecular signature in the early T-cell repertoire following treatment for SCID may help to explain the increased risk of autoimmunity known to occur in these patients.<sup>10</sup>

This study represents the first attempt to apply HTS to measure TCR clonotypic dynamics in a cohort of 27 infants undergoing HCT or GT for SCID. The results show that analysis of *TRB* diversity and composition permits a detailed assessment of T-cell immune reconstitution in response to these therapies. In particular, we have obtained preliminary evidence that a restricted *TRB* diversity at 3 months post-HCT may identify patients at risk for failure of immune reconstitution, thus prompting a second intervention without delay. This study has limitations, including a small sample size, which did not allow adjustment for confounders such as infections, graft versus host disease, and

age at the time of HCT<sup>5</sup>; confinement of TCR analysis to *TRB* only; and unavailability of samples for some of the time points. Nonetheless, our study confirms that treatment strategies ought to be customized to SCID genotype to optimize immune reconstitution. If confirmed in large-scale prospective studies, these data support incorporating HTS of TCR repertoire as a valuable biomarker following cellular therapy for SCID.

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**Clinical implications: HTS of the *TRB* repertoire after HCT for SCID leads to early identification of patients who may benefit from a second intervention.**



**FIG 3.** **A** and **B**, Correlation between (Fig 3, **A**) *TRB* diversity and number of CD4<sup>+</sup> T cells/ $\mu$ L, and (Fig 3, **B**) *TRB* diversity and CD45RA<sup>+</sup>/CD4<sup>+</sup> naive T cells/ $\mu$ L at 6 months post-HCT.<sup>5</sup> Symbols and bars correspond to mean  $\pm$  SEM; *P* values refer to the difference between the 2 groups considering all time points. **C**, Heatmap representing Cysteine index at indicated times after HCT in all patients (n = 27); data are compared with Cysteine index of healthy pediatric controls (n = 7, mean age 15 months). The *P* value refers to difference between patients and controls at the 3-month time point.

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