Supplementary Appendix

Supplement to: Cowan MJ, Yu J, Facchino J, et al. Lentiviral gene therapy for artemis-deficient SCID. N Engl J Med 2022;387:2344-55. DOI: 10.1056/NEJMoa2206575

This appendix has been provided by the authors to give readers additional information about the work.

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Supplementary Methods

T CELL RECEPTOR (TCR) β CHAIN DIVERSITY AND VECTOR INSERTION SITE ANALYSIS

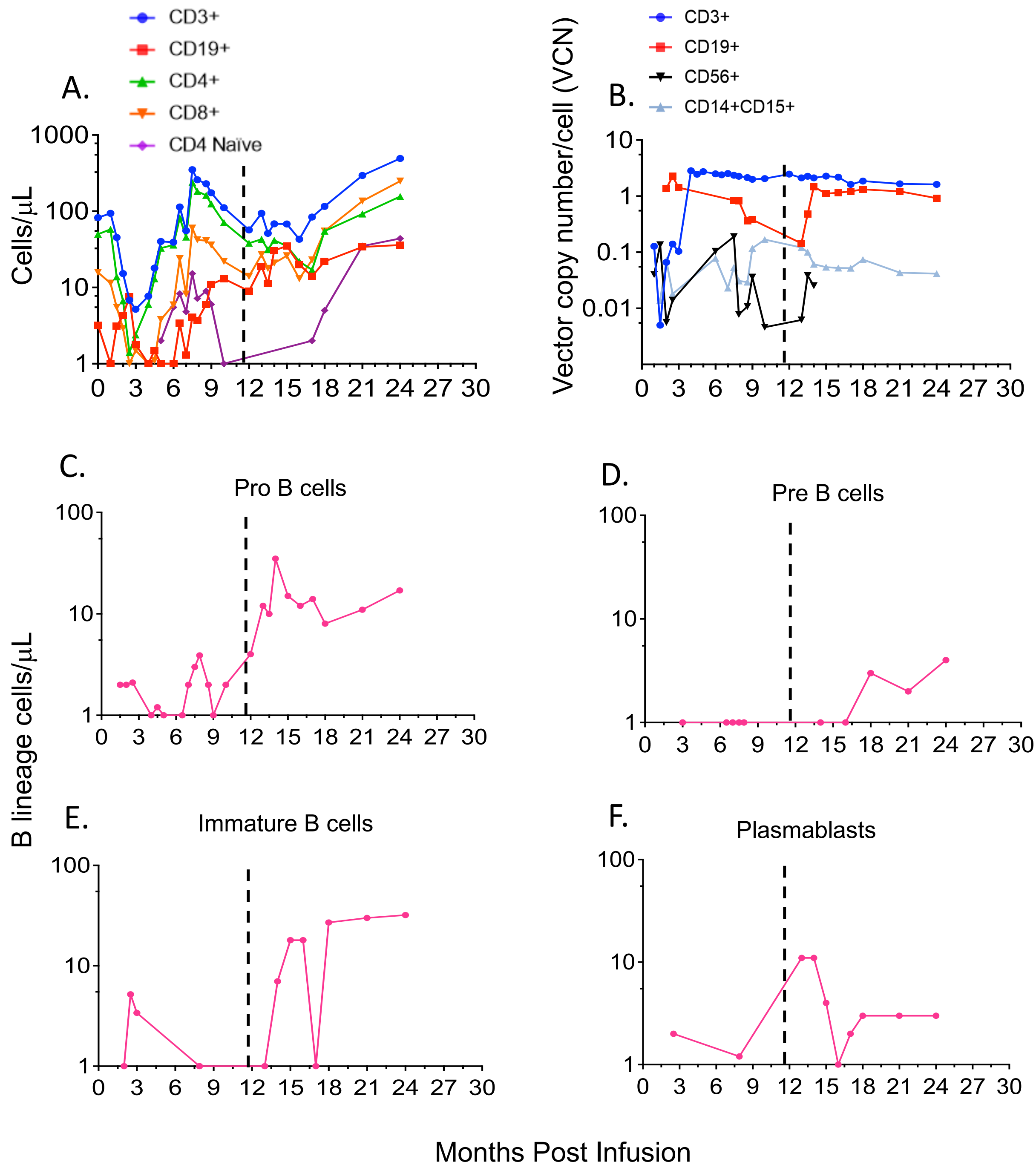
Diversity of the TCRβ repertoire was analyzed by deep sequencing of mRNA encoding the *TCRB* locus as described.[1,2] Sequences identified by LymAnalyzer[3] were used to determine unique sequence frequency and metrics of clonotype diversity.

Vector insertion sites (VIS) were identified in sorted cell lineages by ligation-mediated PCR[4,5] (Lenti-X Integration Site Analysis Kit, Takara Bio, Mountain View, CA), digesting the genomic DNA with restriction enzymes TaqI and HpyCH4IV. Adaptors were ligated to the fragments, and a first PCR was performed with biotinylated primers, followed by capture with Streptavidin beads. Two rounds of nested PCR with customized, bar-coded primers allowed generation of libraries that were sequenced (Illumina, 250 bp paired end reads) and mapped to human genome Release 39.[6-9]

METRICS OF TCRβ DIVERSITY

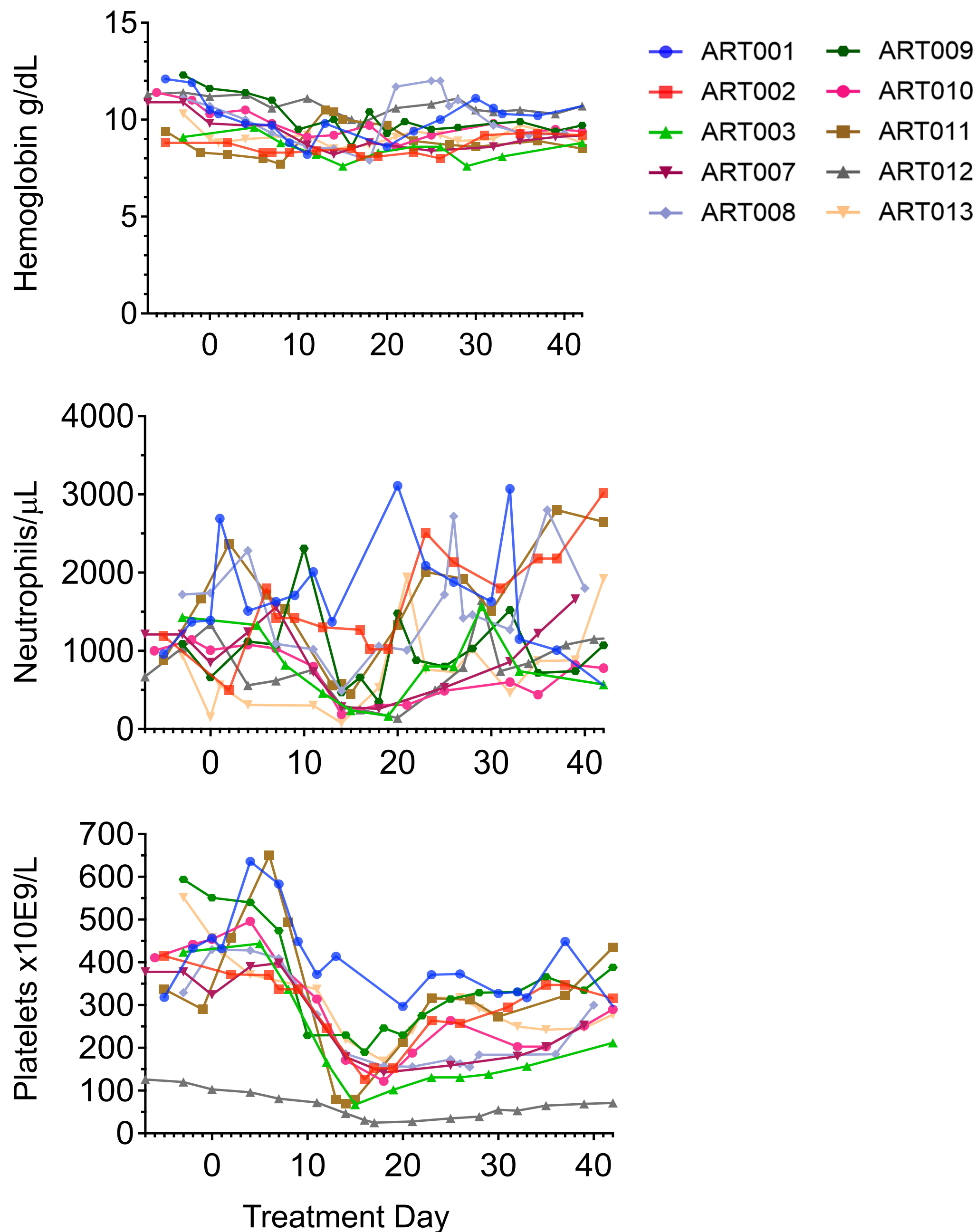
Metrics of TCRβ diversity included the Shannon information index [H], which measures the diversity of the sample on a logarithmic scale, including both the number of variants and their frequency distribution. Two indices were derived from [H]: information density, ID=[H]/ln(number of mapped sequence reads), measuring how close the sample consists entirely of unique reads; and equitability index, E_H=[H]/ln(number of clonotypes), measuring how close the sample consists of equal numbers of reads for each clonotype. See Figure S5 legend for details of the calculations. Normal ranges for E_H and ID were derived from cumulative Beta distributions with parameters matching the mean and variance of a convenience sample of seven healthy adults.[10]

Figure S1: Immune reconstitution in peripheral blood following initial and second infusions of transduced cells in patient ART010.



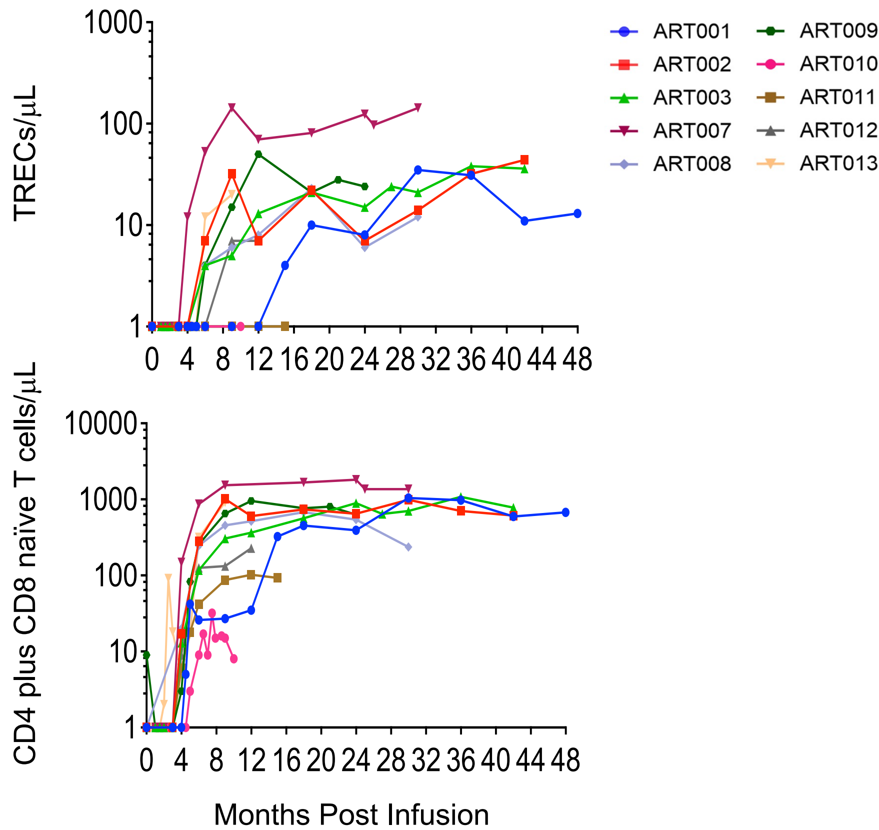
Supplementary Figure S1. Immune reconstitution in peripheral blood following initial and second infusions of transduced cells in patient ART010. A, Numbers of lymphocytes in each subset: $CD3^+$ total T cells, $CD19^+$ B cells, $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells, $CD4^+/CD45RA^+$ naïve T cells. B, Gene marking in the indicated cell subsets. C-F, B lineage cells at sequential stages of maturation. Dashed line represents time of second treatment with busulfan and gene transduced autologous cells. By 12 months after second cell infusion, proliferation to PHA had normalized, cytomegalovirus became undetectable, IgM was present at 17 mg/dL, and isohemagglutinins were positive at 1:4 dilution.

Figure S2: Hemoglobin, Platelet and Absolute Neutrophil Counts in peripheral blood.



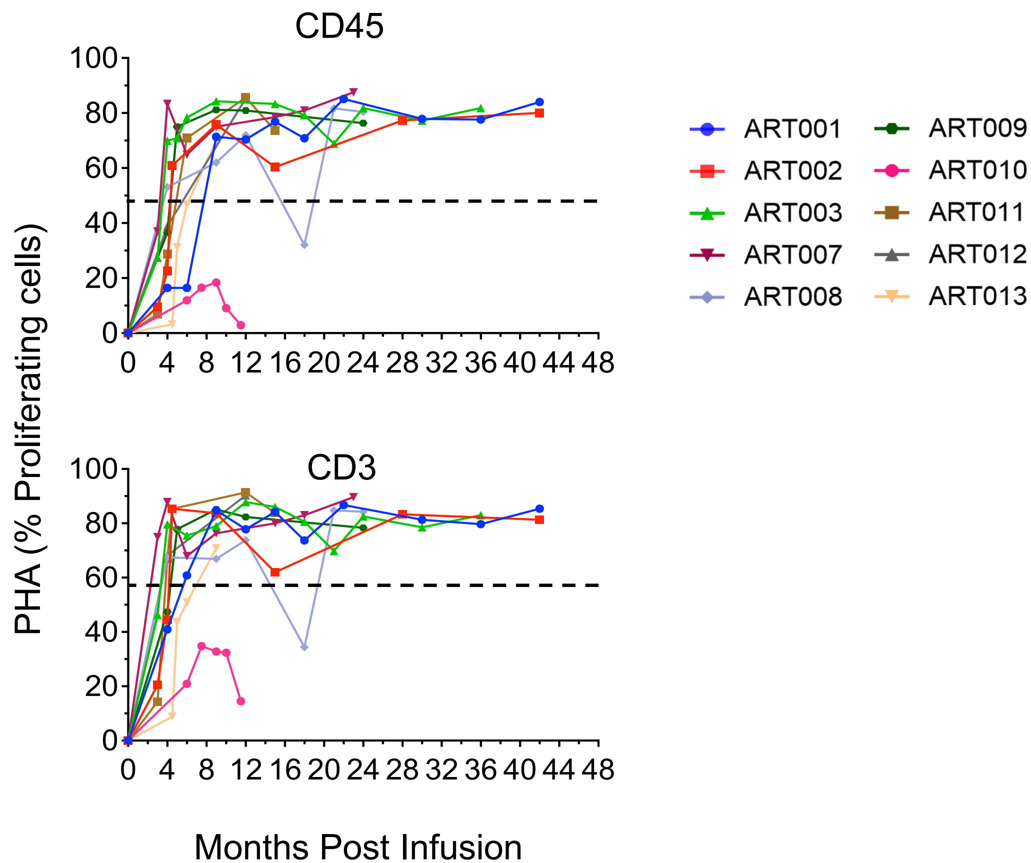
Supplementary Figure S2. Hemoglobin, Platelet and Absolute Neutrophil Counts in peripheral blood. Busulfan was given on days -1 and -2, with gene corrected $CD34^{+}$ cells infused on day 0. Reference Ranges: Hemoglobin: 9.5-13.5 g/dL; Platelets: 140-450x10E9/L; Neutrophils: 500-10000/µL.

Figure S3: Development of T Cell Receptor Excision Circles (TRECs) and naïve T cells post infusion of AProArt-transduced CD34$^+$ cells.



Supplementary Figure S3. Development of T Cell Receptor Excision Circles (TRECS) and naïve T cells post infusion of AProArt-transduced CD34+ cells. Upper graph, TRECs; lower graph, naïve T cells (CD4 plus CD8 T cells that also express CD45RA and CCR7).

Figure S4: Lymphocyte proliferative response to phytohemagglutinin (PHA) following infusion of autologous AProArt-transduced $CD34^{+}$ cells.



Supplementary Figure S4. Lymphocyte proliferative response to phytohemagglutinin (PHA) following infusion of autologous AProArt-transduced $CD34^{+}$ cells. Top, $CD45^{+}$ total lymphocytes; bottom, $CD3^{+}$ T lymphocytes.

- - - , Lower limit of control reference range: CD45: 49.9%, CD3: 58.5%.

Figure S5: T cell receptor Vβ diversity at baseline and following infusion of autologous gene corrected CD34⁺ cells.

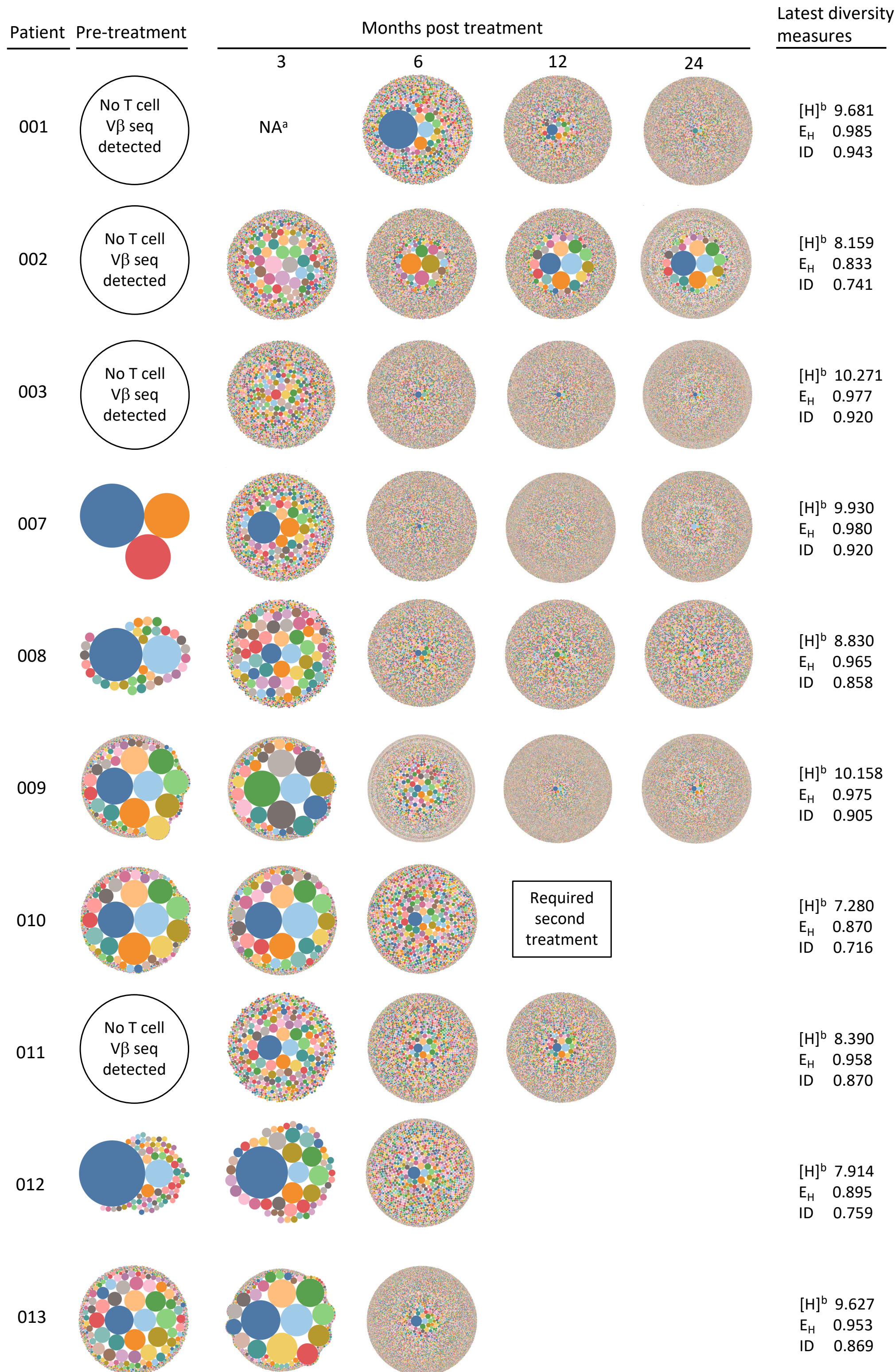


Figure S5. T cell receptor Vβ diversity at baseline and following infusion of autologous gene corrected CD34+ cells. Diversity was assessed by deep sequencing, using the method of Zvyagin et al,[1] modified as in Delmonte et al.[2] Briefly, total blood RNA was reverse transcribed with unique molecular identifiers incorporated into the reverse transcription reaction. After size selection and library synthesis, at least 50,000 sequence reads were obtained from each sample. After excluding duplicate reads, unique sequences aligned to complimentarity-determining regions of the human TCRB locus using LymAnalyzer software were visualized by hierarchical tree maps. The Shannon information index [H], equitability index E_H and information density (ID) were calculated, along with their variances, according to Basharin.[10] We chose to use the equitability index because it is scale independent and measures evenness of the distribution without using the distribution of pairs of variants, as does the alternative Simpson[1-D] index. The Shannon [H] index of information, or entropy, gives the information content of the variants in the sample, but it is dependent on the sample size. Normalizing by dividing by the logarithm of the number of reads gives the information density (ID), which is independent of the number of reads.

Thus, [H] with SD was calculated as: $H = -\sum_{i=1}^{K} p_i \ln p_i$

where N is number of unique reads and p the proportion (n/N) with a particular clonotype sequence in K clonotypes sampled. Equitability index was calculated as: $E_H = H/\ln K$.
Information density was calculated as: $ID = H/\ln N$.

Illustrations and diversity measures are shown for the most recent time point sampled. Initial evaluations at baseline showed no detectable TCR Vβ sequences for patients ART001, ART002, ART003, and ART011, who also had no detectable T cells by flow cytometry and no maternal engraftment. The remaining patients presented with detectable maternal T cells, which could have contributed to the oligoclonal TCR Vβ sequences observed at baseline.

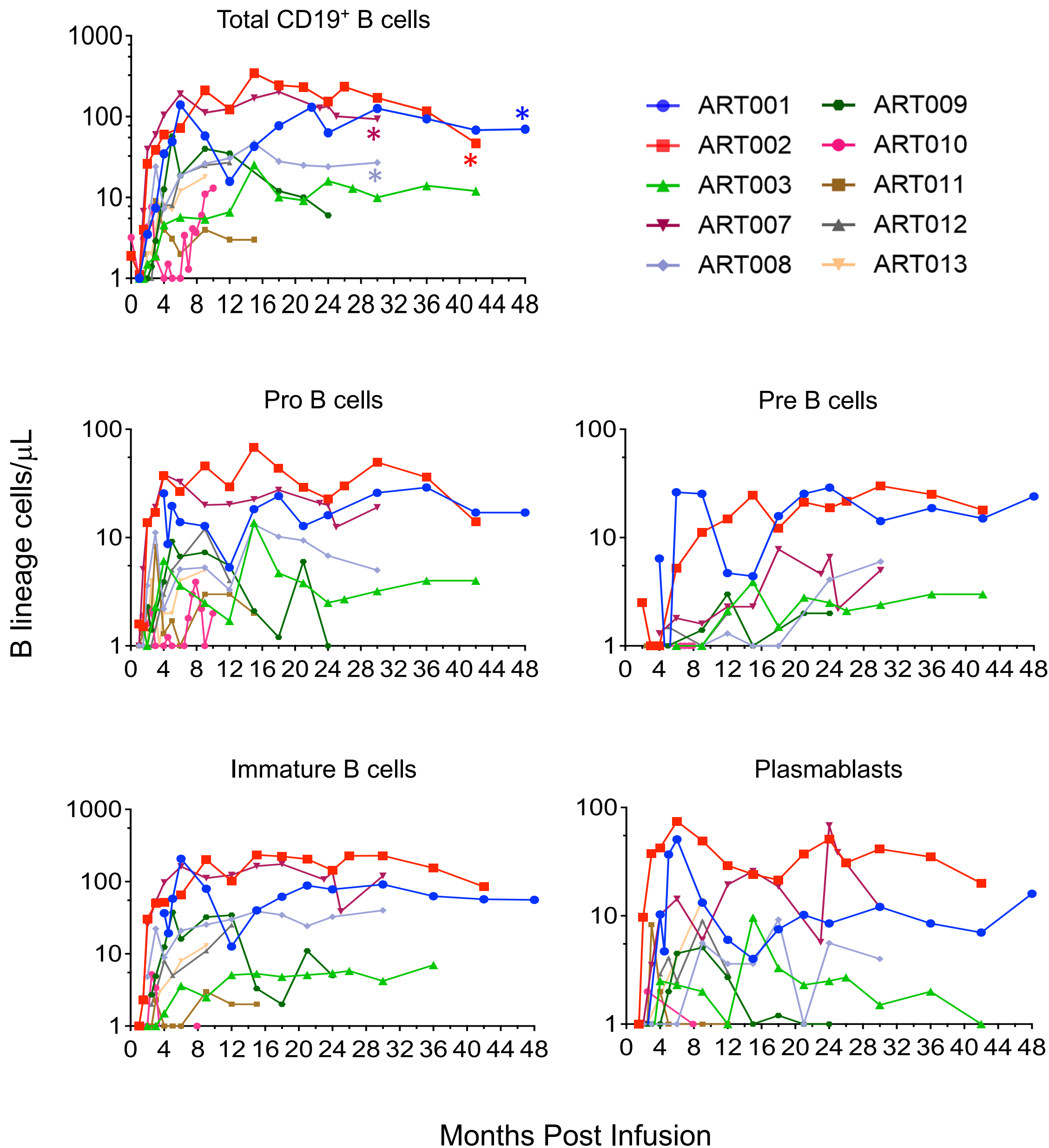
For comparison, diversity metrics and variances of blood samples from 7 healthy adults were obtained, showing mean [H] 9.706 ± SD 0.491, calculated as follows:

$$Var[H] = \frac{1}{N}\left[\sum_{i=1}^{K} p_i \ (\ln p_i)^2 - H^2\right] \qquad SD\ [H] = \sqrt{Var[H]}$$

The mean E_H for the 7 adult samples was 0.935, with 95% confidence interval (CI) 0.840-0.989, derived from fitting to the cumulative Beta distribution (35.65,2.46). The mean ID for the 7 adult samples was 0.882, with 95% CI 0.742-0.972, derived from fitting to Beta(25.05,3.36).

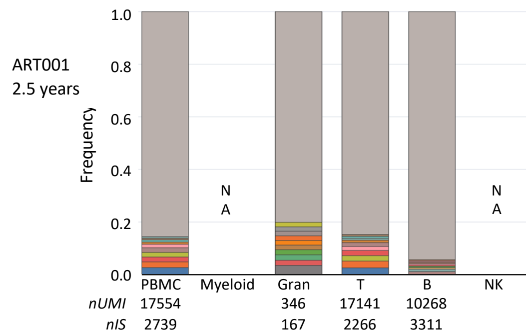
Footnotes: a, no sample available; b, Shannon [H] index, equitability index (E_H) and information density (ID), respectively, at 24 months post treatment or most recent analysis; diversity stabilized by 12 months and has not changed for patients followed longer (not shown).

Figure S6: Sequential stages of B cell maturation following infusion of AProArt transduced CD34$^+$ cells.

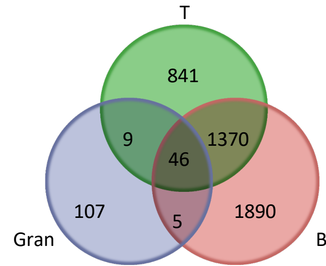


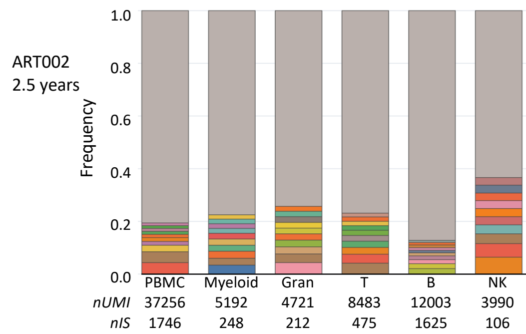
Supplementary Figure S6. Sequential stages of B cell maturation following infusion of AProArt transduced CD34$^+$ cells. Total CD19$^+$ B cells, top graph, with * indicating patients no longer requiring IgG infusions. B lineage cell subsets in peripheral blood were identified by the following cell surface marker combinations: Pro B cells, CD19$^+$, CD43$^+$; Pre B cells, CD19$^+$, CD43$^-$, IgM$^-$; Immature B cells, CD19$^+$, CD43$^-$, IgM$^+$; Plasmablasts, CD19$^+$, CD27$^+$, CD38$^+$.

Figure S7: Vector integration site analysis.

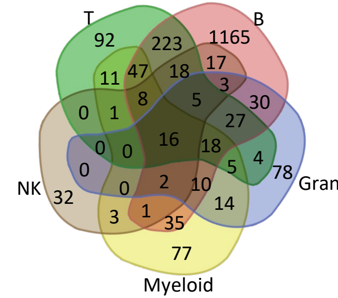


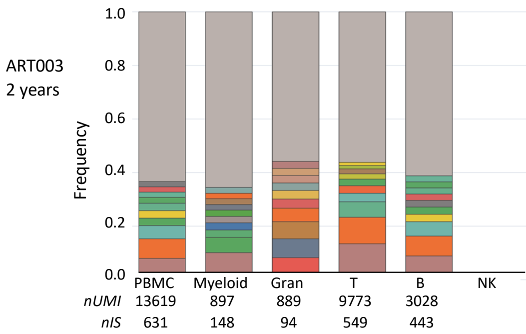
Gene	Frequency
WBP1L	0.026
NF1	0.022
RERE	0.021
ABCA3	0.020
RNF213-AS1	0.016
RNF213	0.016
SNX8	0.013
C1S	0.009
PLVAP	0.007
SKAP1	0.007



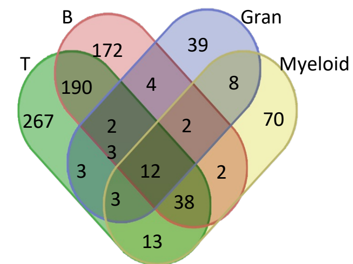


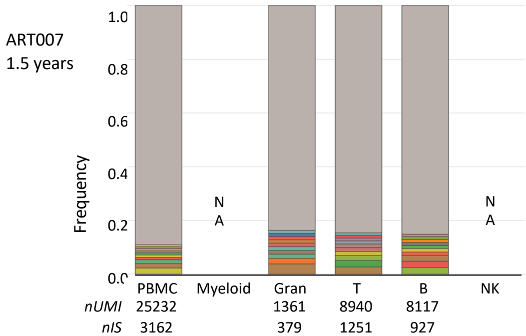
Gene	Frequency
SETD1B	0.045
KLHL3	0.027
RP11-383A19.1	0.016
MAD1L1	0.016
MARK3	0.015
IL21R	0.014
CEP85	0.013
PHF20	0.012
ZFAND3	0.012
TAX1BP1	0.011



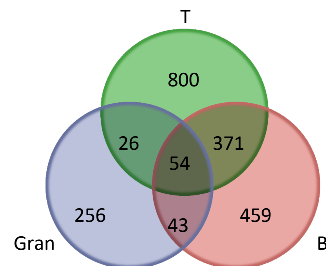


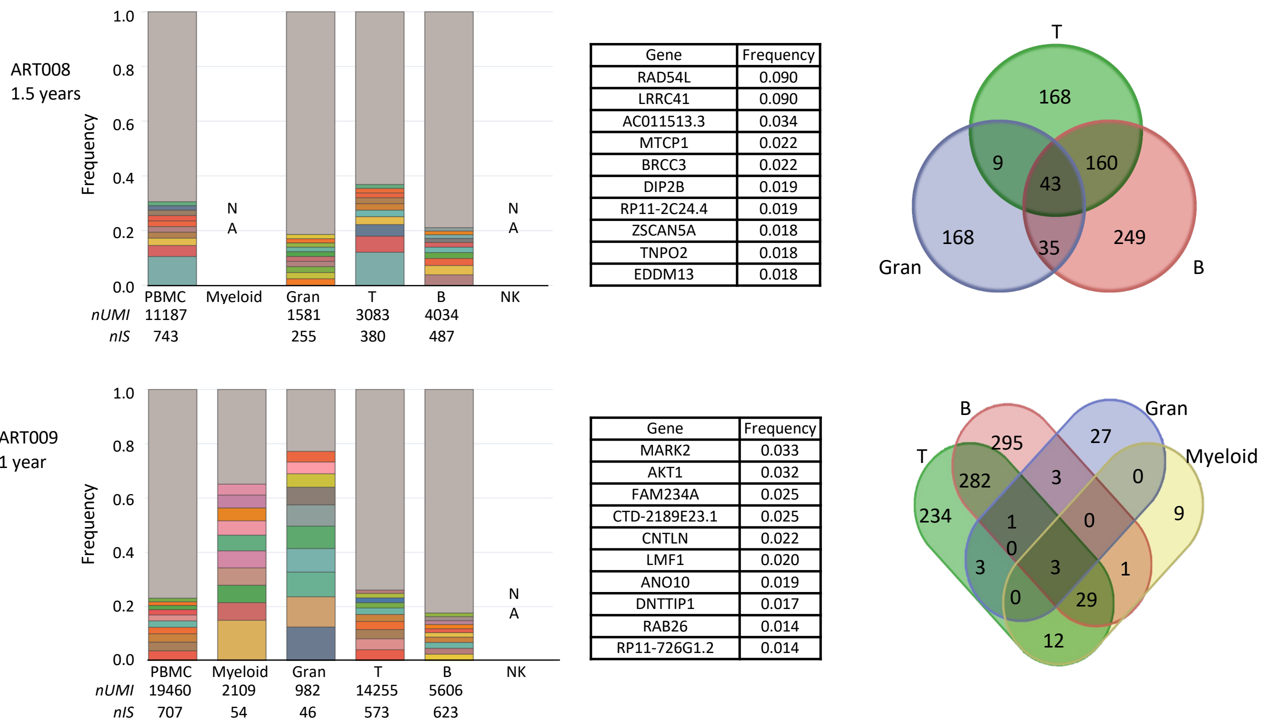
Gene	Frequency
PIP5K1C	0.078
NF1	0.071
INTS11	0.049
EYA3	0.027
CDC5L	0.027
GATAD2B	0.026
RPS6KA1	0.022
SBNO2	0.020
MALAT1	0.019
ENSG10010138868.1	0.019





Gene	Frequency
SLX4IP	0.022
C2CD3	0.016
PGS1	0.014
NF1	0.011
RAB40C	0.009
KDM2A	0.009
ARHGAP35	0.008
FYTTD1	0.008
FBXL20	0.008
UQCR11	0.008





Gene	Frequency
RAD54L	0.090
LRRC41	0.090
AC011513.3	0.034
MTCP1	0.022
BRCC3	0.022
DIP2B	0.019
RP11-2C24.4	0.019
ZSCAN5A	0.018
TNPO2	0.018
EDDM13	0.018

Gene	Frequency
MARK2	0.033
AKT1	0.032
FAM234A	0.025
CTD-2189E23.1	0.025
CNTLN	0.022
LMF1	0.020
ANO10	0.019
DNTTIP1	0.017
RAB26	0.014
RP11-726G1.2	0.014

Supplementary Figure 7. Vector integration site analysis. Isolated cell lineages of sufficient quantity from peripheral blood of the first 6 patients treated were subjected to vector integration site mapping. Years post infusion of transduced $CD34^{+}$ cells are shown. Bar graphs, left: proportion of the 10 most frequent mapped vector-genomic DNA junction sequences in individual colors, with pooled less frequent integration sites in grey. Tables, center: the 10 most frequent genetic loci of vector integration for each patient. Venn diagrams, right: integration sites detected in multiple peripheral blood lineages, with numbers indicating instances of integration sites in common.

PBMC, peripheral blood mononuclear cells; Gran, granulocytes; NA, not available due to insufficient quantity of DNA; *nUMI*, number of unique molecular identifier sequences that contained genomic-AProART provirus junctions; *nIS*, number of distinct vector integration sites detected in the sample.

Table S1. Genotypes and pathogenicity of DCLRE1C variants found in patients in this study.

Patient(s)	Mutation type	cDNA	Protein	ClinVar accession number	Level of pathogenicity (ACMG Criteria)[1]
1, 8, 9, 13	Nonsense	c.597C>A	Tyr199X	VCV000004673.10	Pathogenic
10	Missense	c.47T>C	p.Ile16Thr	VCV001069380.2	Pathogenic
12	Missense	c.346T>C	p.Cys116Arg	SCV002589114	Pathogenic[3]
7	Missense	c.406G>A	Asp136Asn	SCV002598533	Pathogenic
3	Complex	c.492_504 delins13	p.Thr167_Phe168 delins>MetLeu	VCV000649045.1	Pathogenic[3]
12	Splice	c.161+2T>G	splice	SCV002598533	Pathogenic[2]
11	Gross deletion	Del exon 3		VCV001455217.3	Likely Pathogenic
3, 10, 11	Gross deletion	Del exons 1-3		VCV000583822	Likely Pathogenic
2	Gross deletion	Del exons 1-4 (82 kb)		VCV000004666.1	Likely Pathogenic
7	Gross deletion	Del exons 1-5		SCV002589115	Likely Pathogenic

[1]Determination according to Richards, S, Nazneen A, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-424.

[2]Pathogenicity score includes widely accepted functional studies (radiation sensitivity, correction of defect in patient fibroblasts upon addition of normal human cDNA) (M. Cowan and J. Puck, unpublished data).

Table S2. Representativeness of Study Participants

Category	
Disease	Artemis-deficient severe combined immunodeficiency
Special considerations related to	
Sex and gender	Autosomal recessive with males and females being equally affected
Age	Diagnosed by newborn screening (NBS); without NBS patients generally present in the 1st 6 months of life[11,12]
Race or ethnic group	In the U.S. there is a high prevalence of ART-SCID among Navajo and Apache Native Americans[13,14]
Geography	There is a high prevalence in Middle Eastern countries including Saudi Arabia and Israel (Gaza)
Overall representativeness of this trial	Given the small sample size one cannot accurately assess this factor. However, the increased number of Navajo/Apache Native American patients enrolled (40%) is consistent with their increased prevalence of this disease due to a founder mutation. Overall SCID incidence in the U.S. is based on data from universal newborn screening[13]

Table S3. Serious Adverse Events

Patient ID	SAE (month post infusion)[1]	SAE diagnosis[2]	Grade[3]	SAE category	Relationship to study treatment	Unexpected[4]
ART002	-1.2	Processing incident[5]	N/A	N/A	None	Yes
ART001	4.8	AIHA	3	Hospitalization	Probable	No
ART001	6.6	Vomiting	2	Hospitalization	None	No
ART002	5.5	Infection: cytomegalovirus	3	Hospitalization	None	No
ART002	6.5	Rotavirus enteritis	3	Hospitalization	None	No
ART001	11.7	Dehydration	3	Hospitalization	None	No
ART001	14.7	Infection: *C. Difficile*	3	Hospitalization	None	No
ART001	16.2	Infection: Norovirus	3	Hospitalization	None	No
ART001	22.8	Infection: Bacteremia	3	Hospitalization	None	No
ART001	24.1	Poor oral intake related to mouth sores	3	Hospitalization	None	No
ART009	5.9	Rash: suspected systemic staph infection	3	Hospitalization	None	No
ART011	-0.5	Ischemic middle cerebral artery stroke	1	Significant medical event	None	Yes
ART010	10.6	Infection: cytomegalovirus encephalitis	3	Hospitalization	None	No
ART010	14.2 (1st GT)[6] 2.0 (2nd GT)	Fever/infection	3	Hospitalization	None	No
ART012	2.3	GI other: acute onset of blood per rectum	3	Other medically important event	None	No
ART011	7.3	Infection: Urinary tract	3	Hospitalization	None	No
ART008	23.8	Suspected thrombus	3	Hospitalization	None	No

[1] SAE = serious adverse event.
[2] AIHA = autoimmune hemolytic anemia.
[3] Grade 1 = Mild, asymptomatic or mild symptoms; Grade 2 = Moderate, minimal, local or noninvasive intervention indicated; Grade 3 = Severe or medically significant but not immediately life-threatening; Grade 4 = Life-threatening consequences, urgent intervention indicated.
[4.] Not listed in the protocol as consistent with expectations for patients undergoing standard allogeneic hematopoietic cell transplantation for SCID.
[5] Centrifuge tube broke and some cells lost requiring a 2nd bone marrow harvest 3 weeks later.
[6] GT = gene therapy infusion.

Table S4: Adverse Events considered to be possibly, probably, or definitely related to the study treatment.

AE name per CTCAE v4[1]	AE/diagnosis details	Component of study	Grade 1[2] Patients /Events	Grade 2[2] Patients /Events	Grade 3[2] Patients /Events	Grade 4[2] Patients /Events	Total Patients /Events
Anemia	Anemia	Harvest, busulfan	0	3 / 3	3 / 3	0	**6 / 6**
Anorexia	Decreased appetite	Busulfan	1 / 1	0	0	0	**1 / 1**
Fever	Fever	Harvest	2 / 2	0	0	0	**2 / 2**
Hemolysis	Autoimmune hemolytic anemia	Immune reconstitution (dysregulated)	1 / 1	2 / 2	1 / 1	0	**4 / 4**
Hypertension	Hypertension	Stem cell infusion	1 / 1	0	0	0	**1 / 1**
Infections/ infestations: other	Suspected infection; bone marrow culture positive while blood culture negative; no symptoms	Harvest	2 / 2	0	0	0	**2 / 2**
Irritability	Fussiness	Harvest, busulfan	2 / 2	1 / 1	0	0	**3 / 3**
Neutrophil count decreased	Neutropenia	Busulfan	0	0	0	8 / 8	**8 / 8**
Pain	Pain, discomfort	Harvest	3 / 3	2 / 2	0	0	**5 / 5**
Platelet count decreased	Low platelets	Busulfan	0	1 / 1	2 / 2	1 / 1	**4 / 4**
Vomiting	Vomiting	Busulfan	1 / 1	0	0	0	**1 / 1**
Total adverse events in 10 patients			**13**	**10**	**8**	**9**	**40**

[1] AE = adverse event; CTCAE v4 = Common Terminology Criteria for Adverse Events, version 4.

[2] Grade 1 = Mild, asymptomatic or mild symptoms; Grade 2 = Moderate, minimal, local or noninvasive intervention indicated; Grade 3 = Severe or medically significant but not immediately life-threatening; Grade 4 = Life-threatening consequences, urgent intervention indicated.

Supplementary References

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