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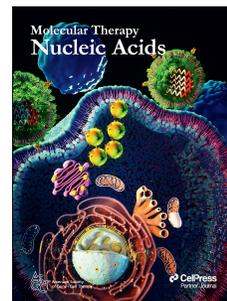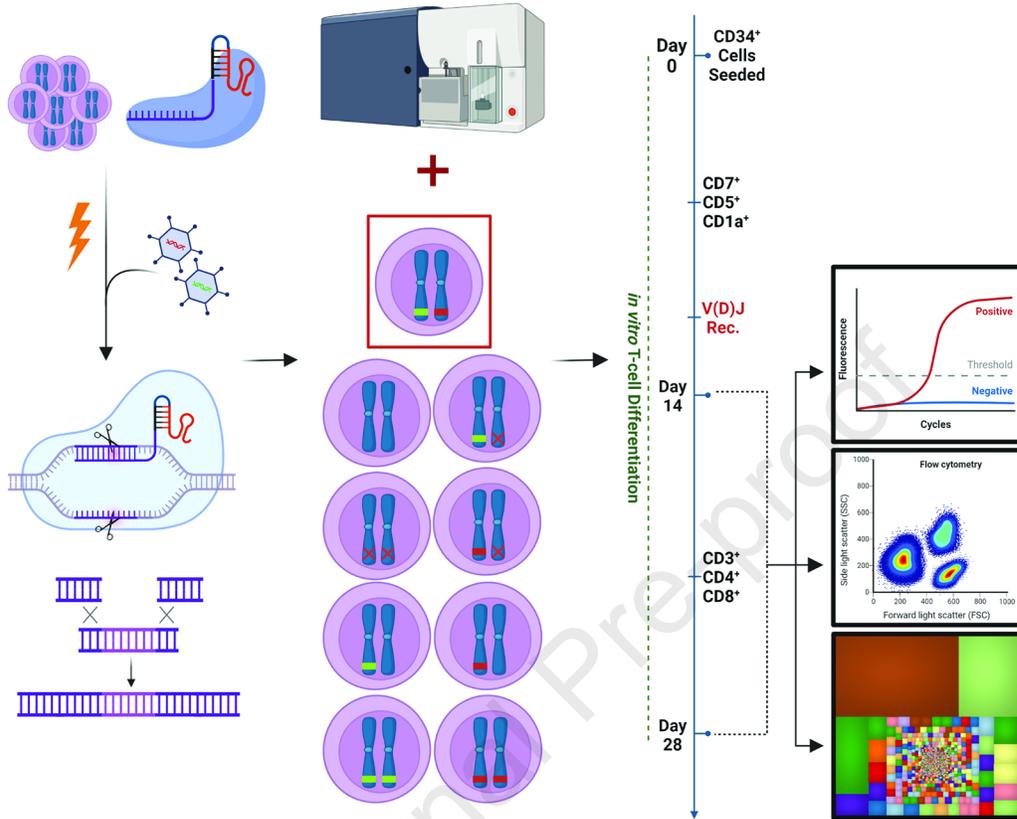
Multiplex HDR for Disease and Correction Modeling of SCID by CRISPR Genome Editing in Human HSPCs

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Running title: CRISPR-Cas9/rAAV6 Genome Editing for Modeling and Correction of SCID Disease

21   **Abstract**

22   Severe combined immunodeficiency (SCID) is a group of disorders caused by mutations in genes

23   involved in the process of lymphocyte maturation and function. CRISPR-Cas9 gene editing of the

24   patient's own hematopoietic stem and progenitor cells (HSPCs) *ex vivo* could provide a therapeutic

25   alternative to allogeneic hematopoietic stem cell transplantation (HSCT), the current gold standard

26   for treatment of SCID. In order to eliminate the need for scarce patient samples, we engineered

27   genotypes in healthy donor (HD)-derived CD34$^+$ HSPCs using CRISPR-Cas9/rAAV6 gene-

28   editing, to model both SCID and the therapeutic outcomes of gene-editing therapies for SCID via

29   multiplexed homology directed repair (HDR). Firstly, we developed a SCID disease model via

30   biallelic *knock-out* of genes critical to the development of lymphocytes; and secondly, we

31   established a *knock-in/knock-out (KI-KO)* strategy to develop a proof-of-concept single-allelic

32   gene correction. Based on these results, we performed gene correction of *RAG2*-SCID patient-

33   derived CD34$^+$ HSPCs that successfully developed into CD3$^+$ T cells with diverse TCR repertoires

34   in an *in-vitro* T-cell differentiation (IVTD) platform. In summary, we present a strategy to

35   determine the optimal configuration for CRISPR-Cas9 gene correction of SCID using HD-derived

36   CD34$^+$ HSPCs, and the feasibility of translating this gene-correction approach in patient-derived

37   CD34$^+$ HSPCs.

38   **Introduction**

39   Severe combined immunodeficiency (SCID) is a group of multiple monogenic disorders

40   characterized by a profound block of T-cell development that harms both cellular and humoral

41   adaptive immunity.[1,2] Depending on the type of SCID, B and NK cells may also be affected. Thus,

42   based on the affected gene, patients can be classified according to the presence or absence of T, B,

43 and NK lymphocytes (T-, B-/+, and NK-/+, respectively). The most common SCID is X-linked

44 SCID (SCID-X1 [from mutations in the *IL2RG* gene]), which presents with a T-B+NK- immune

45 phenotype. Other forms of SCID can develop as a result of mutations in *Recombination-activating*

46 *gene 1* (*RAG1*), *RAG2,* or *DNA Cross-Link Repair 1C* (*DCLRE1C*) and display the T-B-NK+

47 immune phenotype, while other forms develop from mutations in the *Interleukin 7 Receptor*

48 *Subunit Alpha* (*IL7RA*) gene and present with a T-B+NK+ immune phenotype.[3-7] *RAG1* and *RAG2*

49 genes encode proteins that, when complexed together, commence the lymphoid-specific variable

50 (V), diversity (D), and joining (J) gene [V(D)J] recombination process by catalyzing DNA double-

51 strand breaks (DSBs) at the recombination signal sequences (RSSs) which flank the V, D, and J

52 gene segments.[8] The *DCLRE1C* gene encodes the Artemis protein which assists in the functional

53 resection of the V, D, and J gene segments by employing its endonuclease activity on the 5' and 3'

54 overhangs and hairpins of the RAG-complex-induced DSBs.[9] V(D)J recombination is a critical

55 step in the maturation of T and B cells as it is responsible for the generation of a diverse repertoire

56 of T and B cell receptors (TCR and BCR, respectively).[10] Thus, patients with disease-causing

57 variants in the *RAG1*, *RAG2*, or *DCLRE1C* genes typically present with significantly reduced or

58 complete absence of T and B cells. IL7RA signaling plays a major role at various stages of T-cell

59 development, namely ensuring the survival of naive T cells and assisting in the homeostatic

60 expansion of both naive and memory T cells via proliferation.[11] Thus, typical patients with

61 mutations in the *IL7RA* gene will present a lack of T cells. Ineffective expression of any of these

62 genes can lead to SCID, highlighted by severe lymphopenia and lack of cellular and humoral

63 adaptive immunity.[6]

64 Infants born with SCID appear healthy in the first few weeks of life, however, following

65 environmental exposure to pathogens and the decline of maternally transferred antibodies, they

66 become prone to develop life-threatening bacterial, viral, and/or fungal infections. Without early

67 intervention to reconstitute their immune system, patients often do not survive past the first two

68 years of life.[2] The definitive curable treatment for SCID patients is allogeneic HSCT from a human

69 leukocyte antigen (HLA)-matched (related or unrelated) donor.[12] If a full HLA-matched donor is

70 not available, patients may undergo haploidentical HSCT from one of their parents. Successful

71 HSCT promotes lymphoid lineage development resulting in a long-term patient survival rate of

72 >80%, however, there are significant limitations to this approach especially in the absence of a

73 HLA-matched donor where the survival rate decreases to 60-70%.[13,14] These limitations include

74 graft failure after HSCT that leads to poor immune reconstitution as well as potentially fatal graft-

75 versus-host disease (GvHD).[15,16]. Therefore, due to the devastating nature of SCID and the

76 limitations of HSCT, it is crucial to develop new treatment options, such as gene therapy.

77 The ability to genetically edit CD34^{+} hematopoietic stem and progenitor cells (HSPCs) as well as

78 the cells' marked ability to reconstitute the immune system from a small number of original cells,

79 make these stem cells an attractive target for gene therapy.[17,18] Viral vectors can facilitate the

80 delivery of a corrected transgene to autologous HSPCs *ex vivo* as was previously established in

81 gene therapy clinical trials using lentiviral (LV) or gammaretroviral (γRV) transduction.[19,20,21-23]

82 Treatment of Chronic Granulomatous Disease (CGD) , Wiskott-Aldrich Syndrome (WAS), SCID-

83 X1, and most recently of Adenosine Deaminase (ADA)-SCID with γRV resulted in the activation

84 of proto-oncogenes leading to a leukemic transformation in some patients.[24-29] To improve the

85 safety of viral transgene delivery, LVs have replaced γRVs, the viral enhancer sequences

86 responsible for the elevated risk of genotoxicity were removed, and a self-inactivating element

87 was added to the vectors.[30] This resulted in successful therapy for SCID-X1 patients, however,

88 expansion of a single clonal population was reported in one patient case.[31-34] The main

89   disadvantages of using γRV and LV vectors for CD34$^+$ HSPC gene therapy remain the semi-

90   random integration and constitutive expression of the transgene which can lead to incomplete

91   phenotypic correction, dysregulated hematopoiesis, toxicity, and insertional mutagenesis.[35] Thus,

92   delivery of the transgene via a targeted genome-editing approach could prove beneficial.

93   Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated

94   nuclease 9 (Cas9), commonly known as CRISPR-Cas9, has had a tremendous impact on the field

95   of gene editing due to its simplicity, specificity, and applicability in a wide variety of cell types.[36-

96   38] The combination of CRISPR-Cas9 and a donor DNA molecule delivered by recombinant adeno-

97   associated virus serotype 6 (rAAV6) can provide a therapeutic approach to genome editing in

98   CD34$^+$ HSPCs. Although rAAV6 lacks any integration machinery of its own, precise targeting and

99   insertion of the donor DNA payload through the homology-directed repair (HDR) pathway in

100   CD34$^+$ HSPCs can occur following the Cas9-induced site-specific DSB.[39-41] However, the use of

101   rAAV6 vectors is not without challenges. Our group has shown that rAAV6 vectors are recognized

102   by cellular repair proteins triggering a DNA damage response (DDR) proportional to that of the

103   amount of virus used, referred to as the multiplicity of infection (MOI).[42] Therefore, when

104   developing rAAV6-based protocols, reducing the MOI as much as possible, while maintaining the

105   required levels of HDR, is of utmost importance.

106   Developing a gene-therapy strategy requires a thorough understanding of the disease phenotype

107   and extensive assessment of the viability of the respective functional gene correction before it can

108   reach the clinic. To achieve this, large amounts of patient samples are required to model and test

109   the method *ex vivo*. Since SCID patients are infants, neither drawing large amounts of peripheral

110   blood (PB) nor retrieving large samples through invasive bone-marrow procedures are viable

111   options for procuring such samples for pre-clinical proof-of-concept research. Therefore, obtaining

112   sufficient amounts of SCID patient-derived CD34$^+$ HSPCs presents a major challenge. To

113   circumvent the need for large amounts of patient samples, we utilized multiplex HDR, which has

114   been shown to be an effective method for the enrichment of cells with engineered genotypes.[43] We

115   engineered HD-derived CD34$^+$ HSPCs to (1) model SCID and (2) simulate gene-correction

116   therapies for SCID. Via cell sorting, we were then able to enrich for cells with the desired

117   engineered genotype to model and track their T-cell progression.

118   In this study, we accomplished three main goals towards bringing a curative gene therapy for SCID

119   closer to becoming a reality, while providing a more general technique for modeling other

120   recessive blood disorders using HD-derived CD34$^+$ HSPCs. (1) We developed an innovative

121   autosomal recessive SCID disease model via biallelic *knock-out* (*KO*) of *RAG2*, *DCLRE1C,* or

122   *IL7RA* genes in HD-derived CD34$^+$ HSPCs. When *KO* CD34$^+$ HSPCs were subjected to a stromal

123   cell-free, IVTD system,[44] these engineered cells lacked the ability to differentiate into CD3-

124   expressing cells and presented a failure in execution of TCR V(D)J recombination, similar to that

125   of SCID-patient-derived cells. (2) We then utilized a *KI-KO* approach to simulate functional gene

126   correction of *RAG2* in HD-derived CD34$^+$ HSPCs. Due to the recessive nature of SCID, correction

127   of only one allele is sufficient to cure the patient. In this strategy, we mimicked monoallelic

128   correction in SCID-patient cells by *knock-in* (*KI*) of a codon-optimized diverged cDNA cassette

129   into the endogenous *RAG2* loci in one allele (thereby preserving regulatory non-coding elements)

130   and *KO* of the second allele. In contrast to the *RAG2* biallelic KO cells, the *KI-KO* differentiated

131   T cells presented normal CD3 expression and TCR repertoire diversity. (3) Lastly, we showed

132   first-of-its-kind functional gene correction of *RAG2*-SCID patient-derived CD34$^+$ HSPCs. In

133   contrast to the unedited *RAG2*-SCID-patient cells, the corrected population developed into CD3$^+$

134   T cells with diverse TCR repertoires. Overall, our method provides a platform to study the disease

6

135   phenotypes with a multi-parameter readout in the form of immunophenotyping and V(D)J

136   recombination assessment via next-generation sequencing (NGS) and will allow researchers to

137   determine the optimal configuration for gene therapies for other SCIDs and immunodeficiencies.

138   **Results**

139   **Modeling SCID disease with CRISPR-Cas9/rAAV6-mediated biallelic *KO* in HD-derived**

140   **CD34+ HSPCs**

141   In order to generate biallelic *KO* of the *RAG2*, *DCLRE1C,* or *IL7RA* genes individually in HD-

142   derived CD34+ HPSCs, chemically modified sgRNAs were designed to target the genomic DNA

143   a few base-pairs downstream of the respective gene's start codon.[37] We PCR amplified each

144   individual gene's on-target site and quantified the INDEL frequencies of the corresponding Sanger

145   sequences via the tracking of INDELs by decomposition (TIDE) analysis and found that all

146   sgRNAs induced INDELs at high frequencies yet produced distinctly different INDEL patterns

147   (Figure S1A-E). Additionally, we used NGS to map the INDEL events of the *RAG2* sgRNA and

148   found that the distinct pattern was highly reproducible (Figure S1F).

149   The sgRNA was delivered together with the Cas9 endonuclease as a ribonucleoprotein (RNP)

150   complex in conjunction with rAAV6 vectors carrying a DNA template for gene disruption. The

151   template contained a reporter gene flanked by arms of homology for HDR at the aforementioned

152   CRISPR-Cas9 cut-site. Following successful HDR, the integration of the reporter gene into the

153   target gene's open reading frame was expected to abolish the transcription of the coding region of

154   the target gene (Figure 1A). Two DNA donors, each containing a distinct reporter gene, were

155   required for each gene to allow for multiplex HDR and biallelic *KO* enrichment via cell sorting of

156   cells expressing both reporter genes. We used two control groups in the following analyses: (1)

157   cells electroporated without the presence of the RNP complex or rAAV6 (herein referred to as

158   *Mock*) as well as (2) cells with biallelic *KO* of the *C-C motif chemokine receptor 5* (*CCR5*) gene.

159   While *CCR5* is expressed in T cells, its expression does not affect T-cell development, allowing

160   for the determination of the effect of CRISPR-Cas9 and rAAV6 treatments on the T-cell

161   developmental process. Biallelic targeting of *RAG2* and *CCR5* was carried out with green

162   fluorescent protein (GFP) and truncated nerve growth factor receptor (tNGFR) reporter genes

163   (Figure 1A and S2A), whereas biallelic targeting of *DCLRE1C* and *IL7RA* were carried out with

164   GFP and tdTomato reporter genes (Figure S2B and S2C). Immediately following electroporation,

165   cells were exposed to their respective rAAV6 donors. Two days post-electroporation, the cells

166   were sorted for CD34 expression, and double-positive reporter gene expression tNGFR$^+$/GFP$^+$ or

167   tdTomato$^+$/GFP$^+$, indicative of biallelic *KO* (See example for *RAG2* in Figure 1B). Average

168   biallelic HDR frequencies at the different loci ranged from 1.7-4.6% (Figure 1C and S2D). DNA

169   was purified from cells that were cultured in CD34$^+$ HSPCs medium post-sort and the DNA was

170   analyzed by Digital Droplet PCR (ddPCR) for quantification of locus-specific target integration.

171   ddPCR analysis revealed that the *CCR5, RAG2*, and *DCLRE1C* sorted cells contained the *KO*

172   disruption DNA donors in ~100% of the alleles whereas *IL7RA* sorted cells showed slightly less

173   efficient integration (Figure S2E).

Modeled SCID CD34$^+$ HSPCs do not progress to CD3$^+$ T Cells in a cell-free *in-vitro* T-cell

differentiation system

176   CD34$^+$ HSPCs differentiate into CD7$^+$/CD5$^+$ pro-T cells which then continue to develop into T-

177   cell-committed pre-T CD1a$^+$ cells (identifiable in IVTD by immunostaining on day 14). These

178   CD7$^+$/CD1a$^+$ cells can then undergo TCR rearrangement and become immature single-positive

179   (ISP) CD4$^+$ T cells. These give rise to double-positive (DP) CD4$^+$/CD8$^+$ cells that express CD3

180 (identifiable in IVTD by immunostaining on day 28 and 42 [in cases where extensive follow-up is

181 required]). These cells then undergo full maturation into CD3-expressing single-positive (SP)

182 CD8$^+$ or CD4$^+$ cells (i.e., CD3$^+$/CD8$^+$ or CD3$^+$/CD4$^+$ cells [Figure S3A]). CD3 receptors form a

183 complex with the cell's TCR, which serves a fundamental role in the maturation of thymocytes

184 from their immature precursors. Typical SCID is characterized by the absence of or significant

185 reduction in CD3$^+$ T cells (<300 cells/ml).[45] The stromal cell-free IVTD system that we used

186 provided the ability for easy observation and immunophenotyping by flow cytometry of T-cell

187 development at predetermined time points and assessment of V(D)J recombination via NGS

188 analysis.

189 In order to model *RAG2-, DCLRE1C-,* or *IL7RA*-SCID, biallelic KO CD34$^+$ HSPCs for each locus

190 were individually cultured in the IVTD system and compared to *Mock* and *CCR5* KO populations.

191 Consistent with the literature, the *IL7RA* biallelic KO populations presented complete cell death

192 after only 6 days in culture (Figure 2A), whereas cells that had no HDR integration were

193 predominantly viable cells (Figure S3B).[6] In contrast, *Mock* cells and *CCR5*, *RAG2*, and

194 *DCLRE1C* biallelic KO populations were able to proliferate through the 28 day time point (Figure

195 2B). Immunophenotyping by flow cytometry revealed no significant difference in the expression

196 levels of CD5, CD7, or CD1a between the *RAG2* or *DCLRE1C* KO and *CCR5* KO cells after 14

197 days. Additionally, similar expression of both CD4 and CD8 was observed in all three groups after

198 28 days. However, at this stage of differentiation, *CCR5* KO cells developed into CD3$^+$ T cells,

199 while hardly any *RAG2* or *DCLRE1C* KO cells expressed CD3 (Figure 2B and S3C). Furthermore,

200 as expected, the expression of the endogenous *RAG2* and *DCLRE1C* genes were drastically

201 reduced in the *RAG2* and *DCLRE1C* biallelic *KO* populations (Figure S3D-E).

202 Due to their central role in somatic recombination, we sought to determine whether the *RAG2* and

203 *DCLRE1C* KO cells were able to perform functional V(D)J recombination, the lack of which is a

204 hallmark of SCID lymphocytes. To do so, we deep-sequenced the T-cell receptor gamma (TRG)

205 loci, one of the first chains to be recombined among the different TCR chains.[46,47] Preparation of

206 TRG libraries from *RAG2* KO cells revealed no amplification of the recombined locus (Figure

207 S3F) and the rearrangement in *DCLRE1C* KO cells was similarly impaired, noted by standard PCR

208 amplification using primers flanking the V-J regions of the TRG locus (Figure S3G). Taken

209 together, our IVTD approach can be used to accurately model SCID and help us characterize the

210 SCID phenotype for validation of future gene-correction results. This was highlighted by

211 accurately producing the gene-specific disease phenotypes: *IL7RA* KO cells did not proliferate or

212 differentiate at all and *DCLRE1C* KO and *RAG2* KO cells neither developed into CD3+ T cells nor

213 underwent effective TCR V(D)J recombination.

214 **T-cell differentiation phenotype of SCID-patient cells validates the disease model**

215 To validate our use of the IVTD platform as a reliable method to model the SCID phenotype, we

216 subjected SCID-patient-derived CD34+ HSPCs to 42 days of IVTD and examined their

217 differentiation capability (Figure S4A). The SCID-patient-derived CD34+ HSPCs samples were

218 extracted and purified from the PB of *RAG1*- and *RAG2*-SCID patients (Figure S4B-C). Both

219 patients presented with a clinical phenotype suggestive of SCID and subsequent immune workup

220 and genetic testing confirmed the diagnosis. The first patient is homozygous for a 4bp deletion

221 (c.1407-10 del.4bp-TTGC) in the *RAG1* gene, resulting in a frameshift mutation, premature stop

222 codon, and dysfunctional RAG1 protein, while the second patient is homozygous for a missense

223 mutation (c.G104T; p.G35V) in the *RAG2* gene, which reduces the binding capabilities of RAG2

10

224   to RAG1 during V(D)J recombination.[8,48] Alongside the CD34$^+$ HSPCs from each patient, control

225   HD-derived PB CD34$^+$ HSPCs were seeded in the IVTD system.

226   After 14 days, there was no significant immunophenotypic difference in CD5 or CD7 expression

227   between the groups, however, a range of CD1a expression was noticed across the sample

228   populations (0-37%) (Figure S4A). Following 28 and 42 days in the IVTD system, the SCID-

229   patient-derived cells did not differentiate into CD3$^+$ cells and cell viability was still >90% in all

230   samples, indicative of a halt in T-cell progression (Figure S4A). To emphasize the V(D)J

231   recombination impairment in the SCID-patient cells compared to the HD-derived PB control, DNA

232   was extracted and used as a template for TRG recombination PCR. The HD-derived PB cells

233   successfully underwent TRG recombination, whereas the SCID-patient-derived PB cells failed

234   (Figure S4D-F). An additional sample of cells was extracted and purified from PB of the *RAG1*-

235   SCID patient following successful HSCT and subjected to the IVTD system. Consistent with the

236   clinical reports, the *RAG1*-SCID cells post-HSCT showed robust CD3 expression (Figure S4A and

237   Table S1) and completed successful TRG V(D)J recombination by day 14 (Figure S4F).

238   **Biallelic *KI-KO* targeting of *RAG2* in HD-derived CD34$^+$ HSPCs to simulate functional gene**

239   **correction**

240   Since *RAG2*-SCID is an autosomal recessive disorder, correction of only one allele is required to

241   have the patient develop a functional immune system. This is highlighted by the fact that carriers

242   of *RAG2* mutations (e.g., the parents of the *RAG2*-SCID patient) present as fully healthy

243   individuals. We aimed to utilize multiplex HDR for *RAG2 KI-KO* for correction simulation in HD-

244   derived CD34$^+$ HSPCs. This provided three main advantages over alternative editing strategies

245   which requires prolonged culturing of the cells and can be done using induced pluripotent stem

246   cells (iPSCs) (e.g. first actuate *RAG2 KO* and then subsequently correct the previously *KO* cells).

247   (1) In contrast to prior works that have used iPSCs, HD-derived CD34$^+$ HSPCs provided us with

248   the capability of using biologically-authentic CD34$^+$ HSPCs, the same cells that are used in

249   HSCT[49]; (2) Culturing CD34$^+$ HSPCs for too long reduces the cells regenerative potential. CD34$^+$

250   HSPCs maintain their stemness and a balance between proliferation, quiescence, and regeneration

251   as well as their ability to differentiate to all hematopoietic cell lineages for a limited time in culture.

252   Extensive culturing protocols with multiple editing steps have been shown to lead to a loss of the

253   stem cells qualities and engraftment potential in murine models;[50] and (3) It provides the ability to

254   circumvent the need for scarce patient samples to establish the feasibility of our correction strategy.

255   Based on this, we aimed to simulate functional gene correction of *RAG2* in HD-derived CD34$^+$

256   HSPCs via multiplex HDR using two distinct rAAV6 donors to actuate biallelic *KI-KO*. In the

257   HD-derived CD34$^+$ HSPCs, we targeted one allele with a *KI* template containing diverged

258   functional *RAG2* cDNA (along with a [tNGFR] reporter gene cassette under the regulation of a

259   constitutive phosphoglycerokinase (PGK) promoter) (Figure 3A) and the other allele with a *KO*

260   template consisting of a disrupting reporter cassette (*KO* schematic depicted in *Figure 1A* [GFP

261   reporter gene under the regulation of a constitutive spleen focus-forming virus {SFFV}

262   promoter]). In this context, the use of the tNGFR reporter is advantageous since it enables tracking

263   and enrichment of the corrected cells and has been approved for clinical applications[51]. Our *KI-*

264   *KO* approach mimics the correction in recessive SCID patient cells where a single functional allele

265   is enough to confer the host with a normal immune system. The *KI* correction donor DNA

266   contained complete *RAG2* cDNA with silent mutations added to diverge the cDNA sequence from

267   that of endogenous while maintaining codon usage. The resulting diverged cDNA produces the

268   correct protein sequence, while the reduced similarity to the genomic sequence precludes the

269   cDNA sequence from being re-cut by residual Cas9 or from serving as a potential homology arm

270   causing premature cessation of HDR.[52] These *KI-KO* cells, double-positive for GFP and tNGFR,

271   simulate the genotype and therapeutic outcome of *RAG2*-SCID single-allelic correction gene-

272   editing therapy and were analyzed relative to our disease modeled biallelic *RAG2* KO cells.

273   *RAG2 KI-KO* CD34$^+$ HSPCs were sorted for double-positive tNGFR$^+$/GFP$^+$ expression (HDR

274   values in Figure S5A) and sorted cells were subjected to IVTD. To validate *RAG2* biallelic editing

275   efficiency, targeted alleles were analyzed by ddPCR and found to be positive (Figure S5B).

276   Additionally, quantitative real-time PCR (qRT-PCR) analysis revealed that the endogenous gene

277   expression of *RAG2* was markedly reduced (Figure S5C) while robust diverged cDNA expression

278   was found exclusively in the engineered cells (Figure S5D). Importantly, the expression of the

279   diverged *RAG2* cDNA indeed facilitated T-cell development highlighted by the successful

280   differentiation of *RAG2 KI-KO* edited cells into CD3$^+$ T cells relative to *RAG2* KO cells on day

281   28 (*RAG2 KI-KO*: 2.7%; *RAG2* KO: 0.03%) (Figure 3B-C, Figure S5E-F).

282   Deep-sequencing analysis of TRG V(D)J recombination on days 14 and 28 revealed a diverse TRG

283   V(D)J rearrangement repertoire in the *RAG2 KI-KO* population following robust expression of the

284   *RAG2* diverged cDNA (Figure 4A). Moreover, calculation of Shannon and Simpson diversity

285   indices revealed that there were no significant differences between the TRG clonotype diversity

286   richness of *Mock*, *CCR5* KO, and *RAG2 KI-KO* populations (Figure 4B). Lastly, the frequency

287   distribution of complementarity determining region 3 (CDR3) lengths was comparable to that of

288   the control groups, indicating production of a diverse TCR repertoire (Figure S6). The CDR3

289   region is responsible for recognizing processed antigen peptides and the length and sequence of

290   the CDR3 varies by T-cell clone. Thus, the sequence of CDR3 determines the structure and

291   specificity of the TCR, where a unique CDR3 sequence represents a specific T-cell clonotype.

292   Sequencing of the CDR3 region can, therefore, be used as a measurement of TCR diversity.[53]

293   Together, these data indicate that *KI* of the correction diverged cDNA promotes differentiation

294   into CD3^{+} T cells and promotes the development of a highly diverse TRG repertoire.

295   **RAG2 functional gene correction in RAG2-SCID-patient-derived CD34^{+} HSPCs**

296   After determining that integration and expression of our correction donor were effective in

297   facilitating T-cell development, we obtained CD34^{+} HSPCs from the cord blood (CB) of a *RAG2*-

298   SCID patient to actuate *in-vitro* functional gene correction. This patient presented with a clinical

299   SCID phenotype (Omenn phenotype) and had compound heterozygous missense mutations

300   (G95V+E480X) in the *RAG2* gene.[54] In compound heterozygotes, each allele of the gene has a

301   different genetic mutation. In such cases, our approach is uniquely advantageous since we integrate

302   the complete *RAG2* cDNA including the endogenous 3' UTR region at the initiation codon of the

303   endogenous *RAG2* locus, thus resolving all possible mutations in the open reading frame while

304   preserving the endogenous regulation and gene expression with a single rAAV6 donor.

305   Consequently, we aimed to target the *RAG2*-SCID CD34^{+} HSPCs with *RAG2* diverged cDNA

306   (schematic depicted in *Figure 3A*) using a rAAV6 MOI of 12,500 viral genomes (VG) per cell.

307   Notably, however, we observed that when HD-derived CD34^{+} HSPCs were treated with the *RAG2*

308   correction donor at 12,500 VG/cell and subjected to the IVTD system, cellular yield was

309   substantially reduced compared to samples that were not exposed to the rAAV6 vectors (Figure

310   S7A). While the rAAV6 particles were found to be significantly diluted over the course of the

311   IVTD process, they were still present by days 28 and 42 (Figure S7B) leading to increased cell

312   death and/or reduced proliferation in the rAAV-treated populations, both hallmarks of prolonged

313   rAAV-induced toxicity. Moreover, although two days post-electroporation (day 0) effective HDR

14

314 was observed in these cells via both flow cytometry and ddPCR (Figure S7C), we saw a decrease

315 in targeted allele frequency via both methods over the 42 days of IVTD (Figure S7D-E). Therefore,

316 to reduce the potential for similar results in the patient samples, cell sorting was used to enrich for

317 corrected CD34^{+} HSPCs using the tNGFR marker in two samples of *RAG2*-SCID (one treated at

318 a MOI of 12,500 VG/cell and one at 6,250 VG/cell) (Figure S7F). We then subjected the sorted

319 cells to the IVTD system, thus minimizing the risk of positive selection of unedited cells in the

320 IVTD system. In parallel, HD-derived *Mock* CD34^{+} HSPCs, untreated *RAG2*-SCID CD34^{+}

321 HSPCs, and unsorted *RAG2*-SCID corrected CD34^{+} HSPCs (MOI of 12,500 VG/cell) were

322 cultured in the IVTD system. For the sorted cells, ddPCR was performed to determine the extent

323 of *RAG2* cDNA genomic integration which was observed to be ~50% of all targeted alleles for

324 both MOIs (Figure S7G). Following 28 days in the IVTD system, the *RAG2*-SCID corrected cells

325 developed into CD3^{+} cells, in all three corrective populations, compared to *RAG2*-SCID cells that

326 showed impaired progression towards CD3 expression (12,500 VG/cell *RAG2*-SCID unsorted:

327 2.7%; 12,500 VG/cell *RAG2*-SCID sorted: 6.0%; 6,250 VG/cell *RAG2*-SCID sorted: 6.2%; *RAG2*-

328 SCID: 0.2%) (Figure 5). These data were comparable with CD3-expression levels of *Mock* cells

329 (average of 5%) after 28 days in the IVTD system (Figure 5 and S7H). The percentage of CD3^{+}

330 cells in the population continued to rise by day 42 in the sorted populations similar to the *Mock*

331 sample (Figure 5 and S7H). Additionally, an increase in cell death in the rAAV-treated populations

332 was observed via cell viability staining, a hallmark of prolonged rAAV-induced toxicity (Figure

333 S7I).

334 Deep sequencing of the TRG repertoire revealed that each of the two sorted populations developed

335 a diverse TRG repertoire, highlighted throughout the IVTD timeline by treemap, CDR3 length,

336 and Shannon and Simpson diversity indices (Figure 6A-B and Figure S8). We could not, however,

337   evaluate the repertoire of the unsorted *RAG2*-SCID correction cells at day 42 since only a very

338   small number of CD3^{+} cells survived until this time point (Figure 5). The richness of the observed

339   TRG repertoires was comparable to that of the untreated *Mock* populations in the IVTD system

340   (Figure 6B). To further examine the rearrangement capability, copies of T-cell receptor excision

341   circles (TRECs) were quantified. TRECs, are a DNA marker that represents the excision of the

342   delta-coding segments out of the T-cell receptor alpha (TRA) locus, allowing the TRA

343   recombination to occur.[55] According to newborn screening data for SCID, TREC copies are

344   completely undetectable in *RAG2*-SCID samples.[4] In our samples, TRECs were detected only in

345   the sorted *RAG2*-SCID correction samples at day 28 and 42 of differentiation, with a higher copy

346   number in the 6,250 *RAG2*-SCID corrected cells (Table 1). In summary, building off of our success

347   with our correction donor in the *KI-KO* correction simulation approach, we were able to utilize the

348   same donor to correct *RAG2*-SCID-patient-derived CD34^{+} HSPCs *in vitro*, producing CD3^{+} T cells

349   with diverse TRG repertoires.

350   **Discussion**

351   Disease modeling is a fundamental part of understanding the mechanisms underlying the

352   expression and regulation of the affected genes and due to their central roles in the T-cell

353   development process, we chose to focus on the *RAG2*, *DCLRE1C*, and *IL7RA* genes. Patient-

354   derived samples represent a natural system to study these mechanisms and their influence on the

355   various cell processes, however, in diseases such as SCID, obtaining a sufficient quantity of CD34^{+}

356   HSPCs from young patients for scientific studies is not a viable option. In place, some researchers

357   have generated iPSCs derived from dermal fibroblasts or skin keratinocytes of SCID patients in

358   order to build disease models and to study the cells' differentiation potential.[56-59] This option has

359   been widely accepted, however, iPSCs lack the authenticity of unadulterated CD34^{+} HSPCs. We

360   present a novel approach to address this problem by modeling different recessive forms of SCID

361   through biallelic CRISPR-Cas9/rAAV6-mediated gene *KO* in easily-attainable, abundant,

362   biologically-authentic, HD-derived CD34$^+$ HSPCs. This strategy is made possible by multiplexing

363   HDR using distinct reporter genes and sorting via FACS to enrich for complete biallelic *KO* cells.

364   While in this work average biallelic HDR ranged from 1.7-4.6% (depending on the locus), we

365   believe that enhancing HDR by reducing toxic rAAV6-induced DDR and/or inhibiting the NHEJ

366   repair pathway, among other techniques, we can further improve our method.[60,61]

367   We tracked the biallelic *KO* cells in a stromal cell-free IVTD system to better understand the SCID

368   phenotype to subsequently use as confirmation for the success of our proof-of-concept correction

369   phenotype. The readout of our system is multi-faceted, allowing for immunophenotyping via flow

370   cytometry to track T-cell differentiation and NGS for TCR repertoire analysis. We believe that

371   using this system for disease modeling via CRISPR-Cas9 editing has the potential to rapidly

372   accelerate research on suspected disease-causing genes. These disease models can then be

373   phenotypically and functionally validated in various differentiation platforms paving the way for

374   new potential gene therapies.

375   One of the challenges when modeling SCID disease is effectively inducing a reproducible T-cell

376   differentiation phenotype.[62] A recent study by Pavel-Dinu *et al.* presented an approach for *IL2RG*

377   gene correction on male HD-derived CD34$^+$ HSPCs and SCID-X1 patient samples cultured in the

378   *in-vitro* OP9 T-cell differentiation system. A major drawback of the OP9 system is the difficulty

379   of efficiently differentiating PB CD34$^+$ HSPCs while obtaining a consistent differentiation

380   pattern.[62-64] Hence, Pavel-Dinu *et al.* addressed this problem by assessing the differentiation

381   pattern of *IL2RG* HDR-corrected CD34$^+$ HSPCs after transplantation to NSG mice. Through

382   enrichment of edited cells via sorting and subsequent use of a stromal cell-free IVTD system we

383 have overcome the variability challenges encountered in other T-cell differentiation studies. Thus,

384 we established a sustainable, versatile, and reproducible system for evaluating gene-correction

385 efficiency and studying the mechanisms of recessive forms of SCID that allows for easy cell

386 tracking, immunophenotyping, and V(D)J recombination analyses.

387 With the use of the IVTD system, we showed the successful differentiation of HD-derived CD34$^+$

388 HSPCs to CD7$^+$CD5$^+$ cells and eventually to CD4, CD8, and CD3-expressing T cells, with diverse

389 TRG repertoires. *RAG2* biallelic KO and *DCLRE1C* biallelic KO CD34$^+$ HSPCs developed into

390 CD7$^+$CD5$^+$ cells, however, did not develop further to express CD3 and did not exhibit TRG gene

391 rearrangement. Previously, Bifsha *et al.* and Bosticardo *et al.* assessed the T-cell differentiation

392 outcome of SCID-patient-derived PB CD34$^+$ HSPCs via the OP9 IVTD system and the artificial

393 thymic organoids (ATO) system, respectively.[62,65] In their work, the *RAG2* patient cells displayed

394 a developmental failure at the same T-cell differentiation stage (CD3-expressing stage) as we

395 observed in our disease models as well as in the SCID-patient-derived PB CD34$^+$ HSPC samples.

396 Curiously, clinical analysis of *RAG2*-SCID patients c.G104T and p.G95V+E480X showed

397 expression of CD3 (Table S1 and S2) despite the lack of differentiation of the same patients'

398 CD34$^+$ HSPCs into CD3$^+$ cells in the IVTD system. Although patients with SCID typically present

399 profoundly reduced total T cell counts, patients can sometimes display the presence of self-reactive

400 cells and trans-placentally acquired maternal T cells.[66] This could explain the discrepancy between

401 clinical blood analysis and the output of our IVTD method. Taken together, modeling recessive

402 forms of SCID via CRISPR-Cas9/rAAV6 gene editing coupled with the use of the highly

403 reproducible IVTD system can effectively limit the requirement for large quantities of scarce

404 patient-derived samples.

405   As mentioned, we chose to focus on the *RAG2, DCLRE1C,* and *IL7RA* genes which combined are

406   responsible for ~21% of all SCID cases in the United States of America, while *DCLRE1C* and

407   *IL7RA* mutations alone were responsible for 59.3% of all SCID cases in Israel.[3,4,7] Even when a

408   HLA-matched donor is found and allogeneic HSCT is conducted, patient outcomes for *RAG-*

409   SCIDs are significantly worse than for T-B+NK- SCIDs such as SCID-X1.[67,68] *RAG*-SCIDs

410   present a T-/B-/NK+ phenotype and the presence of NK cells has been shown to mediate graft

411   rejection in murine SCID models as well as in human observational studies.[69] Thus, development

412   of other treatment options for these SCIDs such as autologous gene therapy is critical to eliminate

413   the need for finding an HLA-matched donor and to limit the risk of graft rejection.

414   Although great strides have been made in the past few decades, gene therapy using viral vectors

415   in its current form (γRV or LV) could be hazardous for highly controlled and regulated genes such

416   as *RAG1* and *RAG2* due to the constitutive expression of the transgene. In these cases, this can

417   result in genomic instability, and in some cases leukemia, as a result of expression outside of the

418   precise developmental window.[70-72] A recent study by Miyazaki *et al.* demonstrated that the

419   mechanism for developmental regulation of *RAG1/2* gene expression is via cis-elements

420   surrounding the *RAG1/2* coding regions which control the spatial genomic organization inside the

421   locus.[73] Additionally, Rommel *et al.* found that aberrantly functioning RAG1/2 promotes

422   lymphocyte malignancy through the formation of translocations and/or deletions in cancer-causing

423   genes.[74] To address this, we aimed to establish a gene-editing method that addresses gene

424   regulation and integration specificity. In our study, the *RAG2* sgRNA used for gene correction was

425   designed to induce a specific DSB several base pairs downstream to the gene's ATG start codon

426   and the diverged cDNA delivered by the rAAV6 included the gene's endogenous 3' UTR. In

427   contrast to the strategy employed in Pavel-Dinu *et al.*, our method allowed for the subsequent

428   expression of the transgene to rely on the conserved endogenous 5' and 3' transcriptional and

429   translational regulatory elements.[75] To ensure the safety of our gene-editing strategy the *RAG2*

430   sgRNA's off-target potential was previously thoroughly examined, ensuring that it edits only a

431   small number of off-target sites with low editing frequency.[76,77] Our approach highlights the

432   importance of safeguarding the regulatory elements that are located outside of the coding region

433   and the importance of inducing precise integration of transgenic elements.

434   After establishing a novel disease model, we proceeded to evaluate a proof-of-concept CRISPR-

435   Cas9/rAAV6 gene-correction strategy through our *KI-KO* approach in HD-derived CD34+ HSPCs.

436   Since *RAG2*-SCID is an autosomal recessive disorder, correction of only one allele is required to

437   have the patient develop a functional immune system, hence our use of a single-allelic *KI* while

438   the other allele is *KO* (mimicking a mutated allele). While theoretically one could employ a *KI*

439   strategy and rely on Cas9-induced INDELs for the *KO* of the other allele, our *KI-KO* method is

440   more definitive for two reasons. 1) The INDEL efficiencies for sgRNAs although highly-efficient,

441   are not 100%. Thus, even if it is a small amount, there will be cells that will not have a *KI-KO*

442   genotype. 2) Our approach, although applied here for *RAG2* alone is an approach that can be

443   applied universally to any monogenic disorder. In each case, the gene-specific sgRNA would have

444   its own unique INDEL signature, not always leading to gene *KO*. Thus, a uniform HDR-based

445   biallelic *KI-KO* approach is beneficial. Our correction simulation using *RAG2* diverged cDNA

446   expression led to successful T-cell differentiation highlighted by successful TRG gene

447   rearrangement and expression of T-cell markers, CD4, CD8, and CD3. This allowed us to test our

448   correction donor on HD-derived CD34+ HSPCs before utilizing any precious patient-derived

449   samples. With the confidence in our system, we then actuated the first-of-its-kind, successful, *in-*

450   *vitro* correction of *RAG2*-SCID-patient-derived CD34^{+} HSPCs, which, post-correction, produced

451   CD3-expressing T cells with a diverse TRG repertoire.

452   Based on previously studied cases of "naturally-occurring gene therapy" where a revertant

453   mutation led to the unexpected development of T lymphocytes and diverse TCR repertoires in

454   SCID patient, it is assumed that individual corrected cells are capable of at-least partially

455   correcting the SCID phenotype. In these cases, it is estimated that a single cell with the corrective

456   revertant mutation was able to undergo ~11 divisions before undergoing TCR gene arrangements,

457   thus giving way to ~1,000 unique TCR sequences.[78-81] These cases highlighted the fact that even

458   a small number of "corrected" cells can generate a fully diversified TCR repertoire enough to

459   reconstitute the immune system. Additionally, 5-10% donor chimerism is considered to be the

460   threshold for successful clinical improvement for SCID patients post-allogenic HSCT. Dvorak *et*

461   *al.* showed that donor chimerism as low as 3% was sufficient for T- and B-cell recovery in SCID

462   patients in contrast to greater than 20% for diseases such as thalassemia or sickle cell anemia.[82]

463   While rAAV6 is commonly used to deliver the donor DNA in genome-editing experiments, our

464   group has shown in Allen *et al.* that rAAV6 vectors can trigger a potentially toxic DDR after

465   entering cells proportional to the MOI used.[42] In order for CRISPR/rAAV6 treatments to be

466   implemented as a clinical therapy for the purpose of gene correction, reduction of the rAAV6

467   toxicity is required. We demonstrated that lowering the MOI (from 12,500 to 6,250 VG/cell)

468   maintains HDR of ≥17% and allows for potential alleviation of rAAV6-induced genotoxicity as

469   noted by higher levels of TREC copy numbers as well as higher CD3 expression in the 6,250

470   VG/cell sample (Figure 5 and Table 1). Additionally, we noted a difference between the sorted

471   and unsorted 12,500 VG/cell populations on day 28 and day 42 in the frequency of CD3^{+} cells.

472   We hypothesize that this is due to lower input of corrected cells to our IVTD system as well as

473 competition between the corrected and uncorrected cells in our IVTD system in the unsorted

474 sample. Due to these challenges, we observed a drop off in CD3 expression in the unsorted sample

475 while the CD3-expressing population in the comparable sorted sample expanded from 6% on day

476 28 to 19% on day 42. Based on this new understanding of the effects of MOI and cell enrichment,

477 we propose the following possible ways to counter this reduction in efficacy: 1) Improving HDR

478 efficiency by inhibiting the NHEJ repair pathway via molecules such as i53 and/or DNA-PK

479 inhibitors; 2) Transiently suppressing p53 to rescue the toxic DDR; and/or 3) Enrichment of the

480 corrected cell population via cell sorting.[60,61] Additionally, we are currently working towards using

481 good manufacturing practices (GMP) grade rAAV6 preparations, free of potential toxic impurities.

482 This will enable us to expand our research to animal models and eventually to the clinic. Although

483 follow-up studies are ongoing to improve HDR efficiencies, thus increasing the feasibility of

484 achieving high donor chimerism frequencies without cell enrichment, our enrichment strategy

485 enables ~100% HDR input frequency after cell sorting which together with the relatively low

486 threshold for requisite donor chimerism in SCID patients, provides a valuable proof-of-concept

487 correction methodology.

488 In summary, we present a disease model for SCID and proof-of-concept gene therapy using a

489 combination of CRISPR-Cas9, rAAV6 donors, a reproducible cell-free IVTD system, and

490 abundant HD-derived CD34$^+$ HSPCs. This system allows for circumventing the difficulty of

491 obtaining large amounts of patient samples while providing a valuable tool that will allow

492 researchers to find the optimal gene-editing configurations (i.e. engineered nuclease and donor

493 DNA) for SCID and other additional recessive blood disorders. Lastly, since we use authentic

494 CD34$^+$ HSPCs as opposed to iPSCs, cell lines, or cells that no longer maintain their stemness, we

495 believe that the feasibility of translating our gene correction strategy to the clinic will be simpler

496 highlighted by our successful correction of *RAG2*-SCID-patient-derived CD34^{+} HSPCs.

497 **Materials and Methods**

498 **CD34^{+} HSPCs Purification**

499 CD34^{+} HSPCs were isolated from CB samples collected at Sheba Medical Center CB Bank under

500 Institutional Review Board - approved protocols to obtain CB units for research purposes.

501 Informed consent was signed indicating that cord blood specimens that are not suitable for banking

502 will be used for research. Mononuclear cells (MNCs) were separated from fresh CB samples by

503 Lymphoprep™ density gradient medium (STEMCELL Technologies Inc.). Frozen CB samples

504 were thawed and treated with 5,000 U/ml of DNase I (Worthington). Using the CD34^{+} Microbead

505 Kit Ultrapure (Miltenyi Biotec), CD34^{+} HSPCs were purified from the MNCs by CD34 labeling

506 according to the manufacturer's protocol. Enriched CD34^{+} HSPCs were stained with APC anti-

507 human CD34 antibodies (clone 581, Biolegend, San Jose, CA, USA), and sample purity was

508 assessed on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were

509 cryopreserved in CryoStor® CS10 medium (STEMCELL Technologies Inc.) or were cultured for

510 48 hours at a density of 2.5 x 10^5 cells/ml in StemSpan SFEM II (STEMCELL Technologies Inc.)

511 supplemented with Stem Cell Factor (SCF) (100 ng/ml), Thrombopoietin (TPO) (100 ng/ml), Fms-

512 like tyrosine kinase 3 ligand (Flt3-Ligand) (100 ng/ml), Interleukin 6 (IL-6) (100 ng/ml),

513 StemRegenin 1 (SR1) (0.75 mM), UM171 (35 nM) (STEMCELL Technologies Inc.), and 1%

514 Penicillin/Streptomycin (Biological Industries Israel Beit Haemek LTD). Cells were cultured at

515 37°C, 5% CO_2, and 5% O_2. The PB samples were obtained at Sheba Medical Center under

516 Institutional Review Board. Informed consent was signed to ensure that specimens will be used

23

517   only for research purposes. CD34$^+$ HSPCs from PB *RAG1* and *RAG2* SCID patients, and from

518   healthy donors were isolated by negative selection using RossetaSep® (STEMCELL Technologies

519   Inc.). Cells from the fractionated plasma layer were enriched by Lymphoprep™ density gradient

520   medium (STEMCELL Technologies Inc.) and subsequently stained and sorted for APC human

521   CD34$^+$ (clone 581, Biolegend) and FITC human CD45^{low} (clone HI30, BD Pharmingen)

522   expression by Aria III cell sorter (BD Biosciences). Post-sort, cells were stained with PE/Cy7-

523   CD7 (clone:CD7-6B7, BioLegend) and BV421-CD5 (clone: UCHT2, BioLegend) and were

524   analyzed by LSRFortessa™ cell analyzer (BD Biosciences).

rAAV6 Donor DNA Template Design

526   All rAAV6 vector plasmids were cloned using NEBuilder® HiFi DNA Assembly Master Mix (cat

527   # E2621L, New England Biolabs (NEB) Inc.) into the pAAV-MCS plasmid (Agilent Technologies

528   containing inverted terminal repeats (ITRs). Each rAAV6 disruption donor contains a different

529   reporter gene (GFP, tNGFR, or tdTomato) under the control of a SFFV promoter and followed by

530   a BGH polyA sequence. The *RAG2* correction rAAV6 donor was designed and contains diverged

531   *RAG2* cDNA followed by the *RAG2* 3'UTR and a reporter gene (tNGFR) controlled by a PGK

532   promoter and BGH polyA sequence. Each donor DNA was designed with 400bp left and right

533   homology arms flanking the sgRNA cut site. The rAAV6 vectors were produced by Vigene

534   Biosciences in large-scale rAAV6 packaging. See Table S5 for the DNA donor sequences.

CRISPR-Cas9 Genome Targeting and Biallelic Integration

536   *RAG2* and *CCR5* modified sgRNAs, previously described, were synthesized by TriLink

537   BioTechnologies.[37,76] *IL7RA* and *DCLRE1C* modified sgRNAs were synthesized by Integrated

538   DNA Technologies (IDT, Coralville, IA). The 20 bp sgRNA genomic target sequences were as

539 follows: *RAG2*: 5'-UGAGAAGCCUGGCUGAAUUA-3', *CCR5*: 5'-

540 GCAGCAUAGUGAGCCCAGAAG-3', *IL7RA*: 5'-ACAAUUCUAGGUACAACUUU-3', and

541 *DCLRE1C*: 5'-GCGCUAUGAGUUCUUUCGAG-3'. 260 pmol of sgRNA was complexed pre-

542 electroporation with 104 pmol of Alt-R Cas9 protein (IDT, Coralville, IA) forming an RNP

543 complex, at a 1:2.5 molar ratio (Cas9:sgRNA). Electroporation of CD34$^+$ HSPCs was performed

544 with P3 nucleofection solution (Lonza, Basel, Switzerland) in the Lonza Nucleofector 4D

545 (program DZ-100). INDEL quantification was performed via the tracking of INDELs by

546 decomposition (TIDE) analysis platform (available at https://tide.nki.nl/). Electroporated CD34$^+$

547 HSPCs were plated at 4 x 10^5 cells/ml and transduction of disruption *RAG2* donors was performed

548 at a MOI of 6,250 VG/cell for each donor. The MOI for the *RAG2* correction rAAV6 in HD-

549 derived CD34$^+$ HSPCs was 12,500 VG/cell, and for *RAG2*-SCID CD34$^+$ HSPCs the MOI was

550 6,250 or 12,500 VG/cell. For *IL7RA* rAAV6 disruption donors, GFP and tdTomato, MOIs were

551 12,500 VG/cell and 25,000 VG/cell, respectively. For *DCLRE1C* rAAV6 disruption donors, GFP

552 and tdTomato, the MOI was 12,500 VG/cell for each donor. rAAV6 donors were added to the

553 plated cells, within 15 minutes of electroporation. After 24 hours, fresh CD34$^+$ medium was added

554 to form a final concentration of 2.5 x 10^5 cells/ml. 48 hours post-electroporation, cells were

555 collected and prepared for the biallelic enrichment by the Aria III cell sorter (BD Biosciences).

556 Cells were stained with PE human CD34 (clone: 561, BioLegend) antibodies, and when rAAV6

557 tNGFR is transduced APC-tNGFR (clone: ME20.4, BioLegend) staining is performed as well.

558 **INDEL Frequency Quantification via NGS**

559 On-target NGS for the *RAG2* sgRNA was conducted by our group and reported previously and the

560 FASTQ files were downloaded for additional analysis using the CRISPECTOR software tool from

561 the sequence read archive (SRA) under accession number PRJNA628100.[76,83]

562 **Digital Droplet PCR™**

563 The quantification of genomic integration was performed by Digital Droplet PCR™ (ddPCR™,

564 Bio-Rad, Hercules, CA, USA). Genomic DNA was extracted from sorted populations cultured in

565 CD34$^+$ medium and IVTD using GeneJET Genomic DNA Purification Kit (Thermo Fisher

566 Scientific, USA). Each ddPCR reaction mix contains a HEX reference assay detecting copy

567 number input of the *CCRL2* gene to quantify the chromosome 3 input. Separate assays with a set

568 of target-specific primers and a FAM-labeled probe (500 nM and 250 nM, respectively) for each

569 gene were used to detect the locus specific donor integration. The reaction was carried as follows:

570 10 μl ddPCR Supermix for Probes No dUTP (Bio-Rad), 1 μl of each PrimeTime® Standard qPCR

571 Assay (IDT, Coralville, IA), μl of a restriction enzyme mix [1/2 EcoRI-HF® (NEB #R3101, 2/5

572 Nuclease-free water, 1/10 CutSmart Buffer 10X (NEB)], 20 ng genomic template DNA, and

573 supplemented to a total of 20 μl with Nuclease-free water. Droplet samples were prepared

574 according to manufacturer's protocol (Bio-Rad) and 40 μl of the droplets were conveyed to a 96-

575 well plate and amplified in a Bio-Rad PCR thermocycler with the following PCR conditions: 1

576 cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and then 55°C for 3

577 minutes, followed by 1 cycle at 98°C for 10 minutes at a ramp rate of 2.2°C/s. After the PCR, the

578 96-well plate was loaded in the QX200 droplet reader (Bio-Rad). The droplets from each well were

579 analyzed and the concentration of copies/μl of the site-specific donor integration (FAM) and wild-

580 type *CCRL2* (HEX) alleles were calculated using the QuantaSoft analysis software (Bio-Rad).

581 Primers and probes sequences are presented in Table S3.

582 **Estimation of mRNA Levels in Differentiated T Cells**

583 RNA was extracted using Direct-zol™ RNA Miniprep Plus (Zymo Research, cat #R2073) from

584 differentiated T cells obtained on days 14 and 28 of IVTD. cDNA was prepared from 50-150 ng

585 RNA, using Oligo d(T)23 VN- S1327S (NEB), dNTPs 10mM, and M-MuLV Reverse

586 Transcriptase (cat# M0253S, NEB). qRT-PCR reactions were done using TaqMan® Fast

587 Advanced Master Mix (cat# AB-4444557, Thermo Fisher Scientific) and carried out on

588 StepOnePlus™ Real-Time PCR System (cat# 4376600, Thermo Fisher Scientific). PCR

589 conditions were as follows: uracil-N-glycosylase gene (UNG) incubation was 2 minutes at 50°C,

590 polymerase activation was 20 seconds at 95°C, followed by 40 cycles of 1 second at 95°C and 20

591 seconds at 60°C. Primers and probes sequences are presented in Table S4.

592 **_in-vitro_ T-cell Differentiation System**

593 Using StemSpan™ T Cell Generation Kit (STEMCELL Technologies, Inc.), CD34$^+$ HSPCs were

594 cultured in StemSpan™ SFEM II containing Lymphoid Progenitor Expansion Supplement on

595 plates coated with Lymphoid Differentiation Coating Material for 14 days. Subsequently, cells

596 were harvested and re-seeded on coated plates with StemSpan™ T Cell Progenitor Maturation

597 Supplement for an additional 14 days. PB and CB CD34$^+$ HSPCs experiments, were re-seeded on

598 coated plates with StemSpan™ T Cell Progenitor Maturation Supplement at day 28 of culturing

599 and harvested at day 42 of differentiation. Flow cytometry analysis was conducted at each time

600 point of differentiation using the LSRFortessa™ cell analyzer (BD Biosciences). On day 14,

601 collected cells were stained with PE/Cy7-CD7 (clone:CD7-6B7, BioLegend), BV421-CD5 (clone:

602 UCHT2, BioLegend), and CD1a-PE (clone: BL6, Beckman Coulter, USA) or CD1a-APC (clone:

603 BL6, Beckman Coulter, USA) antibodies. On days 28 and 42, collected cells were stained with

604 PE/Cy7-CD4 (clone: RPA-T4, BioLegend), APC-r700-CD8a (clone: RPA-T8, BD Horizon™),

605 and BV421-CD3 (clone: UCHT1, BioLegend) antibodies. BD Horizon™ Fixable Viability Stain

27

606 510 was performed on all the collected cells at each time point. APC-NGFR (clone: ME20.4,

607 BioLegend) staining was conducted on all tNGFR rAAV6 integrated cells. For evaluating the

608 background staining, fluorescence minus one (FMO) + isotype control antibody staining was

609 performed using the following isotypes: PE/Cy7 Mouse IgG2a κ (cat # 400232, BioLegend), BV-

610 421 Mouse IgG1 κ (cat # 400158, BioLegend), PE Mouse IgG1 κ (cat # 400112, BioLegend),

611 PE/Cy7 Mouse IgG1 κ (cat # 400126, BioLegend), APC-R700 Mouse IgG1 κ (cat #564974, BD

612 Horizon™), and APC Mouse IgG1 k (cat # 400122, BioLegend).

613 **Identification of TRG Gene Rearrangements**

614 gDNA from differentiated T cells and CD34$^+$ HSPCs was extracted by the GeneJET Genomic

615 DNA Purification Kit (Thermo Fisher Scientific). TRG rearrangement was assessed by PCR

616 amplification of 12 possible CDR3 clones, using combinations of 4 primers for Vγ and 3 primers

617 for Jγ regions in each reaction (IdentiClone™ TCRG Gene Clonality Assay, Invivoscribe, Inc.).

618 TRG clonality was ran and analyzed on 2% agarose gel. For deep sequencing of the TRG

619 repertoire, the TRG rearranged genomic products were amplified using a single multiplex master

620 mix LymphoTrack® TRG assay (Invivoscribe, Inc.). PCR amplicons were purified and sequenced

621 using the Miseq V2 (500 cycles) kit, 250-bp paired-end reads (Illumina, San Diego, CA). FASTQ

622 files were analyzed by LymphoTrack Software (Invivoscribe, Inc.). LymphoTrack Software

623 unique sequences generated data of each sample that was further analyzed by the IMGT® software,

624 the international ImMunoGeneTics information system® (HighV-QUEST, http://www.imgt.org).

625 The analysis of the incidence of productive and unproductive TRG rearrangements sequences were

626 performed amidst the total sequences and presented visually by TreeMap 2019.3.2 software

627 (Macrofocus GmbH). Unique sequences and CDR3 length analysis were obtained from the total

628 productive sequences. The Shannon's H and Simpson's D diversity indices were calculated by

629 PAST software.[84] The sequencing data were deposited to the Sequence Read Archive (SRA), under

630 accession number: PRJNA838341.

Quantification of TRECs

632 TREC copy numbers were analyzed by qPCR, as previously described.[85] DNA samples were

633 examined in triplicates, 100 ng for each replicate. qPCR reactions were carried out on

634 StepOnePlus™ Sequence Detector System (Applied Biosystems). A standard curve was

635 assembled by utilizing serial dilutions with 10^3-10^6 copies of TREC plasmid. RNase P

636 amplification (TaqMan assay, Applied Biosystems) was conducted for quality control of the DNA

637 TREC amplification.

Acknowledgments

Author Contributions

648 O.I., D.A., O.K., Y.Z., D.B., A.A., and A.L. designed and conducted the experiments, evaluated,

649 and analyzed the data; O.I. and D.B. performed the bioinformatics analyses, with the help and

650   guidance of Y.N.L.; K.B., and A.N. provided cord blood samples and R.S. provided PB samples;

651   K.B., Y.N.L., A.N., and R.S. critically reviewed the experiments and provided important advice;

652   A.H. supervised and conceived the research and planned the experiments and the approaches;

653   A.H., D.A., O.I., and Y.Z. wrote the manuscript, with contributions from all authors.

654   **Declaration of Interests**

655   The authors declare that they have no known competing financial interests or personal

656   relationships that could have appeared to influence the work reported in this paper.

657   **Supplemental Information**

658   Supplemental data. Figures S1–S7; Tables S1-S4.

659   Table S5. rAAV6 donor DNA sequences.

660   **Data Availability Statement**

661   The authors declare that all data supporting the findings of this study are available within the paper

662   and its supplemental information files.

663   **Key Words**

664   CRISPR-Cas9, rAAV6, HSPCs, genome editing, gene regulation, *RAG2*, SCID

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972   transplantation in RAG-2-deficient SCID patients. PLoS One *7*, e30494.
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974

975   **List of Figure Captions**

976   **Figure 1. Biallelic targeting of SCID-related genes via CRISPR-Cas9/rAAV6 in HD-derived**

977   **CD34$^+$ HSPCs. (A)** Schematic representation of *RAG2* disruption donors containing tNGFR or

978   GFP selectable markers driven by an SFFV promoter and two 400 bp arms of homology for direct

979   recombination repair (left homologous arm [LHA]; right homologous arm [RHA]). Successful

980   HDR results in *KI* of the reporter gene approximately 43 bp downstream from the *RAG2* ATG start

981   codon. *DCLRE1C* and *IL7RA* biallelic editing (not shown here) utilize tdTomato and GFP reporter

982   genes under the regulation of a SFFV promoter and a BGH polyA sequence. **(B)** FACS approach

983   for enrichment of biallelic *RAG2* gene-targeted CD34$^+$ HSPCs 2 days after CRISPR-Cas9 and

984   rAAV6 editing. Representative HSPC FACS plots of cells transduced with rAAV6 only (*left*) and

985   cells treated with CRISPR-Cas9 and rAAV6 (*right*) are shown. The double-positive tNGFR$^+$/GFP$^+$

986   population indicative of biallelic integration of two different DNA donors was isolated. Gating

987   determination is based on cells treated with only the rAAV6 vector (left) to compensate for the

988   episomal expression determined to be around 1%. **(C)** GFP and either tNGFR or tdTomato biallelic

989   targeting frequencies (determined as described above in *Figure 1B*) at *CCR5* (N=10), *RAG2*

990   (N=11), *DCLRE1C* (N=3)*,* and *IL7RA* loci (N=3). Data are represented as mean ± SEM.

991

992   **Figure 2. SCID modeling using biallelic *KO* of HD-derived CD34$^+$ HSPCs in a cell-free IVTD**

993   **assay. (A)** Flow cytometry analysis of *IL7RA* biallelic KO in IVTD. *IL7RA* biallelic KO cells

37

994 showed no survival by day 6 of IVTD, based on flow cytometry viability stain out of the double-

995 positive tdTomato$^+$/GFP$^+$ biallelic enriched population. Double-negative (tdTomato$^-$/GFP$^-$) cells

996 showed 100% survival on day 6. Gating was determined by unstained cells. The data represent one

997 of 3 independent experiments. **(B)** Flow cytometry analysis of T-cell developmental progression.

998 *Mock*, *CCR5* biallelic KO, *RAG2* biallelic KO, and *DCLRE1C* biallelic KO cells developed normal

999 expression of early markers of T-cell differentiation (CD7, CD5, and CD1a) upon analysis at 14

1000 days of IVTD. Following 28 days of IVTD, *Mock* and *CCR5* biallelic KO cells expressed mature

1001 T-cell markers: CD4, CD8, and CD3, while *RAG2* biallelic KO and *DCLRE1C* biallelic KO cells

1002 lack CD3 expression. Gating was determined by FMO + isotype controls. The data represent one

1003 of 3-10 independent repeats (*Mock* [day 14 N=10 and day 28 N=7], *CCR5* KO [day 14 N=10 and

1004 day 28 N=6], *RAG2* KO [day 14 N=10 and day 28 N=6], and *DCLRE1C* KO [day 14 N=3 and day

1005 28 N=3]). Cell viability for all samples remained >98% through day 28.

1006

1007 **Figure 3. *RAG2 KI-KO* gene correction simulation strategy in HD-derived CD34$^+$ HSPCs. (A)**

1008 Schematic representation of *RAG2* correction donor containing diverged codon-optimized *RAG2*

1009 cDNA and 3' UTR followed by a tNGFR selectable marker under the regulation of a PGK promoter

1010 and BGH polyA sequence. This is flanked by two 400 bp arms of homology for direct

1011 recombination repair, labeled LHA and RHA, on the left and right side of the CRISPR cut site,

1012 respectively. **(B)** Flow cytometry analysis of T-cell developmental progression of *Mock*, *CCR5*

1013 biallelic KO, *RAG2* biallelic KO, and *RAG2 KI-KO* correction simulation cells. All of the groups

1014 showed normal expression of early markers: CD7, CD5, and CD1a (upon 14 days of IVTD), as

1015 well as mature T-cell markers: CD4 and CD8 (upon 28 days of IVTD). CD3 expression was

1016 observed in *Mock*, *CCR5* biallelic KO, and *RAG2 KI-KO* correction simulation cells, yet not in

1017   *RAG2* biallelic KO cells. (**C**) Summary of CD3 expression by *Mock*, *CCR5* biallelic KO, *RAG2*

1018   biallelic KO, and *RAG2 KI-KO* correction simulation populations at day 28 of IVTD. (N=7, N=6,

1019   N=6, and N=4, respectively). The data for *Mock*, *CCR5* KO, and *RAG2* KO are taken from *Figure*

1020   *S3C* and are presented here for comparison to the *RAG2 KI-KO* correction simulation population.

1021   ** p<0.005 (Mann-Whitney test). Data are represented as mean ± SEM.

1022

1023   **Figure 4. Expression of diverged *RAG2* cDNA in *KI-KO* cells leads to the development of**

1024   **normal TCR repertoire.** (**A**) Representative tree map depiction of the clonal complexity of the

1025   TRG deep-sequencing repertoire of differentiated T cells from *Mock*, *CCR5* biallelic KO, and

1026   *RAG2 KI-KO* correction simulation groups (due to lack of CD3 expression in the *RAG* biallelic

1027   KO population, sequencing and mapping of the repertoire were impossible). Each square

1028   represents a unique V-J pair, and the size of each square represents the clone's frequency. (**B**)

1029   Shannon and Simpson diversity indices of TRG repertoire on days 14 and 28 of *Mock*, *CCR5*

1030   biallelic KO, and *RAG2 KI-KO* correction simulation groups. (N=3). Data are represented as mean

1031   ± SEM.

1032

1033   **Figure 5. *RAG2* gene correction in *RAG2*-SCID patient-derived CD34$^+$ HSPCs facilitates**

1034   **normal T-cell differentiation.** Flow cytometry analysis of T-cell developmental progression, on

1035   days 14, 28, and 42, of the following cell groups: *Mock*, *RAG2*-SCID, unsorted *RAG2*-SCID

1036   correction with 12,500 VG/cell, sorted *RAG2*-SCID correction with 12,500 VG/cell, and sorted

1037   *RAG2*-SCID correction with 6,250 VG/cell. Corrected cells were treated with the same rAAV6

1038   *RAG2* correction donor depicted in *Figure 3A*. Robust CD3 expression was found on day 28 in all

39

1039   *RAG2* correction populations, and on day 42 only in the sorted populations. Due to the scarcity of

1040   patient-derived sample, N=1 for the *RAG2*-SCID samples and the *Mock* plots are from a

1041   representative donor.

1042

1043   **Figure 6. Corrected *RAG2* gene expression in *RAG2*-SCID patient-derived CD34$^+$ HSPCs**

1044   **allows for normal TCR repertoire development. (A)** Representative tree map depiction of the

1045   clonal complexity of the TRG deep-sequencing repertoire of differentiated T cells from *Mock*

1046   and*RAG2*-SCID correction populations, over the 42 days in the IVTD system (days 14, 28, and

1047   42). **(B)** Shannon and Simpson diversity indices of TRG repertoires on days 14, 28, and 42 of

1048   *Mock* and *RAG2*-SCID correction groups (*Mock* [day 14 N=5, day 28 N=4, day 42 N=3]; and

1049   *RAG2*-SCID correction groups [day 14, day 28, and day 42 N=1]). Data are represented as mean
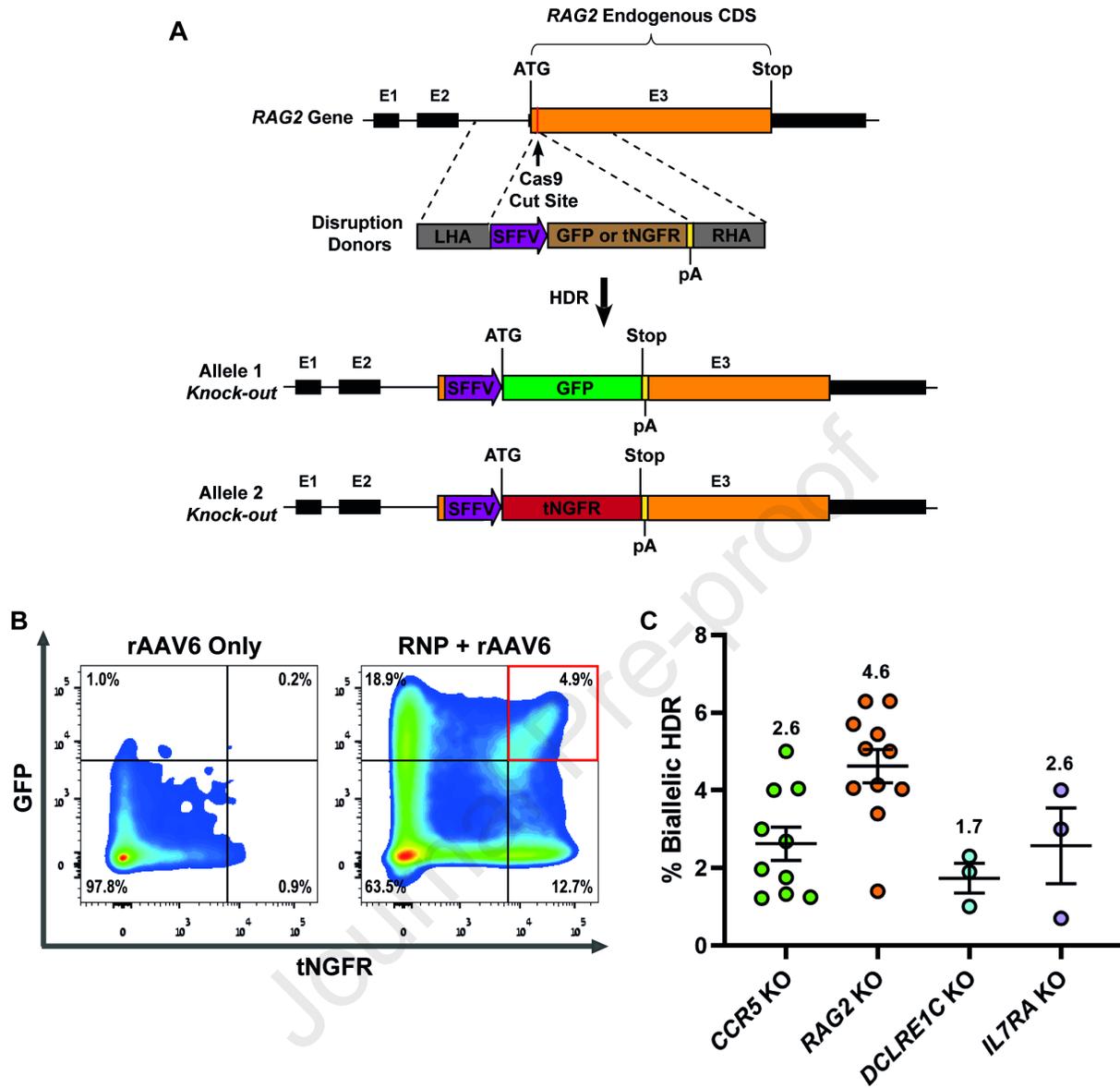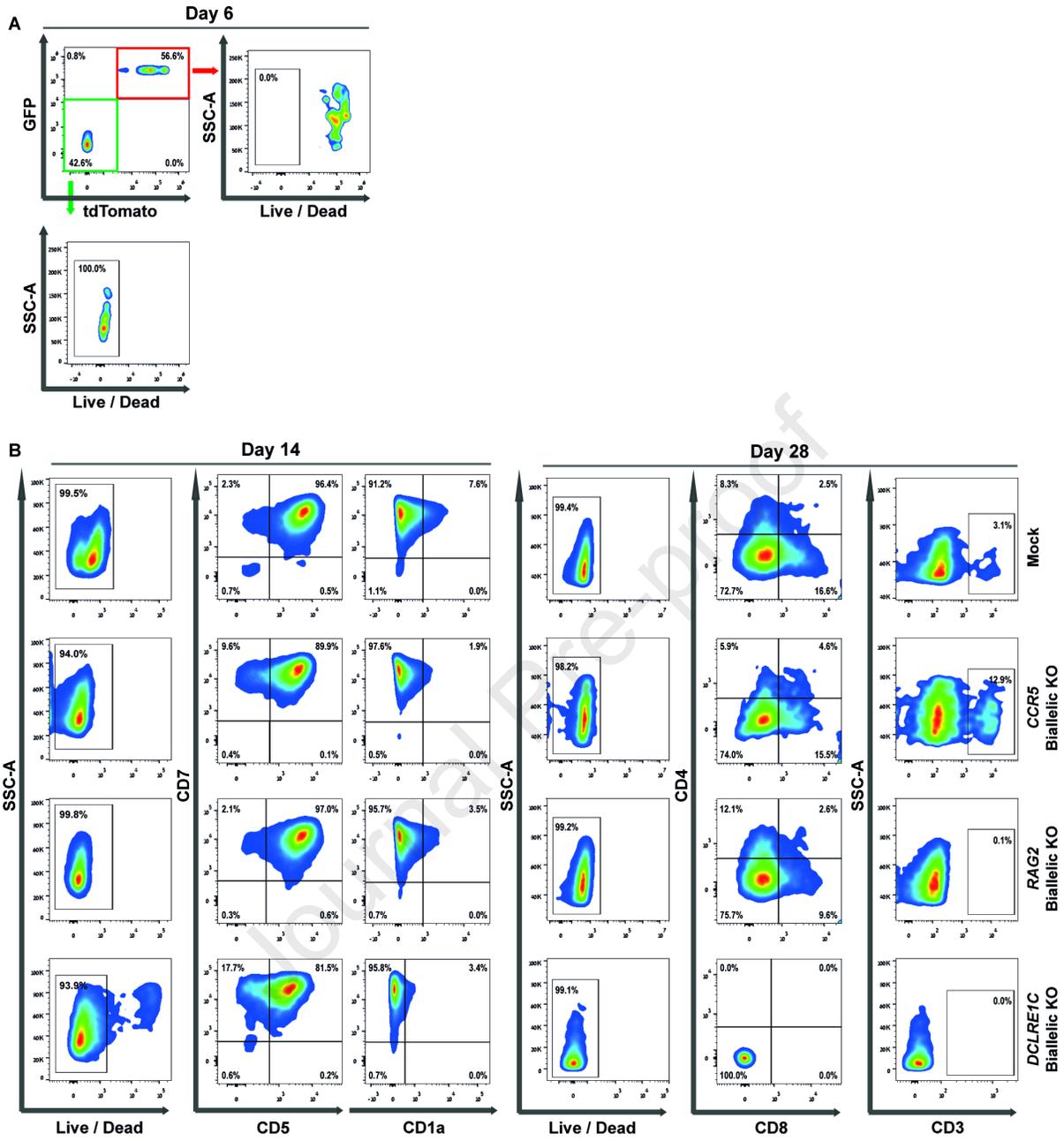
1050   ± SEM.

1051

1052   **Table 1. Quantitative PCR analysis of TRECs in *RAG2*-SCID and *RAG2*-SCID correction**

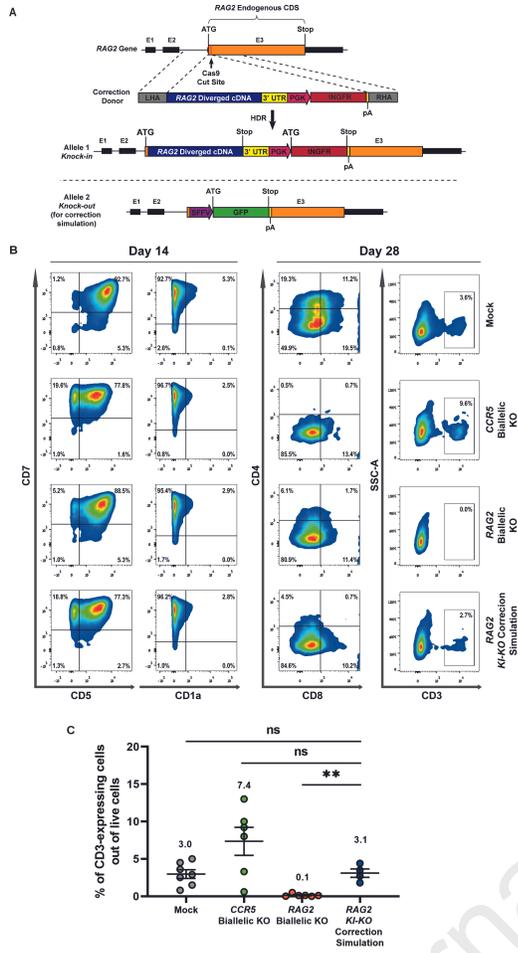1053   **cells over the 42 days in the IVTD system.**

		TREC (copies)	RNase P (average C_T values)
Day 14	*RAG2*-SCID	UN	24.9
	Correction *RAG2*-SCID unsorted	UN	24.8
	Correction *RAG2*-SCID sorted 12,500 VG/cell	UN	24.3

40

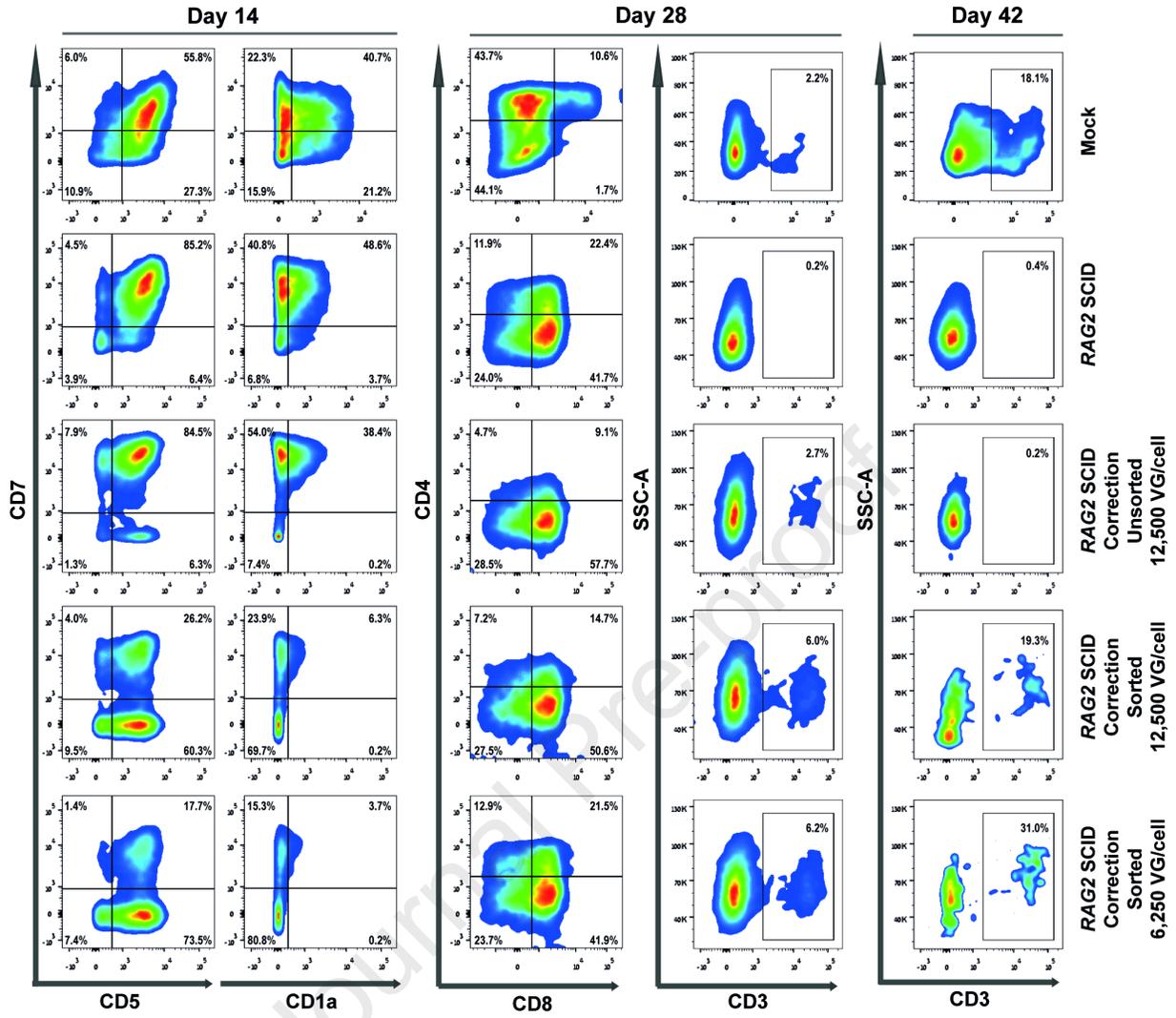|			
	Correction *RAG2*-SCID with sorted 6,250 VG/cell	UN	24.7
Day 28	*RAG2*-SCID	UN	25.1
	Correction *RAG2*-SCID unsorted	UN	24.7
	Correction *RAG2*-SCID sorted 12,500 VG/cell	5.4	25.1
	Correction *RAG2*-SCID sorted 6,250 VG/cell	22.4	24.9
Day 42	*RAG2*-SCID	UN	24.7
	Correction *RAG2*-SCID unsorted	UN	24.9
	Correction *RAG2*-SCID sorted 12,500 VG/cell	8.3	25.6
	Correction *RAG2*-SCID sorted 6,250 VG/cell	31.5	25.1

1054 RNase P was used as an internal control for quality of genomic DNA amplification. TRECs were

1055 detectable solely in the sorted *RAG2*-SCID correction groups on days 28 and 42 in the IVTD

1056 system. UN = undetermined.

A

RAG2 Endogenous CDS

ATG   Stop

E1 E2

RAG2 Gene

E3

↑ Cas9
Cut Site

Disruption
Donors   LHA SFFV GFP or tNGFR RHA

pA

HDR ↓

Allele 1
Knock-out

E1 E2   SFFV   GFP   E3

ATG   Stop

pA

Allele 2
Knock-out

E1 E2   SFFV   tNGFR   E3

ATG   Stop

pA

B

rAAV6 Only

GFP

1.0%   0.2%

97.8%   0.9%

RNP + rAAV6

18.9%   4.9%

63.5%   12.7%

tNGFR

C

% Biallelic HDR

2.6   4.6   1.7   2.6

CCR5 KO RAG2 KO DCLRE1C KO IL7RA KO

A

TRG Day 14 | TRG Day 28

Mock

CCR5 Biallelic KO

RAG2 KI-KO Correction Simulation

B

Mock   *CCR5* Biallelic KO   *RAG2 KI-KO* Correction Simulation

Shannon's H Diversity Index

Day 14: 4.26, 3.48, 3.54
Day 28: 4.90, 4.22, 4.27

Simpson's Diversity Index 1- D

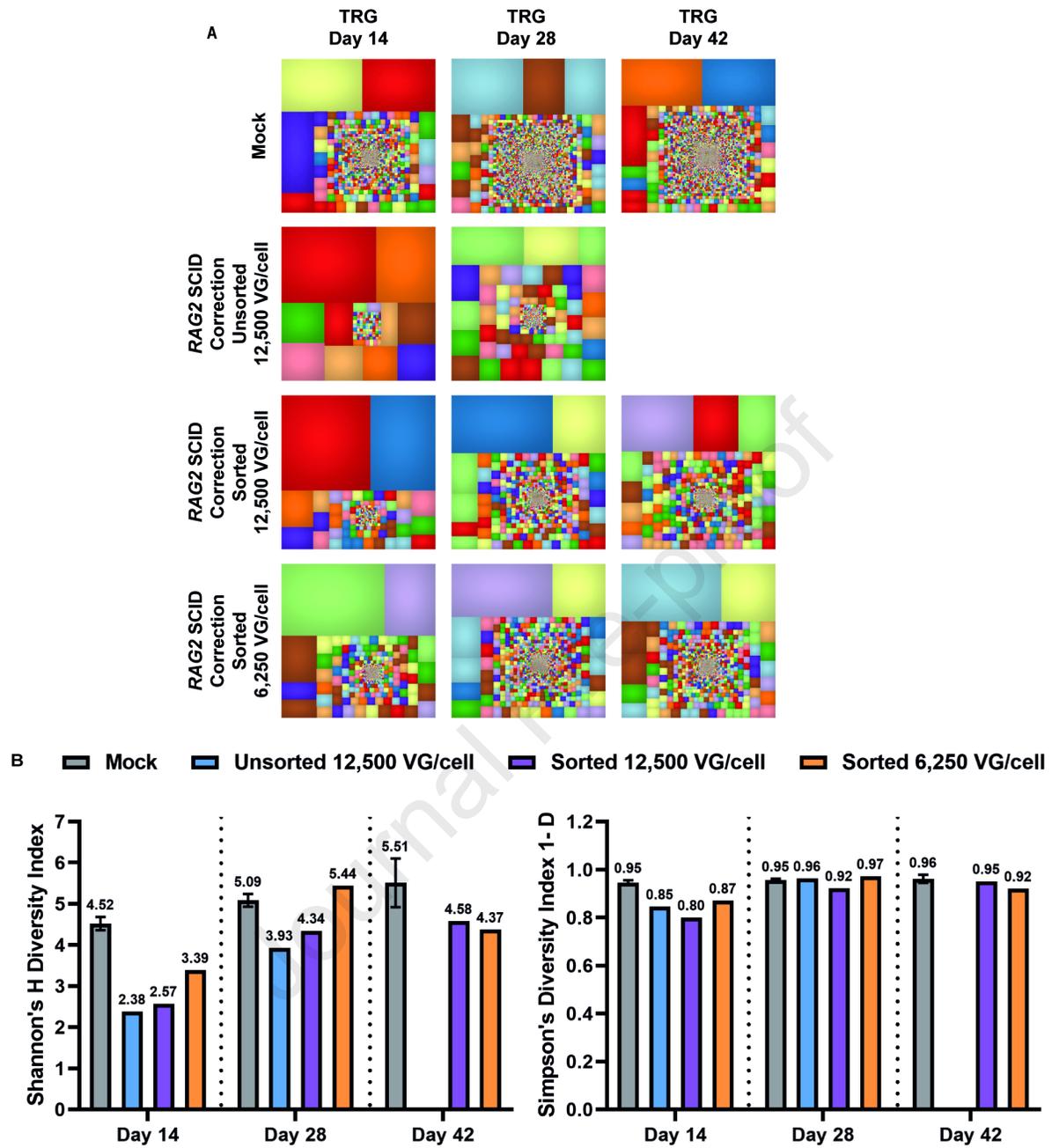Day 14: 0.93, 0.89, 0.91
Day 28: 0.95, 0.92, 0.92

eTOC Synopsis (50 words)

We present a platform using CRISPR-Cas9/rAAV6 editing and an *in vitro* T-cell differentiation system where we model SCID disease and present a proof-of-concept gene therapy for treatment of *RAG2*-SCID. This technique eliminates the need for large quantities of patient-derived samples and can be easily translated to other genetic blood disorders.