Article Title: Genetically Corrected RAG2-SCID Human Hematopoietic Stem Cells Restore V(D)J-Recombinase and Rescue Lymphoid Deficiency

Short Title: Gene correction for RAG2-SCID deficiency

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KEY POINTS

- Human hematopoietic stem cells can be corrected to restore endogenous RAG2 gene expression while preserving durable engraftment potential.
- Gene-corrected RAG2 locus restores V(D)J recombination in RAG2-SCID patient stem cells, promoting T and B-cells’ receptor formation.
ABSTRACT

Recombination-activating genes (RAG1 and RAG2) are critical in lymphoid cell development and function for initiating the V(D)J-recombination process to generate polyclonal lymphocytes with broad antigen-specificity. Clinical manifestations of defective RAG1/2 genes range from immune dysregulation to severe combined immunodeficiencies (SCID), causing life-threatening infections and death early in life in the absence of hematopoietic cell transplantation (HCT). Haploidentical HCT without myeloablative conditioning carries a high risk of graft failure and incomplete immune reconstitution. The RAG complex is only expressed during the G0-G1 phases of the cell cycle at the early stages of T and B cell development, underscoring that a direct gene correction would capture the precise temporal expression of the endogenous gene, is a promising therapeutic approach for RAG1/2 deficiencies. Here, we report a feasibility study using the CRISPR/Cas9-based “universal gene-correction” approach for the RAG2 locus in human hematopoietic stem/progenitor cells (HSPCs) in healthy donors and one RAG2-SCID patient. V(D)J recombinase activity was restored following gene correction of RAG2-SCID-derived HSPCs, resulting in the development of TCR αβ and γδ CD3+ cells and single-positive CD4+ and CD8+ lymphocytes. TCR repertoire analysis indicated a normal distribution of the CDR3 length and preserved usage of distal TRAV genes. We confirmed in vivo rescue of B-cell development, with normal IgM surface expression and a significant decrease in CD56bright NK cells. Together, we provide specificity, toxicity, and efficacy data supporting the development of a gene-correction therapy to benefit all RAG2-deficient patients.

INTRODUCTION

Severe Combined Immunodeficiencies (SCID) are genetic diseases caused by inherited mutations in genes required for T-, B-, and occasionally natural killer (NK)-cell development and function1. Defects in the Recombination Activating Genes 1 and 2 (RAG1/2) are the second most common cause of SCID. RAG1 and RAG2 form a heterotetrameric complex (with two copies of each protein) that initiates the V(D)J recombination process: it mediates DNA cleavage at recombination signal sequences (RSS) flanking the Variable (V), Diversity (D), and Joining (J) coding elements of the T-cell receptor (TCR) and immunoglobulin (Ig) receptor loci. The non-homologous end-joining (NHEJ)-mediated DNA repair of the RAG-induced DNA breaks joins the coding elements that generate functional TCR and Ig molecules.

Over 200 unique pathogenic mutations have been identified to disrupt the RAG1/2 genes causing T- B- NK+ SCID phenotype2,3 and a spectrum of immune dysregulation4-5: Omenn Syndrome (OS), atypical SCID (AS)6, 7 and delayed-onset combined immunodeficiency with granulomas and/or autoimmunity (CID-G/AI)8-10. Patients with severe forms of RAG-deficiency, including SCID, OS, and AS, achieve immune reconstitution with high survival rates (>90%) following allogeneic hematopoietic cell transplantation (allo-HCT) from HLA-matched donors11-14. In contrast, patients who rely on haploidentical HCT without myeloablative conditioning experience high graft failure rates (>75%) or poor lymphoid reconstitution when sustained engraftment is achieved11,12,15. This is due to the host’s genetically defective common lymphoid progenitors (CLPs) occupying the bone marrow and thymus niches16 and preventing donor’s CLPs from establishing lymphopoiesis.

To identify therapeutic options beneficial to all RAG-deficient patients, feasibility studies using lentivirus-based gene therapy were performed in Rag1/2 disease mouse models17-19. These studies showed variable therapeutic outcomes suggesting that overexpressing Rag genes could
not robustly and faithfully recapitulate the levels of regulation and expression necessary to support immune system development and function, thus presenting significant concerns for clinical translation.

Advances in gene-editing technology\textsuperscript{20-22} have allowed gene-correction of human HSPCs. This strategy offers significant therapeutic benefits for diseases like \textit{RAG1}/\textit{RAG2}-deficiency, where the underlying gene relies on strict spatiotemporal gene regulation and expression\textsuperscript{23}. The RAG protein complex is only expressed in G\textsubscript{0}/G\textsubscript{1} in early T and B-cell development, thus demonstrating precise cell cycle and developmental specificity. Cluster Regulatory Interspaced Short Palindromic Repeats-associated Cas9 nuclease (CRISPR/Cas9) is a genome engineering platform\textsuperscript{21-23} that has been successfully applied to primary human cells\textsuperscript{24-26}, including HSPCs\textsuperscript{26-30} to introduce a desired genomic modification through homologous recombination-mediated gene-targeting (HR-GT)\textsuperscript{23}. We have previously demonstrated \textit{in vitro} rescue of the T-cell developmental blockage using CRISPR/Cas9-AAV6 based correction of a patient’s \textit{RAG2} homozygous null mutation (c.831T>A; p.Y277*)\textsuperscript{31}. However, the \textit{in vivo} efficacy of the gene-correction approach remains to be determined. Here, we develop and test the safety and effectiveness of a novel approach for correcting \textit{RAG2}-deficiency in a patient’s own HSPCs. We used the CRISPR gene-correction platform to repair the DNA sequence of a \textit{RAG2}-deficient patient’s stem cells carrying two null compound heterozygous variants causing \textit{RAG2-SCID} and show that it can restore \textit{V(D)J} activity, rescue T- and B-cell development, and correct the immature NK cell phenotype \textit{in vivo}, following long-term engraftment into an immunodeficient NSG-SGM3 mouse model.

METHODS

\textbf{Human CD34\textsuperscript{+} HSPCs.} PB-CD34\textsuperscript{+} cells from a \textit{RAG2-SCID} subject were collected following informed consent (protocol NCT00001405, approved by the NIH IRB as NIH protocol 94-I-0073). Additional information is detailed in supplemental methods.

\textbf{Off-target activity assay.} The top forty-eight putative off-target sites for \textit{RAG2} were identified using COSMID\textsuperscript{32} and tested using rhAmpSeq\textsuperscript{33,34} (Integrated DNA Technologies). Additional information is described in supplemental methods.

\textbf{Colony-forming units (CFU) assay.} Single live HSPCs were sorted and analyzed, as previously described\textsuperscript{27}. Colony genotyping is detailed in supplemental methods.

\textbf{Transplantation of human gene-modified CD34\textsuperscript{+} HSPCs and engraftment assessment.} Rescue experiments, detailed in supplemental methods, were done using frozen PB-HSPCs from one \textit{RAG2-SCID} patient with compound heterozygous null variants (c.296C>A, c.1324C>A; p.P99Q, A442T).

\textbf{T and B-cell receptors repertoire analysis.} High-throughput sequencing of TCR\textalpha{} and TCR\beta{} and immunoglobulin M (IgM) heavy chain (Vh) analysis was performed as described in the supplemental methods. PCR assay for the seven Vh chains was performed as described\textsuperscript{35}.

\textbf{Karyotype analysis.} \textit{RAG2-SCID} patient-derived PB-HSPCs mock or RNP only treated were subjected to G-band karyotyping analysis on 20 cells derived from each condition [WiCell Cytogenetics (Madison, WI, USA)].

\textbf{Statistical analysis.} Statistical analysis was done with Prism 9 (GraphPad software).
Ethics and animal approval statement. All the work described in this study was carried out in compliance with all relevant ethical regulations. The animal studies were reviewed, approved, and monitored by the Stanford University IACUC committee.

Data sharing statement.
Off-target sequencing data have been deposited at the BioProject (ID PRJNA817273) under accession code SAMN26753110 (https://www.ncbi.nlm.nih.gov/biosample/26753110). The authors declare that the data supporting this study are available within the paper and its supplementary information files or from the authors upon reasonable request.

RESULTS

Efficient “universal” gene-targeting strategy of RAG2 to the endogenous locus in human HSPCs.

Two central concepts of the corrective therapeutic approach for RAG2-deficiency are that hematopoietic stem cells (HSCs) and their progeny will preserve the physiological gene regulation necessary to achieve temporal and lineage-specific RAG2 protein expression and activity (Figure 1A-B) and that most of the RAG2-causing mutations, including deletions, will be corrected. We screened and selected a RAG2-specific guide (sgRNA-3, supplemental Figure 1A-B) that when complexed with HiFi Cas9 nuclease achieves a median INDEL frequency (gene-editing mediated NHEJ; GE-NHEJ) of 52.6% (range: 27.1-91.0%) in CB-HSPCs and 68.0% (range: 31.0-81.0%) in PB-HSPCs. The guide’s INDEL profile is characterized by 2-17 bp deletions (supplemental Figure 1C, 1F), which abrogate RAG2 protein expression in vivo (supplemental Figure 1D-E).

We used adeno-associated virus 6 (AAV6) to deliver a codon-optimized (coRAG2) transgene bearing homology arms to the guide’s cut sites (Figure 2A). Absolute quantification showed an average of 40.6 ± 3 (s.e.m, n=13 unique HSPCs donors) alleles carrying the coRAG2 (Figure 2B) or higher (1.4 -1.7-fold increase) when total alleles were quantified at single-cell resolution (Figure 2C, green and hashed bars combined; supplemental Figure 2A-B; supplemental Figure 3A-B): 58.0% [(5,000 MOI, n = 5 donors); 56.0% (2,500 MOI, n = 4 donors); 68.7% (1,250 MOI, n = 2 donors). We estimated the distribution of cells with one (mono-allelic) or two (bi-allelic) alleles targeted as a function of viral multiplicity of infection (MOI) and observed an increase in mono-allelic HR-GT with decreased MOI (Figure 2C): 5,000 MOI 25.6 ± 2.0 (s.e.m.) mono-allelic and 32.4 ± 8.7 (s.e.m.) bi-allelic; 2,500 MOI 31.7 ± 4.5 (s.e.m.) mono-allelic and 24.5 ± 7.4 (s.e.m.) bi-allelic; 1,250 MOI 38.6 ± 14.2 (s.e.m.) mono-allelic and 30.1 ± 4.6 (s.e.m.) bi-allelic. Importantly, correction of a single allele is sufficient to revert the disease phenotype.

We report no significant bias in the coRAG2-modified HSPCs’ (coRAG2-HSPCs) ability to generate all four early myeloid and erythroid hematopoietic progenitor cells compared to control conditions, demonstrating an absence of unintended perturbations in the non-lymphoid lineages (supplemental Figure 2A-C). To address the virus-related toxicity at higher MOIs, we tested p53 binding protein 1 (53BP1) inhibitor (i53) in combination with a lower AAV6 concentration. Treatment with i53 inhibits the NHEJ pathway and tips the balance towards homology direct repair (HDR) pathway. We observed a 1.5-fold higher HR-GT without diminishing the modified cells’ differentiation potential (supplemental Figure 2B). Overall, we show that coRAG2-HSPCs support early hematopoietic progenitor development.

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Specificity and safety of the RAG2 gene-correction approach.

To evaluate the off-target profile of the RAG2 lead guide (sgRNA-3), we used COSMID32, a bioinformatic tool to predict the putative guide’s binding sites, and rhAMP-Seq33,34 to confirm them. Off-target activity at a total of 48 predicted loci was quantified38 in the genome of PB-derived HSPCs from a RAG2null patient carrying compound heterozygous missense mutations (c.[296C>A; 1342C>A]) (Figure 2D). We compared the percent INDELs (GE-NHEJ) detected in cells electroporated with the ribonucleoprotein (RNP) to mock-treated (control) cells. Sites considered true off-target (OT) were those with GE-NHEJ > 0.1% in the RNP-treated condition relative to the mock sample. None of the sites were valid off-targets (Figure 2D; Table 1). While OT-11 and OT-14 are not bona fide off-target sites because there is no increased frequency of INDELs compared to Mock, in Table 1, we annotate these sites for completeness. These results, combined with no chromosomal abnormalities detected by karyotype analyses (supplemental Figure 4A-B), suggest a high degree of specificity by the lead RAG2 guide (sgRNA-3) when complexed with HiFi S.p.Cas932 (RNP).

Cumulatively, we transplanted 63 mice with 28 million gene-modified HPSCs (3.5 million modified cells derived from healthy donors and 24.5 million modified cells derived from RAG2-SCID patients) and analyzed them 18-22 weeks following transplantation. We observed no hematopoietic skewing or detectable gross tumors. In sum, these data offer strong genetic and functional evidence for the safety of our gene-correction strategy for RAG2-deficiency.

Human hematopoietic system is derived from coRAG2-HSPCs.

To determine if gene-modified stem cells can support long-term normal hematopoiesis, we performed xenotransplantation studies into immunodeficient NSG or NSG-SGM3 mice (Figure 3A). We first tested the hematopoietic reconstitution of coRAG2-HSPCs in fresh CB-HSPCs using intra-hepatic (IH) injections into NSG neonates because this system has been shown to best support lymphopoiesis39. Primary human engraftment was measured 18 weeks post-transplant by FACS analysis for human chimerism (% hCD45+ HLA A-B-C+ cells) in the bone marrow (Figure 3B left panel; supplemental Figure 5A) and spleen (Figure 3B right panel; supplemental Figure 5B), as follows: unmodified 28.6% (range: 10.3-39.4%), RNP 15.4% (range: 4.4-41.8%), 15.8% (range: 7.0-40.3%). We observed no difference in the repopulation capacity of HSPCs that underwent HR-GT or GE-NHEJ compared to cells that did not (mock or wild type). Within the hCD45+HLA+ cells, the mean frequency of cells expressing coRAG2 purified from bone marrow (BM) was 19.5 ± 6.5 (s.e.m.; n = 10) and from spleen was 20.7 ± 5.3 (s.e.m., n = 11), as compared to 21.0% bulk targeted alleles before engraftment (Figure 3D -Tx vs +Tx). coRAG2 cDNA was also detected in sorted lymphocytes (CD19+ B cells and CD3+ T cells) from BM (Figure 3D, yellow squares and triangles) and spleen (Figure 3D, red squares and triangles). The quantification only showed a proliferative advantage in T-cells sorted from spleen (53.5 ± 6.1 (s.e.m., n = 4)) with the remaining sorted cells expressing the transgene at levels comparable to bulk (not sorted) human hCD45+HLA+ cells: 16.6 (n = 1; sorted CD19+ from BM); 27.9 ± 9.9 (s.e.m., n = 3 sorted CD19+ from spleen); 53.6 ± 6.2 (s.e.m., n = 4; sorted CD3+ from BM); 34.4 ± 11.5 (s.e.m., n = 5; sorted CD3+ from spleen), (Figure 3D).

To assess the efficacy of the gene-correction strategy in a cell source of similar derivation as that of RAG2-deficient patients, we repeated the xenotransplantation studies using frozen PB-HSPCs derived from healthy donors. An equal number of unmodified (wild type and mock) and HR-GT PB-HSPCs treated (+i53) or not (-i53) were transplanted intra-femoral into sub-lethally irradiated 8 weeks-old NSG-SGM3 mice (Figure 3A, 3C). Twenty-two weeks post-
transplantation, the median frequencies of hCD45\(^+\)HLA\(^+\) cells in BM were as follows: unmodified 6.8% (range: 4.0-14.2); HR-GT (+i53) 1.12% (range: 0.2-25.0); HR-GT (+i53) 5.9% (range: 3.6-11.4). We observed no statistical difference in the repopulation capacity of cells that underwent HR-GT compared to cells that did not, nor among HR-GT cells treated or not with p53 inhibitor (i53) in BM (Figure 3C left panel; supplemental Figure 6A-B) or SP (Figure 3C right panel). Human cells carrying the coRAG2 cDNA were detected among sorted mature B cells: CD19\(^+\) [-i53: 11.5 ± 4.1 (s.e.m., n = 4); +i53: 18.8 ± 9.6 (s.e.m., n = 5)] and CD20\(^+\)IgM\(^+\) [-i53: 10.9 ± 0.4 (s.e.m., n = 2); +i53: 9.9 ± 5.2 (s.e.m., n = 4)], providing the first line of evidence that coRAG2 cDNA drives productive V(D)J recombination reactions (Figure 3E).

To assess the HR-GT cells’ ability to differentiate into multiple hematopoietic lineages, we used human CD3, CD19, CD14, and CD235a markers to identify lymphoid (B, T), myeloid (monocytes), and erythroid population, respectively. Compared to unmodified (mock) cells, coRAG2-modified cells showed no statistical difference skewing towards a particular lineage in BM (supplemental Figure 5C left panel), in circulating cells in the peripheral blood (supplemental Figure 5C right panel) or spleen (supplemental Figure 5D left panel; supplemental Fig. 7). Together, these data demonstrate that the coRAG2 transgene can support durable hematopoiesis and drive lymphoid development, in vivo, without lineage skewing.

**In vivo B-cell lineage reconstitution from gene-corrected RAG2\(^{null}\) HSPCs.**

We performed xenotransplantation experiments into NSG-SGM3 immunodeficient mice to assess the phenotypic correction of the B-cell developmental defect in RAG2\(^{null}\) patient HSPCs (RAG2\(^{null}\) HSPCs). Four different non-GMP (good manufacturing practice) grade AAV6 virus batches carrying the coRAG2 transgene (AAV6\(^{\text{coRAG2}}\)) were purified and tested (5,000 MOI) on frozen RAG2\(^{null}\) HSPCs (Figure 4A). Total HR-GT (green bars) or GE-NHEJ (grey bars) generated by three different virus batches in the absence of i53 treatment showed a mean % INDEL of 54.9 ± 3.7 (s.e.m.), mean %HR of 29.9 ± 5.1 (s.e.m.), respectively, with 15.2% alleles (± 5.6 s.e.m.), remaining unmodified. These viruses, however, decreased cellular viability, diminished CFU, and long-term engraftment potential of gene-corrected RAG2\(^{null}\) HSPCs (data not shown). We purified a fourth AAV6\(^{\text{coRAG2}}\) batch and used it to transduce the patient cells at lower MOI (2,500) and in the presence of p53 inhibitor (+i53). Under these optimized conditions, we achieved a 19.2% HR-GT and 37.0% GE-NHEJ with no observed toxicity.

Two different cell doses (5.0 x 10\(^5\) and 1.0 x 10\(^6\)) of HR-GT RAG2\(^{null}\) HSPCs were transplanted intra-femoral into NSG-SGM3 mice and unmodified controls (mutant and wild type cells). Human chimerism (hCD45\(^+\)HLA\(^+\)) was quantified at 22-weeks post-transplant in the BM and resulted as follows: 4.4% (range: 0.9-21.7; Tx = 1.0 x 10\(^6\) HR-GT) in mice transplanted (Tx) with 1.0 x 10\(^6\) HR-GT; 4.1% (range: 0.9-12.3; Tx = 5.0 x 10\(^5\) HR-GT); 9.6% (range: 7.78-12.5; Tx = 1.0 x 10\(^6\) RAG2\(^{null}\) HSPCs); and 43.5% (range: 16.4-67.3; 1.0 x 10\(^6\) healthy donor HSPCs) (Figure 4B). A two-fold decrease in human chimerism was observed between the healthy donor and HR-GT RAG2\(^{null}\) HSPCs, regardless of the cell dose transplanted. We observed no difference in the level of engraftment between the healthy donor and unmodified RAG2\(^{null}\) HSPCs (Figure 4B white and grey circles), suggesting an increased sensitivity of RAG2\(^{null}\) HSPCs to the gene-correction reagents. Transplanting NSG-SGM3 mice with a lower dose of HR-GT RAG2\(^{null}\) HSPCs generated a statistically significant increase in the fractions of small pre-B II (light blue bar) cells, immature (white bar), and mature (burgundy bar) cells when compared to uncorrected mutant cells (Figure 4C-D). The triple-positive CD19\(^+\)CD20\(^+\)IgM\(^+\) mature B cells derived from HR-GT RAG2\(^{null}\) HSPCs (Figure 4E, red/white and black squares) developed at comparable
levels to healthy donor engrafted cells (Figure 4E, white squares). When sorted (supplemental Figure 8) and analyzed for IgM immunoglobulin heavy chain repertoire, the HR-GT RAG2null HSPCs engrafted with a higher cell dose (1.0 x 10⁶) showed expression of all 7-immunoglobulin heavy chain Variable gene families (Figure 4F, black bottom panel). The lower cell dose (5.0 x 10⁵) expressed 5 out of 7 heavy chain variable regions in the (Figure 4F, middle red panel). Together, this data shows that up to 84.8% of RAG2null alleles are gene-modified, of which up to 29.9% alleles are corrected and up to 54.9% alleles are disrupted. Furthermore, HR-GT RAG2null HPSCs can restore the B-cell development block.

**Transplantation of RAG2null gene-corrected HSPCs rescues T-cell development in NSG-SGM3 mice.**

Since we observed correction in the B-cell compartment, we also assessed the potential of the HR-GT RAG2null HSPCs in rescuing the T-cell developmental defect. Despite lower human chimerism in the spleen (Figure 4B, 5A), corrected RAG2null HSPCs gave rise to CD3+ T-cells at levels comparable to healthy donor-derived T-cells (in 3 mice) (Figure 5B-C). The derived CD3+ T-cells express αβ and γδ T-cell receptors (TCR) and gave rise to single-positive CD4+ and CD8+ T-cells, demonstrating that CLPs derived from corrected RAG2null HSPCs undergo normal lymphopoiesis and achieve maturation.

We used high-throughput sequencing (HTS) to quantify the diversity and composition of the TCR repertoire and determine the clonality at the complementary determining region 3 (CDR3) level. CDR3 is the region that promotes TCR-peptide MHC complex formation. Using a treemap profile analysis of the CDR3 of sorted HR-GT RAG2null derived CD3+ T cells and healthy donor control, we determined an oligoclonal pattern of TCR CDR3 specificity, with Shannon’s H entropy index of 6.4 and 5.2, respectively (Figure 5E-F). Virtual spectratyping analysis revealed similar CDR3 lengths of productive rearrangements in bulk T-cells from HR-GT RAG2null and healthy donor control (supplemental Figure 9).

It has been reported that mature T cells derived from RAG2 patients carrying hypomorphic mutations have an abnormal TRA repertoire composition defined by a markedly reduced usage of the most 5’ TRAV and 3’ TRAJ genes⁴⁰. HTS of TCR rearrangements at the TCRα/TCRδ (TRA/TRD) loci were demonstrated. HR-GT showed a similar rearrangement pattern of TRAV and TRAJ gene usage in RAG2null-derived CD3+ T cells and healthy donor-derived CD3+ T-cells (supplemental Figure 10A-B). Analysis of TRB rearrangements showed comparable patterns in HR-GT RAG2null derived CD3+ T cells to healthy donor control (supplemental Figure 10C-D). Together, the data demonstrate that the function of the RAG complex is restored following endogenous genomic integration, promoting T-cell development with a normal VJ pairing in gene-edited cells, supporting the previously published in vitro results³¹.

**Correction of the immature NK CD56bright phenotype in RAG2null patient**

It was reported that RAG-deficient mice display an increased proportion of natural killer (NK) cells that have an immature phenotype, with heightened cytotoxic activity and limited survival potential⁴¹. These observations were also reported in patients with SCID caused by defects in RAG and NHEJ genes⁴². The FACS analysis of human chimerism in mouse BM confirmed that NK cells derived from RAG2null HSPCs expressed significantly higher CD56brightCD16- cell surface markers than healthy donors (supplemental Figure 11A). Following gene-correction, the CD56brightCD16− population was reduced, although not to the
same levels observed in healthy donors (supplemental Figure 11B). These results show that gene-corrected HSPCs rescue both the adaptive and innate immune defects.

**DISCUSSION**

We describe a proof-of-concept study for a novel therapy for RAG2 deficiencies. Through a broad and diverse set of preclinical studies performed in vitro, in vivo, in immunodeficient mice, using healthy donor and RAG2null patient-derived HSPCs, we have demonstrated the application of CRISPR/Cas9-AAV6 platform in correcting a RAG2null patient’s hematopoietic stem cells to restore the V(D)J activity in support of lymphopoiesis. For primary immunodeficiencies (PIDs) like RAG2-SCID, where a strict spatiotemporal level of gene expression and regulation is required for normal immune development and function3,43, a gene-correction approach has several safety and therapeutic benefits when compared to semi-random gene addition strategy: (1) it restricts the gene activity to the lymphoid lineage and the G0-G1 cell cycle phase [safety], (2) it circumvents the risk of genotoxicity from ubiquitous RAG activity [safety], (3) it allows for physiological gene expression [efficacy], and (4) it is easily adaptable to correct other forms of RAG2 deficiencies, such as OS, AS and CID-G/AI for which disrupting and correcting the mutant alleles both alleviate disease burden and achieves cure [efficacy].

While lentiviral (LV) gene therapy is being developed for RAG1-SCID44 (a closely but genetically distinct from RAG2-SCID) and the safety and efficacy of delivering the RAG1 nuclease in HSPCs are being tested (NCT04797260), no alternative therapies, other than allo-HSCT are currently available for patients with RAG2 deficiencies. We report the first feasibility study of gene-correction at the RAG2 locus in human HSCs derived from healthy and RAG2null patients to assess the safety and efficacy of this strategy. Allo-HCT for RAG1/2-deficient patients shows clinical improvement if a minimum of 20% donor chimerism is achieved11, an allele correction threshold that we have achieved and surpassed. We demonstrate that coRAG2-HSPCs can support durable hematopoiesis (18-20 weeks in immunodeficient mice), with only a 2-fold decrease in engraftment potential of corrected RAG2null patient’ cells. This decrease could be explained by a heightened sensitivity to non-GMP grade virus, an observation confirmed by the CFU assay (data not shown), or a reduced regenerative potential of patients’ cells. In support of the latter, we show that the unmodified RAG2null HSPCs have a 4.5-fold lower engraftment capacity than healthy-donor HSPCs controls. Alternatively, in the absence of RAG2 expression, patient cells with lower DNA repair capacity are not selected against, resulting in an overall decrease in the fitness of the hematopoietic progenitors (HPCs), necessary for supporting HSCs engraftment after conditioning42.

We demonstrate in vivo rescue of B-cell development following gene-correction, but no detectable IgG heavy chain transcripts in the corrected and wild type sorted mature B cells, possibly due to the absence of a foreign antigen challenge to stimulate class switch recombination (CSR) in immunodeficient mice housed under pathogen-free conditions. When translating results from humanized mouse models to clinical settings, we acknowledge the microbiome differences between mice and humans and the critical role it plays in shaping the immune system’s development and function45. Humanized mice models have an underdeveloped immune system, which could underestimate the potency of the therapeutic product we are testing.

Gene-targeted RAG2null HSPCs allowed correction of the peripheral T cell compartment, as documented in the spleen of transplanted mice. This was especially evident when a higher cell dose was transplanted into mice. It is possible that with lower dose cells, fewer corrected early
thymic progenitors (ETP) survive the mouse thymic selection in support of lymphopoiesis. Delivering a higher dose of corrected cells could compensate for this limitation. We show that the ETP that survived the thymic selection developed into mature SP CD4+ and CD8+ T lymphocytes expressing αβ γδ T-cell receptors that can propagate developmental signals, supporting single-positive CD4+ and CD8+ T-cells. The newly derived receptors have an oligoclonal rearrangement pattern at the TRA/TRD locus with a similar pattern in usage of TRAV and TRAJ genes to those derived from healthy controls. This contrasts with the previous findings, where we demonstrated polyclonal TCR repertoire in T-cells, derived in vitro, from gene-corrected RAG2null HSPCs31. It is reasonable to assume that of the gene-corrected and transplanted HSPCs clones, only a limited number repopulated the mouse thymus, thus restricting the number of stem cells that can contribute to the T-cell lineage development. This is supported by a recent study whereby using lentivirus cellular barcoding and purified human BM- or CB-derived HSPCs transplanted into NSG mice, less than 10 HSC clones contributed to the repopulation of the mouse thymus microenvironment46. Although the study further showed that even with a limited clonal contribution to the T-cell lineage, a diverse and polyclonal TCR repertoire can be generated46, we reason that the fresh CB- or BM-derived HSPCs used in their study holds greater lymphoid potential than frozen PB healthy or patient-derived HPSCs used in the study.

In the absence of myeloablative conditioning, RAG-deficient patients show a high graft failure rate, and those who engraft often have poor lymphoid reconstitution11. It has been speculated that genetically defective early CLPs occupy the bone marrow and thymic niches16. In the absence of niche clearing, the donor functional CLPs must compete with the endogenous genetically defective progenitors for space to engraft and develop. Moreover, we and others have recently shown that CD34+ cells from RAGnull patients can differentiate up to CD4+CD8+ double-positive (DP) cells in vitro47,48, suggesting that after HCT, competition between host and donor-derived cells might exist up to the DP stage. Additionally, it has been reported that the RAG-patient derived NK cells have higher perforin expression and increased degranulation potential41,49. These cytotoxic NK cells can attack the graft in the absence of conditioning. We show that there is a shift in the predominant NK population following gene-correction from a CD56bright CD16- to a CD56dim CD16+. We thus propose that an autologous HCT using gene-corrected HPSCs, combined with reduced intensity myelosuppressive conditioning alone or in combination with antibody-based depletion, such as anti-CD45 50,51, would conceivably provide therapeutic benefits by eliminating the competitive environment in the hematopoietic niches and assuring graft survival through correction of the cytotoxic function of the derived NK cells.

Finally, using i53 treatment resulted in ~1.5-fold higher gene-targeting (HR-GT) without altering engraftment potential. We anticipate that a GMP-grade virus would decrease cellular toxicity and eliminate the use of i53, simplifying the path to clinical translation. Our studies provide the rationale for further testing our strategy to correct the immune dysregulations in RAG2-deficient patients.

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AUTHORSHIP

M.P.-D. and C.L.G, designed and performed most of the experiments. Y.G.N carried out the IF engraftments with RAG2-SCID patient cells and, together with R.M., provided thoughtful comments on the manuscript. T.K. carried out the BCR analysis. O.M.D., B.P., M.B., and F.P. carried out TCR analysis. S.V. assisted with mouse studies and provided thoughtful comments on the manuscript. H.G. assisted with AAV6 virus production and testing. N.B., G.L., and C.V. carried out the off-target analysis, designed and purified i53 protein. A.S. and T.M. obtained and purified CD34+ HSPCs from donated cord blood. H.L.M obtained patient cells and provided thoughtful comments on the manuscript. L.D.N. and M.H.P. supervised the work, assisted with experimental design, and provided thoughtful feedback and discussion of the manuscript. M.P.-D., L.D.N., and M.H.P are responsible for all the data.

CONFLICT OF INTEREST STATEMENTS

Disclosure: M.H.P serves on the SAB for Allogene Tx and is co-founder and Board of Director for Graphite Bio.; R.M. is on the Board of Directors of Beyond Spring Inc, the Scientific Advisory Boards of Zenshine Pharmaceuticals, and Kodikaz Therapeutics Solutions Inc.; None of these companies had input in the design, execution, interpretation, or publication of the work in this manuscript. Other authors declare no competing interests.

REFERENCES


Recognition, Binding, and Cleavage

**Disease State**

- Defective RAG2 gene expression and function
- No T-cell and Ig Receptors rearrangement
- Absence of T- and B-cells
- Abnormal hematopoiesis
  - No lymphoid lineage
  - Immature NK cells phenotype

**Gene Corrected Healthy State**

- CRISPR/Cas9-AAV6 gene correction of RAG2-SCID Patient’s Stem Cells
- Productive V(D)J rearrangement
- High Receptor Clonality
- Normal hematopoiesis
  - Rescue of T-/B- cells lineages
  - Mature NK cells phenotype

**Severe Combined Immunodeficiency (SCID)**

- Disease State Gene Corrected
  - Healthy State

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Figure 1. Schematics of the hematopoietic developmental defect in RAG2-SCID patients. (A) Overview of the human T-cell (top panel) and (B) B-cell (bottom panel) developmental stages in thymus and bone marrow from common lymphoid progenitor (CLP). Dotted red squares mark the developmental block in RAG2-SCID patients. RAG2, recombination activating gene 2; DN, double negative stage; ISP, immature single positive stage; EDP, early double-positive stage; DP, double-positive stage.
Figure 2. Efficacy and safety of genome targeting the RAG2 locus using “universal” correction strategy.  
(A) Schematics of gene-targeted integration of codon-optimized RAG2 cDNA (coRAG2) and expression cassette. coRAG2 sequence is under the control of the endogenous promoter. (B) Percent coRAG2 gene-targeted (coRAG2-GT) in healthy donor (HD)-purified hematopoietic stem cells (HSPCs) from fresh cord blood (CB) and frozen peripheral blood (PB). Each circle represents a unique human HSPC donor. Genome targeted integration was quantified by digital-droplet PCR (ddPCR). (C) Frequency of cells with one (mono-allelic) or two (bi-allelic) alleles targeted, as a function of virus’ MOI. Analysis was done on single cells sorted onto methylcellulose plates (n = 5 - 5,000 MOI; n = 4 - 2,500 MOI; n = 2 - 1,250 MOI). Bars ± s.e.m. (D) Off-target analysis using RAG2-SCID (c.296C>A; c.1342C>A) patient-derived HSPCs. Next-generation sequencing (NGS) of 48 COSMID predicted off-target (OT) sites in edited-only (RNP-sgRNA guide #3 and HiFi Cas9 nuclease) or electroporated-only (mock, nucleofected without RNP). Shown INDELs reads for on target (RAG2 gene, white circle), OT sites below the limit of detection (grey circles), and above the limit of detection (green circles).
Table 1. Description of off-target (OT) sites above limit of detection (>0.1)

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>% INDELs</th>
<th>Mock RNP Feature</th>
<th>Distance Gene Type</th>
</tr>
</thead>
</table>

Mismatched bases from RAG2 guide sequence are shown in red and the PAM sequences are shown in bold. When normalized to mock, all % INDELs are < 0.1 using high fidelity (HiFi) Cas9 protein. Distance from transcription start site is shown in base pairs. ncRNA: non-coding RNA.
Figure 3. In vivo lymphoid lineage development from coRAG2-targeted healthy donor-derived HSPCs.
(A) Schematic of engraftment protocol of human HSPCs into NSG immunodeficient mice and secondary analysis.
(B) Percent human cell chimerism (CD45+ HLA A-B-C+ double-positive cells) in bone marrow (BM) and spleen (SP) of mice 22-weeks post-transplant with CB (intra-hepatic injection) or (C) PB-derived HSPCs (intra-femoral injection) edited with RAG2-sgRNA guide 3 (RNP, light grey circles in panel B) or targeted with coRAG2 cassette (GT, yellow and red circles in panel B, dark grey and red circles in panel C). Each dot represents an individual mouse; Panel B: WT (n=10), RNP (n=5), GT (n=11); Panel C: WT (n=5), GT-i53 (n=5), GT+i53 (n=6). Bars = median (D) Percent coRAG2-GT by ddPCR before (-Tx) and after (+Tx) engraftment of CB and (E) PB-derived CD34+ HSPCs into NSG mice and FACS sort, as marked. Bars: mean ± s.e.m.; -i53, without p53 inhibitor; +i53, with p53 inhibitor; stats. One-Way ANOVA, non-parametric test, Kruskal-Wallis test, Dunn’s multiple comparisons test.
Figure 4. Ex vivo gene-targeted RAG2null HSPCs correct in vivo B-cell developmental block.

(A) Percent of total genome editing (INDELs and HR) in RAG2null patient-derived HSPCs, using four different AAV6 production lots. AAV6 lot D (asterisk marked) was used for all subsequent engraftment studies.

(B) Percent human cells (CD45+ HLA A-B-C+) engrafted in BM after transplanting (22 weeks post-Tx) 0.5 x 10^6 un-corrected RAG2null patient-derived HSPCs (n=4 mice, grey circles), 0.5 x 10^6 coRAG2-GT HSPCs (n=15 mice, half red circles) or 1.0 x 10^6 coRAG2-GT HSPCs (n=15 mice, black circles). Healthy donor (HD) HSPCs were used as control (n=6, white circles). RAG2null patient genotype: c.296C>A; c.1342C>A.

(C) FACS-based quantification is shown in (D) of large Pre-B I, small Pre-B II, immature, and mature B cells derived from coRAG2-GT HSPCs. Each population is graphed as a percent of total B cells. Bars: mean ± s.e.m. (D) Representative FACS plots of B-cell developmental stages derived from a healthy donor (left panel), RAG2null patient (middle panel), and coRAG2-GT RAG2null HSPCs. FACS-based quantification of percent cells in each developmental stage is shown.

(E) FACS-based quantification of CD19+CD20+IgM+ triple-positive B cells derived from each condition tested.

(F) PCR-based sequencing of immunoglobulin M (IgM) heavy chain (Vh) families from sorted triple-positive B-cells.
Figure 5. Correction of RAG2 gene function in RAG2null HSPCs restores V(D)J activity and normal T-cells development. (A) Percent human cells (CD45+ HLA A-B-C+) detected in spleen (SP) (22-weeks post-Tx) with coRAG2-GT RAG2null HSPC (0.5 x 10⁶, half-colored red circles or 1.0 x 10⁶, full-colored red circles). HD (white circles) and un-corrected RAG-/- (grey circles) HSPCs-derived human cells were engrafted and analyzed in parallel. (B) Human CD3+ T-cells detected in the spleen (SP) and (C) bone marrow (BM) derived from coRAG2-GT RAG2null HSPCs. (D) FACS plots showing T-cells analysis derived from 3 mice with the highest level of human CD3+ cells (dotted squares). Functional V(D)J rearrangement is demonstrated by the presence of CD3+ TCR α/β, CD3+ TCR γ/δ, and single-positive CD4+ and CD8+ derived from coRAG2-GT RAG2null HSPCs. (E) Treemap diversity analysis for TCRA/TCRD CDR3 sequences from sorted CD3+ cells from (C). Each circle is a unique CDR3 sequence, and the size of the circle represents the frequency out of the total number of reads. (F) Shannon H index score quantification of CDR3 sequence from (E-F), showing oligoclonal repertoire. Shannon index score of 8 indicates polyclonal repertoire. Stats. One-way ANOVA, nonparametric, Kruskal-Wallis test. Median plotted in A-C.