Targeted gene addition in human CD34+ hematopoietic cells for correction of X-linked chronic granulomatous disease

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Gene therapy with genetically modified human CD34+ hematopoietic stem and progenitor cells (HSPCs) may be safer using targeted integration (TI) of transgenes into a genomic ‘safe harbor’ site rather than random viral integration. We demonstrate that temporally optimized delivery of zinc finger nuclease mRNA via electroporation and adeno-associated virus (AAV) delivery of donor constructs in human HSPCs approaches clinically relevant levels of TI into the AAVS1 safe harbor locus. Up to 58% Venus+ HSPCs with 6–16% human cell marking were observed following engraftment into mice. In HSPCs from patients with X-linked chronic granulomatous disease (X-CGD), caused by mutations in the gp91phox subunit of the NADPH oxidase, TI of a gp91phox transgene into the AAVS1 resulted in ~15% gp91phox expression and increased NADPH oxidase activity in ex vivo-derived neutrophils. In mice transplanted with corrected HSPCs, 4–11% of human cells in the bone marrow expressed gp91phox. This method for TI into AAVS1 may be broadly applicable to correction of other monogenic diseases.

Ex vivo transfer of therapeutic transgenes into autologous HSPCs using viral vectors has benefited patients with rare genetic conditions, such as the leukodystrophies1 and immunodeficiencies2,3, but has been associated with oncogenesis4,5 due to random genomic integration. Genome editing with engineered nucleases6 permits targeted rather than random genetic manipulation and is being tested in clinical trials of autologous genome-edited T cells7. For treatment of monogenic recessive diseases, insertion of transgenes at a safe harbor locus could substantially reduce the risk of insertional mutagenesis. The AAVS1 locus8 on chromosome 19 has been shown to be a safe harbor for several transgenes in a broad range of primary and transformed cell types9. Here we describe gene addition to the AAVS1 locus in human HSPCs using a zinc finger nuclease (ZFN) pair and AAV6 donor. Genome editing with engineered nucleases begins with a sequence-specific double-strand DNA break (DSB) that is most commonly repaired by nonhomologous end-joining (NHEJ), which yields either an error-free repair or small insertions and deletions (indels) at the break site10. Alternatively, the DSB undergoes homology-directed repair (HDR) using the sister chromatid or donor sequence as a repair template, enabling targeted gene correction11 or gene addition12 if a DNA substrate that bears a small genetic change or a longer fragment flanked by homology arms to the nuclease target site is provided.

Gene correction and gene addition are usually less efficient than gene disruption, particularly in quiescent human CD34+ hematopoietic stem cells (HSCs), in part due to cell-cycle-imposed control on the DSB-repair pathway choice9. With the goal of developing a clinically relevant strategy for gene addition at a safe harbor locus in human CD34+ HSPCs, we focused first on identifying the optimal ZFN design. We used a ZFN pair targeting the AAVS1 locus combined with a promoterless donor construct in which the Venus fluorescent marker cDNA is preceded by a splice acceptor and a ribosome stuttering signal, and flanked by regions homologous to the AAVS1 locus (SA-2A-Venus) (Fig. 1). In this genome editing approach, following HDR-driven targeted addition of cDNA to the AAVS1 locus, the cell becomes marker-positive because the nuclelease target site at the AAVS1 locus lies within the first intron downstream of exon 1 of the PPP1R12C gene. PPP1R12C gene promoter capture is required to drive expression of the Venus coding sequence using the promoterless SA-2A-Venus donor. Based on a previously described and characterized ZFN heterodimer for targeted addition to the AAVS1 safe harbor region with a composite 24-bp-recognition site8, we generated and screened a panel of ZFN variants with longer composite recognition sites. A heterodimer of 6-finger ZFNs with the same cut-site but a longer (36 bp) recognition site was selected and described and characterized ZFN heterodimer for targeted addition to the AAVS1 locus using a composite 24-bp-recognition site8, we generated and screened a panel of ZFN variants with longer composite recognition sites. A heterodimer of 6-finger ZFNs with the same cut-site but a longer (36 bp) recognition site was selected and used for all subsequent experiments, delivered to cells in the form of in vitro transcribed, capped, polyadenylated mRNAs13. To enhance HDR efficiency and minimize cytotoxicity of repair construct delivery to the CD34 cells, we explored a recombinant adeno-associated...
virus (AAV), a largely nonintegrating vector that exists transiently in various episomal single- and double-stranded forms—in principle, optimal substrates for HDR. Recombinant vectors based on this nonpathogenic human virus have been used effectively and safely in recent clinical trials, with the AAV6 serotype showing the highest efficiency for transducing human HSPCs. 14,15

Next, we optimized parameters for ZFN mRNA electroporation and AAV6 delivery, using healthy donor peripheral blood mobilized CD34+ HSPCs obtained by apheresis and immunoselection. Delivery of the ZFN mRNA was done in a clinical-grade electroporator (MaxCyte GT) which has a Device Master File with the US Food and Drug Administration and is scalable for processing of clinically relevant numbers of HSPCs. To determine the amount of ZFN mRNA required to promote integration of a transgene delivered by AAV6, HSPCs were electroporated with ZFN mRNA concentrations ranging from 6.25 μg/ml to 100 μg/ml for each ZFN, each titrated against incremental amounts of AAV6-SA-2A-Venus. As gauged by cell viability, percentage of live cells expressing Venus and cell proliferation (Fig. 2 and Supplementary Fig. 1), 25 μg/ml ZFN mRNA was selected for subsequent experiments. Cells were then infected...
with varying amounts of AAV6 bearing a promoterless Venus construct and electroporated with the optimal (25 µg/ml) ZFN mRNA dose. Increased Venus expression in transduced HSPCs was associated with reduced cell viability and proliferation (Fig. 3), illustrating the importance of balancing transduction efficiency from increasing AAV6-SA-2A-Venus virus input with cytotoxicity. Finally, because the donor-delivering AAV is replication-incompetent and diluted upon mitosis, and because donor-dependent HDR-directed targeted addition occurs preferentially in S/G2 phase, we reasoned that the timing of the delivery of the virus bearing a repair donor template is critical. We evaluated the timing of ZFN mRNA transfection by electroporating HSPCs with ZFN mRNA following 1, 2 or 3 d of in vitro culture, followed by AAV6-SA-2A-Venus delivery. Optimal integration of promoterless Venus into the AAVS1 site occurred if electroporation is done following 2 d of in vitro culture (Supplementary Fig. 2). The timing of AAV6 addition relative to the timing of electroporation of HSPCs with ZFN mRNA was also investigated, and robust rates of TI were consistently achieved when the cells were transduced with the AAV6 donor immediately after ZFN electroporation (data not shown).

Under optimal ZFN electroporation and AAV6 donor delivery conditions, we observed high levels of Venus marker-positive cells in HSPCs with robust viability as assessed by flow cytometry after 10 d of culture (Fig. 1b), averaging 55.4% Venus⁺ positive, 54.7% viability (Fig. 1d, n = 6 experiments). The AAV6-Venus construct contains a splice acceptor (SA) and a 2A ribosome stuttering signal preceding the Venus cDNA but no promoter, thus ensuring that marker expression in the target cell is driven by activity of the PPP1R12C gene promoter following HDR-driven TI into the AAVS1 locus. To determine the efficiency of TI into the AAVS1 locus in human HSPCs, we used PCR employing two primers located in the AAVS1 locus outside the region of homology present in the donor12,16 (Fig. 1c, schematic in Supplementary Fig. 3a), and a semiquantitative TI deep sequencing assay (MiSeq) to determine the ratio of the AAVS1 alleles containing the integrated transgene to that of alleles containing the wild-type locus (Fig. 1d, 38.8% TI and 15.2% NHEJ-derived indels). Because AAVS1 is on an autosome, TI into just one of the two alleles of the target locus is sufficient to yield a marker-positive cell. Thus, if all cells bore an editing-driven transgene at only one AAVS1 allele, a TI efficiency of 38.8% TI would yield 77.6% Venus⁺ cells. We observed 55.4% Venus⁺ cells (Fig. 1d); our previous data and Poisson distribution statistics indicate that a fraction of cells bear a transgene at both alleles of the nuclease-targeted locus8,11,12.

We next used long-read next-generation sequencing (PacBio, Supplementary Fig. 3b) to characterize the integration junctions between the chromosomal and donor-specified sequences. This analysis showed that 1,083 out of 1,085 (>99.7%) AAVS1 integration events resulted from bona fide HDR (Supplementary Fig. 3c) rather than NHEJ. Further, a junction PCR assay failed to reveal end-capture-based integration of the repair construct into the two most common off-target sites for the ZFNs used in these experiments (data not shown).

We also assessed ZFN-driven targeted integration of a Venus transgene into the subset of CD34⁺ HSCs capable of long-term engraftment in nonobese (NOD) severe combined immunodeficient (scid) IL2Rγnull (NSG) mice preconditioned with busulfan. For our initial mouse transplant experiments, in which in vitro Venus expression averaged 30% of the human HSPCs injected into NSG mice, engraftment of human cells at 8 weeks was 15% in mouse bone marrow, and 12.4% of these human cells were Venus⁺ (Supplementary Fig. 4a,b). Significant Venus expression was observed in the human cells present in spleen and peripheral blood of these mice (Supplementary Fig. 4a ii,iii), demonstrating efficient differentiation of TI-targeted HSCs into mature blood cells.

Longer-term experiments of 4 months showed similar Venus targeting efficiency to the 8-week results. In the representative flow cytometry analysis, engraftment of human cells in the NSG bone marrow was similar for untreated and treated human HSPCs, where 15.9% of human CD45⁺ cells were Venus⁺ (Fig. 1e). Long-term marking of human cells in NSG mouse peripheral blood was also observed, where the similar levels of Venus expression in peripheral blood and bone marrow suggest efficient engraftment and differentiation into multiple peripheral blood lineages of the human HSPCs bearing the
transgene at the safe harbor locus (Fig. 1e). Overall, average CD45+ human cell engraftment in the bone marrow \((n = 16)\) was 40.1 ± 14.6% (mean ± s.d.), of which 10.8 ± 4.2% (mean ± s.d.) were Venus+ (Fig. 1g, first two bars on the left).

Further we investigated the efficiency and molecular structure of targeted and potentially random integration events in the long-term NSG transplants by sorting for human CD45+ and Venus expression (positive and negative). Targeted integration into the AAVS1 locus was measured on DNA from all sorted fractions from each animal using a conventional PCR-based (Fig. 1f) as well as a deep sequencing–based assay (Fig. 1g). The large band size in the Venus+, but not the Venus−, cells is consistent with a substantial fraction of HDR-mediated TI in AAVS1 locus (Fig. 1f). These data indicate that Venus TI in AAVS1 maintains expression during long-term engraftment in immune-deficient mice. Further, these data argue that, at least under these experimental conditions, end capture–based integration of the donor construct into the targeted locus (and, by inference, into potential off-target sites that are cut at significantly lower efficiency) occurs at a level below the limit of detection of the assay shown in Figure 1f.

Deep sequence analysis of the same fractions was performed to assess frequency of TI versus NHEJ-induced indels (Fig. 1g, 3rd to 8th bars from the left). For all 16 mice, sorted Venus+ CD45+ bone marrow cells averaged 61.5% versus 3.7% of AAVS1 alleles with TI versus NHEJ, respectively, with the remaining alleles retaining wild-type sequence. For 14 of the 16 mice (2 failed PCR amplification), the MiSeq analysis showed that sorted Venus− CD45+ bone marrow cells had an average of 0.7% versus 6.2% of AAVS1 alleles with TI versus NHEJ, respectively, with the remaining alleles retaining wild-type sequence. This confirms that there is minimal, if any, functional silencing of the Venus TI over 4 months in vivo. In further support of our notion that end capture–based integration into the AAVS1 target as well as potential off-target sites occurs at a level below the limit of detection of our assays, a deep-sequencing assay for the transgene itself in the marker-negative cell fraction yielded signal indistinguishable from background levels (data not shown).

As noted above, when human CD34+ HSPCs that are 30% Venus+ from gene targeting were transplanted into NSG mice, the average marking of 4-month engrafted human cells from the mouse marrow was 10.8%. Our deep sequence data show that the observed decrease of marking in vivo represents real differences in initial TI marking of short-term, medium-term and long-term repopulating HSPCs initially transplanted, rather than functional silencing of TI at the AAVS1 site. Further, our data argue against the likelihood that the genome of the edited cells bear a substantial burden of randomly integrated, nonexpressing Venus transgenes. In sum, this protocol achieved efficient TI of a repair-construct–specified transgene into the AAVS1 locus in primary human HSPCs.

X-linked chronic granulomatous disease (X-CGD) is caused by mutations in the CYBB gene that encodes the gp91phox subunit of the phagocyte NADPH oxidase, resulting in impaired production of antimicrobial reactive oxidative species. Consequently, patients with X-CGD suffer from severe bacterial and fungal infections with excessive inflammation. To date, 3 of the reported 13 X-CGD patients who have undergone gene therapy have developed myelodysplasia related to integrations in MDS-EVI1 (ref. 5), thus prompting us to test whether TI of functional gp91phox into the AAVS1 safe harbor locus of HSPCs from patients with X-CGD could correct the defect in reactive oxidative species generation.

We applied the optimized delivery protocol for ZFN mRNA electroporation and AAV6 delivery described above to deliver a therapeutic cassette to CD34+ HSPCs obtained from X-CGD patients using AAV6 donors that contained either an MND-promoter–driving gp91phox cDNA or a promoterless construct with a splice acceptor and a 2A ribosomal starter sequence preceding gp91phox cDNA. The MND (myeloproliferative sarcoma virus MPSV enhancer, negative control region NCR deleted, d158rev primer–bining site substituted) promoter has been shown to be suitable to drive high levels of transgene expression in HSCs. Further optimization of AAV6 donor delivery into HSPCs from CGD patients to balance effects on cell growth and transfection efficiency achieved 15% gp91phox protein expression in vitro from both the MND-gp91 donor and the SA-2A-gp91 donor. Deep sequencing assays (not shown) demonstrated a TI efficiency of 7.1% TI (a result consistent with most or all of the gp91+ cells having gene targeting of only one of the two AAVS1 alleles), with NHEJ rate of 20.5%. Despite similar TI efficiencies with both MND-gp91phox and SA-2A-gp91 donors, corrected cells produce substantially more gp91 from the MND promoter (MFI 107) versus from the captured PPP1R12C promoter (MFI 49), though both are less than the native production of gp91 in the normal control (MFI 187). Flow cytometry dihydrorhodamine (DHR) assay (Fig. 4b) showed that MND-gp91 TI corrected CGD HSPCs at this stage of differentiation have NADPH oxidase activity approaching normal levels on a per cell basis. When the culture was allowed to mature further and a chemiluminencescence assay performed (Fig. 4) the MND-gp91 TI-corrected CGD HSPCs generated reactive oxidative species that was approximately 20% of the normal control. The low amount of gp91 produced per cell following SA-2A-gp91 donor correction (Fig. 4a, iv) was associated with very low oxidase activity, whether by DHR analysis (Fig. 4b, iv) or by chemiluminencescence (Fig. 4c).

CGD patient CD34+ HSPCs transfected with the AAVS1-targeting ZFNs and transduced with AAV6-MNDgp91 or AAV6-SA-2A-gp91 were transplanted into NSG mice, and following 8-week engraftment were analyzed for expression of gp91 in the human CD45+ cells from NSG mouse bone marrow (Fig. 2c, d). A representative set of flow cytometry analyses of gp91phox expression in human CD45+ cells engrafted in NSG marrow is shown in Figure 2c following transplant of normal, CGD nontreated and CGD TI-corrected HSPCs. In this example, there is negligible expression of gp91 from the untreated CGD HSPC transplant, but the TI-corrected CGD transplants result in rates of gp91+ cells that are >20% of the normal control. The aggregate data for these studies from the bone marrow of transplanted NSG mice are shown in Figure 2d where it can be appreciated that healthy volunteer HSPCs transplanted into NSG mice result in 35.6 ± 2.8%
The human CD45+ cells in bone marrow from NSG mice 8 weeks after transplant of untreated healthy human donor HSPCs (normal), untreated XCGD patient HSPCs (CGD naive), ZFN plus AAV6-SA-2A-gp91 TI-treated XCGD patient HSPCs (MNDgp91), and ZFN plus AAV6-S2-2A-gp91 TI-treated XCGD patient HSPCs (SA-2Agp91). Indicated at the top of the gated area of each panel is the percent of gp91phox positive cells, where the mean fluorescence intensity (MFI) indicates the average per cell expression of gp91phox positive cell. (b) DHR analysis of NADPH oxidase activity in 14-d-cultured untreated healthy human donor HSPCs (normal), untreated XCGD patient HSPCs (CGD naive), ZFN plus AAV6-MND-gp91 TI-treated XCGD patient HSPCs (MNDgp91), and ZFN plus AAV6-SA-2A-gp91 TI-treated XCGD patient HSPCs (SA-2Agp91). The mean chemiluminescence intensity units are shown at the top of each bar. Error bars, mean ± s.d. (Fig. 2d). (c) Flow cytometry analysis of gp91phox expression of the human CD45+ cells in bone marrow from NSG mice 8 weeks after transplant of untreated healthy human donor HSPCs (normal), untreated XCGD patient HSPCs (CGD naive), ZFN plus AAV6-MND-gp91 TI-treated XCGD patient HSPCs (MNDgp91), and ZFN plus AAV6-SA-2A-gp91 TI-treated XCGD patient HSPCs (SA-2Agp91). The mean chemiluminescence intensity units are shown at the top of each bar. Error bars, mean ± s.d. (Fig. 2d).

A fundamental challenge in the clinical management of genetic diseases is the gap between recognition of the causative genetic lesion and the ability to functionally repair cells harboring the mutation. Using optimized delivery of ZFN and AAV6 to repair the genetic defect in HSPCs, we demonstrate >50% targeted genomic incorporation of a marker transgene in vitro. While this manuscript was under review, a related approach for targeted modification of HSPCs using electroporated ZFN mRNA and AAV6 donor was reported. We applied our method to repair of HSPCs from patients with X-CGD and observed persistent gp91 expression and restoration of NADPH activity in neutrophils derived from these cells following engraftment into NSG mice, which approaches clinically relevant levels. Because all genome editing reagents used here can be deployed in a GMP setting, this approach may enable therapy for X-CGD and other monogenic diseases.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
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COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Approvals for human blood and animal use. Human CD34+ HSPCs from healthy volunteer donors and patients with CGD purified from G-CSF mobilized peripheral blood were obtained after written informed consent under the auspices of National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board–approved protocols 05-1-0213 and 94-1-0073. The conduct of these studies conforms to the Declaration of Helsinki protocols and all US federal regulations required for protection of human subjects.

Use of immune deficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SjI (NSG) mice (from The Jackson Laboratory) for xenotransplant studies was approved by the NIAID Institutional Animal Care and Use Committee under animal use protocol LHD 3E. The conduct of these studies conforms to AAALAC International guidelines and all US federal regulations required for protection of research animals.

ZFN reagents. ZFNs targeting the AAVS1 locus have been described previously8. An optimized pair of the AAVS1-targeting ZFNs with ELD:KKR obligate heterodimeric FokI variants were used in this study.

**AAVS1 ZFN SBS#30054 (NELD):**

1 MDYKHDDGYY KHDIDYKDD DDKMAPPKRR KVQIKRPVAA MAEFPFQCR1 CMNRFPSDRN
61 LSRRHHTHTG KEPFACICRG RKFKALQHLS RHTKHTHPR AP1PKPFQCR1 CMNRFPSDRN
121 NLRHHTHTH GEKPFACICRG GFRKAFDWR RDHTKHTHGS QK1PFQCR1 CMNRFPSDRN
181 EHRHTHTGGE PAICICRGR1 FARLDNRTAH TK1HLRSGLV KESEKEELKS ELRKLYKVP
241 HEYEELIBIA RNSQTQRILE MKVEFMPKCV YGVYRKLHGLG SRKPDKAYT7 VGPSIDYVTK
301 VDTKAYSYY NLPIPGADMM QRYVKENCPT3 NKHNIPNWEV KVYPSSTKEF KLFVSVHFK
361 GNYQAGLLRT NKRNCTNGAV LSVEELIIQG EMIKAGTLT LEVEKRFKKNF EinF*

**AAVS1 ZFN SBS#30054 (KKR):**

1 MDYKHDDGYY KHDIDYKDD DDKMAPPKRR KVQIKRPVAA MAEFPFQCR1 CMNRFPSDRN
61 LSRRHHTHTG KEPFACICRG RKFKALQHLS RHTKHTHPR AP1PKPFQCR1 CMNRFPSDRN
121 NLRHHTHTH GEKPFACICRG GFRKAFDWR RDHTKHTHGS QK1PFQCR1 CMNRFPSDRN
181 EHRHTHTGGE PAICICRGR1 FARLDNRTAH TK1HLRSGLV KESEKEELKS ELRKLYKVP
241 HEYEELIBIA RNSQTQRILE MKVEFMPKCV YGVYRKLHGLG SRKPDKAYT7 VGPSIDYVTK
301 VDTKAYSYY NLPIPGADMM QRYVKENCPT3 NKHNIPNWEV KVYPSSTKEF KLFVSVHFK
361 GNYQAGLLRT NKRNCTNGAV LSVEELIIQG EMIKAGTLT LEVEKRFKKNF EinF*

mRNA production. Plasmids encoding the ZFNs with 64 alanines 3’ of the coding sequence were linearized with SpeI (New England BioLabs) digestion and purified by phenol:chloroform before use as a template for mRNA synthesis by in vitro transcription. mRNA was prepared using the mMESSAGE mMACHINE T7 ULTRA Kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol and was cleaned up with the RNeasy MinElute Cleanup Kit (Qiagen). Alternatively, mRNA was purchased from TriLink Biotechnologies (San Diego, CA).

AAV6 donor construction and virus production. The AAV donor plasmid construct for the Venus fluorescent marker has the cDNA preceded by a splice acceptor (SA) sequence and 2A peptide sequences, and flanked by homology arms to the human AAVS1 gene locus8. Two versions of the AAV donor construct for gp91 were designed; the first one resembles the AAV Venus construct with a SA and 2A elements preceding the gp91pbox cDNA; and a second one that contains a MND promoter driving the gp91 cDNA, both constructs are flanked by AAVS1 homology arms. AAV viruses were produced using a previously described triple plasmid transfection method12. Briefly, HEK 293 cells plated in 10-layer CellSTACK chambers (Corning, Acton, MA) were cultured for 3 d until cells were 80% confluent before calcium phosphate transfection of an AAV helper plasmid expressing AAV2 Rep and AAV6 serotype specific Cap genes, an adeno-virus helper plasmid, and the inverted terminal repeat (ITR)-containing AAV vector genome donor plasmid. 3 d later, the cells were harvested, lysed by repeat freeze/thaw, and the virus precipitated using polyethylene glycol, followed by overnight ultracentrifugation on a cesium chloride gradient, and final formulation by dialysis and filter sterilization. For some experiments (the Venus targeting experiments in the 17-week-engrafted NSG mice), the virus preps were generated by Virovec (Hayward, CA) using their commercial proprietary methodology that is similar to the details provided above.

Human CD34+ HSPC culture, electroporation and transduction for in vitro analysis, and transplant into NSG mice. Cryopreserved human CD34+ HSPCs were thawed at 37 °C, washed in phosphate-buffered saline (Life Technologies) and prestimulated in complete culture medium (Stemspan supplemented with stem cell factor (SCF), fms-related tyrosine kinase 3 ligand (Flt-3-l), and thrombopoietin (TPO), each at 100 ng/ml (Peprotech, NJ). For electroporation, 3–6 × 10^6 cells were spun at 180–300g for 10 min, followed by resuspension in 100 μl of MaxCyte electroporation buffer. The cell suspension was mixed with ZFN mRNA and pulsed with HSC-CL1 program (MaxCyte Systems, Gaithersburg, MD). Following electroporation, the cells were transduced with AAV6-donor (MOI 1–3 × 10^5 vector genomes/cell) and washed after 3 h. Treated cells were kept in culture and either transplanted into NSG mice the following day (day 3 of culture), or maintained in vitro for analyses. For transplant studies, following overnight recovery, treated cells (1–2 × 10^6 per mouse) were transplanted into 6- to 8-week-old NSG mice (Jackson Laboratory) pre-conditioned with intraperitoneal busulfan (20 mg/kg) 24 h prior. The number of mice transplanted depended on amount of material available, particularly with patient CD34+ HSPCs. Following 6–8 weeks, the mice were euthanized for analysis.

Analysis of human cell engraftment and transgene expression by flow cytometry. Fluorochrome-conjugated anti-human monoclonal antibodies were used to identify human hematopoietic cells (CD45-PE (phycoerythrin), human myeloid cells (CD13-APC (allophycocyanin). Human gp91phox expression was determined by indirect staining with murine monoclonal antibody 7D5 (ref. 24) followed by FITC-conjugated goat anti-mouse immunoglobulin G antibody. For flow cytometry, a FACSort (Argon laser; Becton Dickinson, San Jose, CA) was used and the data analyzed using Flowjo version 9.7.6 software.

Dihydrorhodamine flow cytometric assay. We measured phorbol 12-myristate 13-acetate (PMA)–stimulated ROS production either in NSG mice bone marrow cells, or in human myeloid cells differentiated in ex vivo culture from naive and gene-edited X-CGD CD34+ HSPCs or normal donor CD34+ HSPCs. Analysis of gene modification. Several molecular assays were used to detect integration at the AAVS1 locus. Out/Out PCR used primers that recognize human AAVS1 genomic sequences outside of homology regions present in the donor. Targeted integration in the target AAVS1 locus will result in PCR product that is larger than the wild-type product, and the relative integration frequency can be estimated using densitometry based on the relative intensity of the bands. The primers used are listed in Supplementary Table 1 and the approximate location of each of these primers is shown in Supplementary Figure 5a. For the analyses shown in Figure 1c,f the Out/Out PCR primers HDR-F4 and HDR-R5 were used. For a more robust semiquantitative estimate of the frequency of targeted integration, we developed an assay based on Illumina MiSeq deep sequencing. For the molecular analysis shown in Figure 1d DNA was first amplified using the Out/Out PCR as described above in order to avoid amplification analysis of contaminating AAV6 donor, then amplified using a target-specific MiSeq adaptor primer pair containing a set of MiSeq primers recognizing the AAVS1 locus sequences flanking the ZFN binding site (Mi-F and Mi-R) as well as a third primer recognizing sequence from within the transgene (2A-R or PolyA-F) (the size of TI PCR product generated by the
Mi-F/2A-R pair or PolyA-F/Mi-R pair is very similar in size to the NHEJ or wild-type product generated by the Mi-F/Mi-R pair, Supplementary Table 1). Barcodes were then added in this secondary PCR reaction. For the molecular analyses of the 17-week-engrafted NGS mouse bone marrow fractions shown in Figure 1g an Out/Out primary PCR was not needed because it was presumed there was no contaminating AAV6 donor. Only the MiSeq primers Mi-F, 2A-R and Mi-R were used. Also as a result of not first using Out/Out primary PCR, the molecular analysis shown in Figure 1f would detect not only TI at the AAVS1 site, but also any nonhomologous insertion of Venus donor throughout the genome. The final PCR products were cleaned and sequenced in an Illumina MiSeq sequencer using manufacturer’s protocols (Illumina, San Diego, CA). For analysis of gene modification levels, a custom computer script was used to merge paired-end 150-bp sequences, and adapter trimmed via SeqPrep (John St. John, https://github.com/jstjohn/SeqPrep, unpublished) and aligned to the wild-type template sequence. Merged reads were filtered using the following criteria: the 5′ and 3′ ends (23 bp) must match the expected amplicon exactly, the read must not map to a different locus in the target genome as determined by Bowtie2 (ref. 22) with default settings, and deletions must be <70% of the amplicon size or <70 bp long. Indel events in aligned sequences were defined as described previously23, with the exceptions that indels of 1 bp in length were also considered true indels to avoid undercounting real events, and true indels must include deletions occurring at or near the predicted ZFN cut site. Targeted integration TI events were defined based on perfect alignment with the DNA sequence containing the expected integrated sequences (2a or gp91 polyA).

Extended sequencing of AAVS1 locus. We investigated the integration mechanism at the AAVS1 site using PacBio single molecule sequencing. PCR primers were designed with one inside the vector specific region and the other outside the vector in the nonoverlapping genomic region of the AAVS1 locus. This allowed us to amplify the junctions of the integrated vector. Following HDR-mediated integration, the ITR is lost due to homologous recombination since it is located outside the homologous region. Therefore the NHEJ junction sequence did not contain any ITR sequence. In contrast, NHEJ mediated integration retains an ITR in the junction sequence. The expected amplicon sizes range from 1.1 kb to 2.5 kb depending on whether the ITR is retained. We amplified both AAVS1I junction and AAVS1R junction. PacBio long single DNA molecule sequencing identified 1,082 integration events without ITR sequence and 3 integration events retaining ITR sequence. The results suggest that the vast majority of AAV integration at the AAVS1 locus occurred through homologous recombination between vector bearing-AAVS1 sequence flanking the transgene and host AAVS1 sequence.

Primers for Pac-Bio sequencing. AAVS1-l: CTGCCGTCTCTCTCTCCTGAGT. Venus_AAVS1-l: GCTGAACTTGTGGCCGTTTA. Venus_AAVS1-R: GAGCAAAGACCCCAACGAG. AAVS1-R: AAAAGGCAGCCTGCTGAGACA.

Superoxide measurement. A quantitative ferricytochrome C reduction assay was used to measure superoxide production at 10 and 60 min after stimulation.

Randomization and blinding. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

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