CRISPR-Mediated Knockout of *Cybb* in NSG Mice Establishes a Model of Chronic Granulomatous Disease for Human Stem-Cell Gene Therapy Transplants

Colin L. Sweeney,^{1,†} Uimook Choi,^{1,†} Chengyu Liu,² Sherry Koontz,¹ Seung-Kwon Ha,³ and Harry L. Malech^{1,*}

¹Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases; ²Transgenic Core, National Heart, Lung, and Blood Institute;

³Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke; National Institutes of Health, Bethesda, Maryland.

[†]These authors contributed equally to this work.

Chronic granulomatous disease (CGD) is characterized by defects in the production of microbicidal reactive oxygen species (ROS) by phagocytes. Testing of gene and cell therapies for the treatment of CGD in human hematopoietic cells requires preclinical transplant models. The use of the lymphocyte-deficient NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ (NSG) mouse strain for human hematopoietic cell xenografts to test CGD therapies is complicated by the presence of functional mouse granulocytes capable of producing ROS for subsequent bacterial and fungal killing. To establish a phagocyte-defective mouse model of X-linked CGD (X-CGD) in NSG mice, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 was utilized for targeted knockout of mouse *Cybb* on the X-chromosome by microinjection of NSG mouse zygotes with Cas9 mRNA and CRISPR single-guide RNA targeting *Cybb* exon 1 or exon 3. This resulted in a high incidence of indel formation at the CRISPR target site, with all mice exhibiting deletions in at least one *Cybb* allele based on sequence analysis of tail snip DNA. A female mouse heterozygous for a 235-bp deletion in *Cybb* exon 1 was bred to an NSG male to establish the X-CGD NSG mouse strain, NSG.Cybb[KO]. Resulting male offspring with the 235 bp deletion were found to be defective for production of ROS by neutrophils and other phagocytes, and demonstrated increased susceptibility to spontaneous bacterial and fungal infections with granulomatous inflammation. The establishment of the phagocyte-defective NSG.Cybb[KO] mouse model enables the *in vivo* assessment of gene and cell therapy strategies for treating CGD in human hematopoietic cell transplants without obfuscation by functional mouse phagocytes, and may also be useful for modeling other phagocyte disorders in humanized NSG mouse xenografts.

Keywords: chronic granulomatous disease, CRISPR/Cas9, *Cybb*, knockout, NSG mouse

INTRODUCTION

CHRONIC GRANULOMATOUS DISEASE (CGD) is an immune deficiency caused by mutations in the genes encoding the components of phagocytic oxidase (phox), resulting in defects in the production of microbicidal reactive oxygen species (ROS) for killing microorganisms by phagocytes, primarily neutrophils and monocytes/macrophages. Although phagocytes also possess non-oxidative killing mechanisms, these can be insufficient to control infections by a subset of microorganisms. Consequently, CGD patients are susceptible to recurring, life-threatening infections, particularly by catalase-positive bacteria,

including *Staphylococcus aureus*, *Burkholderia cepacia*, *Escherichia coli*, and *Klebsiella* species, and catalase-positive fungi, including some *Aspergillus* and *Candida* species.¹ Patients also experience hyper-inflammation, resulting in granulomatous complications. The X-linked form of CGD (X-CGD) is the most common, and is caused by mutations in the *CYBB* gene on the X-chromosome, which encodes the gp91^{phox} protein.

Allogeneic transplant of hematopoietic stem/progenitor cells (HSPCs) can cure CGD, but many patients lack a suitable donor, and graft-versus-host-disease remains a significant risk. Gene-therapy

*Correspondence: Dr. Harry L. Malech, LHD/NIAID/NIH; Bldg 10, Rm 5-3750, 10 Center Dr, MSC-1456, Bethesda, MD 20892-1456. Email: hmalech@nih.gov

approaches for the correction and transplant of autologous CGD patient HSPCs lack these issues. Such approaches include retrovirus²⁻⁴ and lentivirus vectors^{5,6} and, more recently, nuclease-mediated methods of targeted gene insertion or correction.⁷⁻⁹ For X-CGD, autologous HSPC gene therapy using retrovirus vector derived from murine spleen focus forming virus has demonstrated clinical benefit for life-threatening infections, but long-term gene marking was low, and myelodysplasia due to retroviral insertional mutagenesis was observed.^{3,4} This demonstrates the need for additional gene-correction strategies, which require preclinical models of X-CGD gene therapy in patient cells to test their efficacy in a transplant setting.

An *in vivo* model of X-CGD was previously established in the C57BL/6 mouse background by Dinauer's group through homologous recombination-mediated disruption of exon 3 of the mouse *Cybb* gene,¹⁰ which resulted in defective ROS production and increased susceptibility to experimental infection with *Staphylococcus aureus* and *Aspergillus fumigatus* in affected *Cybb* knockout male mice. Although this mouse strain allows for modeling of the X-CGD phenotype and gene and cell therapy approaches for treatment using mouse hematopoietic cells,¹¹⁻¹⁴ alternate models are necessary for preclinical testing of human gene and human cell therapies for CGD, such as targeting human HSPCs as tested *in vivo* in a xenograft animal model.

The use of lymphocyte-deficient mouse strains, such as NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice,¹⁵ enables the transplant of human HSPCs to reconstitute mice with human hematopoietic cells, including mature functional neutrophils and other phagocytic cells. However, NSG mice also produce functional mouse neutrophils and other phagocytes, which complicate the functional analysis of engrafted human phagocytes. Crossbreeding of the already established X-CGD C57BL/6 mouse strain into the genetically complex NSG background would be a lengthy and complicated breeding program. Alternatively, here, the generation of an X-CGD mouse model is described in the NSG mouse strain by using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease¹⁶⁻¹⁸ to knock out the mouse *Cybb* gene in zygotes from NSG mice through induction of indels (insertions or deletions) at the CRISPR target site. *Cybb* knockout NSG mice exhibited the defect in ROS production and susceptibility to microbial infections with granulomatous inflammation that are characteristic of CGD, and maintained the capacity for human HSPC engraftment, enabling human CGD therapeutic strategies to be tested

without the background of functionally oxidase-positive mouse phagocytes.

MATERIALS AND METHODS

Approvals for human blood and animal use

CD34⁺ HSPC-enriched granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood was obtained from healthy volunteers and X-CGD patients after written informed consent following the Declaration of Helsinki under the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board-approved protocol 94-I-0073.

The use of lymphocyte-deficient NSG mice (JAX Stock No: 005557; obtained from The Jackson Laboratory, Bar Harbor, ME) and *Cybb* knockout NSG (NSG.*Cybb*[KO]) mice was approved by the NIAID Animal Care and Use Committee (ACUC) under animal use protocol LHD 3E and by the National Heart, Lung, and Blood Institute ACUC under animal use protocol H-0125R2.

Generation of *Cybb* knockout NSG mice

Cybb knockout mice were generated directly on the NSG genetic background using CRISPR/Cas9 methodology.¹⁹ Briefly, two CRISPR guide RNA sequences, targeting *Cybb* exon 1 (GGTACTTACA ATGACAAAGA) or exon 3 (GATTTTCGACACACTG GCAGC), were separately cloned into the pDR274 vector (Addgene plasmid #42250).²⁰ Corresponding sgRNAs (single-guide RNAs) were synthesized by *in vitro* transcription using MEGAshortscript T7 transcription kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA). Cas9 mRNA was synthesized by *in vitro* transcription from the plasmid pMLM3613 (Addgene plasmid #42251)²⁰ using the mMACHINE mMESSAGE T7 kit (Invitrogen). Either exon 1 or exon 3 sgRNA was individually mixed with Cas9 mRNA at a concentration of 50 ng/ μ L of Cas9 mRNA and 20 ng/ μ L of sgRNA in nuclease-free microinjection buffer (10 mM of Tris, pH 7.5, 0.1 mM of EDTA) and microinjected into the cytoplasm of fertilized eggs collected from NSG mouse mating pairs. The injected zygotes were cultured overnight in M16 medium (EMD Millipore, Billerica, MA) at 37°C in 5% CO₂. The next morning, embryos that had reached the two-cell stage of development were implanted into the oviducts of pseudopregnant foster mothers (Swiss Webster mice; Taconic Biosciences, Hudson, NY).

The mice born to the foster mothers were genotyped by polymerase chain reaction (PCR) of *Cybb* exon 1 or 3 using tail snip DNA using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA), and PCR products were sequenced

commercially (Macrogen, Rockville, MD). Primers used for exon 1 PCR and sequencing included mCybb1-565-FWD: 5'-GTTGGAAGAGCCTGTGA GAAGA-3', mCybb1-851-FWD: 5'-GGGAACAGC CTTTCAGTTGG-3', mCybb1-908-FWD: 5'-CCTAT TGCCCCAAGCTGCT-3', mCybb1-1192-BWD: 5'-ACCGAATCTACCTGCAAGCA-3', mCybb1-1232-BWD: 5'-AAGGCGTGCTGGGATTAAGA-3', mCybb1-1467-BWD: 5'-TGCACTCTGGTAAATGCTGG-3'. Primers used for exon 3 PCR and sequencing included mCybb3-3905-FWD: 5'-AGGATAGGAGTT CTTGCCGC-3', mCybb3-4213-FWD: 5'-AACTGTG GTCTGGGAGGTGA-3', mCybb3-4431-FWD: 5'-TG GGGAAGAGGAATACGGGT-3', mCybb3-4694-BWD: 5'-ACAGAGCATTGCTTGGCTCT-3', mCybb3-4858-BWD: 5'-TCCAGGAACCTTTTGCCTCA-3', mCybb3-5156-BWD: 5'-TGCTTGTCTCAGGCTCCT TA-3'.

A female mouse heterozygous for a 235 bp deletion in *Cybb* exon 1 was bred to an NSG male to establish the NSG.Cybb[KO] mouse strain. Genotyping of NSG.Cybb[KO] mice was performed using the mCybb1-565-FWD and mCybb1-1192-BWD primer set, resulting in a 628 bp product for the wild-type allele and a 393 bp product for the 235 bp deletion. NSG.Cybb[KO] mice were maintained by crossing of heterozygous females with NSG males both to avoid genetic drift from the NSG background and to protect breeders from spontaneous bacterial or fungal infections to which CGD mice are susceptible. In order to reduce the incidence of infections, breeding mice and offspring were provided with water containing 0.67 mg/mL of sulfamethoxazole and 0.13 mg/mL of trimethoprim (Sulfatrim; STI Pharma, Langhorne, PA), and regular cage changes were performed for these mice before those of other mice housed in the facility.

Predictions of likely deletions resulting from microhomology-mediated end joining²¹ (MMEJ) at the exon 1 and exon 3 CRISPR cut sites were performed using the Microhomology-Predictor tool of the Center for Genome Engineering, Institute for Basic Science, Korea (www.rgenome.net).

Dihydrorhodamine assessment of ROS activity in NSG.Cybb[KO] mice

For dihydrorhodamine (DHR) assay of ROS activity on mouse peripheral blood, 100–200 μ L of mouse blood was collected by tail venisection, lysed with ACK lysis buffer (Quality Biological, Gaithersburg, MD) for 5–7 min at 37°C, and washed with Hank's balanced salt solution without calcium or magnesium (HBSS; Thermo Fisher Scientific). Cells were resuspended in 400 μ L of HBSS, 130 μ M of DHR (Molecular Probes; Thermo Fisher Scientific), and

500 IU of catalase (Sigma–Aldrich, St. Louis, MO), and samples were incubated at 37°C for 5 min. Samples were then stimulated for ROS production by addition of 100 μ L of 400 ng/mL phorbol myristate acetate (Sigma–Aldrich) in HBSS containing calcium and magnesium (Thermo Fisher Scientific), followed by incubation at 37°C for 14 min. Cells were analyzed by flow cytometry for DHR fluorescence using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), as previously described.²²

NSG.Cybb[KO] mouse pathology analysis

For histopathology, mouse tissues were fixed in 10% buffered formalin and embedded in paraffin, cut at a thickness of 5 μ m on a microtome, placed on slides, and stained with hematoxylin and eosin. Each section was examined by a board-certified veterinary pathologist. Images were obtained using a Zeiss Observer 1 microscope (Zeiss, Thornwood, NY) and ZEN blue software (Zeiss).

For bacterial and fungal identification from mouse infections, isolates were cultured on blood agar, phenylethyl alcohol (PEA) agar, MacConkey agar, chocolate agar, anaerobic reducible blood agar, anaerobic reducible PEA agar, anaerobic reducible LKV agar, Sabouraud dextrose agar, inhibitory mold agar slants, thioglycollate broth, or brain heart infusion broth (Remel; Thermo Fisher Scientific), and were further identified based on analysis using spot indole reagent (Remel), Biolog GEN III MicroPlates (Biolog, Hayward, CA), or Vitek 2 identification cards for Gram positive bacteria, Gram negative bacteria, or yeast (bioMérieux, Durham, NC), according to the manufacturers' protocols.

Transduction of human CD34⁺ HSPCs

CL20i4r-EF1a-gp91OPT lentiviral vector²³ for constitutive expression of gp91^{phox} was produced through transient transfection of 293T cells (ATCC, Manassas, VA) with pCL20i4r-EF1a-gp91OPT plasmid and gag-pol, rev-tat, and VSV-G packaging plasmids.²⁴ Virus was harvested daily for 3 days, filtered (0.22 μ m; EMD Millipore), concentrated by centrifugation at 18,600 *g* for 3 h at 4°C, and reconstituted in X-VIVO 10 medium (Lonza, Walkersville, MD). Titer of CL20i4r-EF1a-gp91OPT lentivirus was measured in transduced K562 cells (ATCC) based on flow cytometry analysis of antibody staining for gp91^{phox} expression, as described below.

Cryopreserved mobilized human CD34⁺ HSPCs were thawed 24 h prior to first transduction, and cultured in X-VIVO 10 medium containing 1% human serum albumin (Talecris Biotherapeutics, Research Triangle Park, NC) and 50 ng/mL each

of human stem cell factor, Flt3-ligand, and thrombopoietin (PeproTech, Rocky Hill, NJ). Up to 5 million mobilized human CD34⁺ HSPCs from a patient with X-CGD were transduced twice on consecutive days with CL20i4r-EF1a-gp91OPT lentivirus at a multiplicity of infection of 10 viruses per cell, by spinoculation on RetroNectin (Lonza)-coated plates at 1360 *g* for 30 min at 32°C, followed by overnight culture at 37°C and 5% CO₂, and replacement with fresh HSPC medium the following morning for approximately 8 h of recovery between transductions. HSPCs from the same patient were left untransduced as an uncorrected naïve control. Mobilized CD34⁺ HSPCs from a healthy donor were used as a normal control without transduction for ROS and gp91^{phox} analysis and transplant.

For *in vitro* analysis of transduction efficiency at 3 weeks after the final transduction, HSPCs (naïve X-CGD, transduced X-CGD, or normal donor) were cultured in medium containing 50 ng/mL of G-CSF (Neupogen; Amgen Mfg., Thousand Oaks, CA), then fixed with 2% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 5–10 min, permeabilized with 0.1% saponin (Sigma-Aldrich) in PBS, and stained with an unconjugated antibody to human gp91^{phox} (7D5 antibody)² for 15–20 min, followed by staining with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 5–10 min. Analysis of gp91^{phox} expression was then performed with a FACSCalibur flow cytometer.

Transplant of human CD34⁺ HSPCs in NSG.Cybb[KO] mice

NSG.Cybb[KO] mice at 6–10 weeks of age were treated with 20 mg/kg of busulfan by intraperitoneal injection 24 h before transplant. Thereafter, mice were provided with water containing 2.5 mg/mL of neomycin (VetOne, Boise, ID) instead of Sulfatrim. After the final round of overnight transduction (*i.e.*, no more than 3 days total culture after thaw), one million human HSPCs (naïve X-CGD, transduced X-CGD, or normal donor) were transplanted per mouse by tail-vein injection. At 6 weeks post transplant, blood was collected, and mice were euthanized for analysis of bone marrow.

Analysis of human CD34⁺ HSPCs engraftment and phagocyte activity

Blood and bone-marrow cells were collected from transplanted mice for analysis of human cell engraftment and human X-CGD donor cell correction. Blood samples were lysed with ACK lysis buffer prior to analysis, as described above. Blood cells were stained for ROS production by DHR assay, as described above, to detect functional human

phagocytes. In parallel, marrow cells were co-stained with allophycocyanin (APC)-conjugated antibody to human CD45 pan-leukocyte marker (BD Biosciences) to detect engrafted human hematopoietic cells, or phycoerythrin (PE)-conjugated antibody to human CD45 together with APC-conjugated antibody to human CD13 myeloid marker (BD Biosciences) and unconjugated human gp91^{phox} antibody with FITC-conjugated secondary antibody, as described above. For some transplanted mice, additional marrow cells were stained with FITC or APC-conjugated antibodies to CD13, CD14, CD19, or CD56 (BD Biosciences) for analysis of hematopoietic reconstitution of mice with human myeloid cells, monocytes/macrophages, B cells, or NK cells, respectively. Peripheral blood and spleen cells were collected from additional mice at 10 weeks post transplant and stained with PE-Cy5, APC, or FITC-conjugated antibodies to human CD3, CD4, and CD8 (BD Biosciences), for analysis of reconstitution with human T cells. Samples were analyzed with a FACSCalibur flow cytometer.

RESULTS

Establishment and characterization of NSG.Cybb[KO] mouse strain

Injection of NSG mouse zygotes with CRISPR/Cas9 targeting either exon 1 or exon 3 of the mouse *Cybb* gene on the X-chromosome resulted in highly efficient induction of deletions in *Cybb* at the CRISPR target site in the resulting mice (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/hum), including larger deletions encompassing exon and intron or 5' untranslated regions (Fig. 1A and B) and smaller deletions (≤12 bp) occurring entirely within the target exon (Fig. 1C and D); no insertions were identified at either target site. A number of the smaller deletions matched predicted sequences for deletions caused by the MMEJ DNA repair pathway²¹ through annealing of microhomology sequences of 2–3 bp located on both sides of the DNA double-strand break at the CRISPR target site. These predicted MMEJ-induced mutations included three in-frame deletions for the exon 1 target that were detected in 3/6 mice, one in-frame deletion for exon 3 that was detected in 4/7 mice, and one frameshift deletion for exon 3 that was detected in 1/7 mice (Fig. 1C and D).

One female mouse was heterozygous for a 235 bp deletion encompassing all of exon 1 and 190 bp of the upstream *Cybb* promoter, with the second allele containing a 240 bp deletion (Fig. 1A and Supplementary Table S1). Breeding of the female *Cybb* knockout founder mouse to an NSG male re-

Figure 1. *Cybb* mutations identified in mice arising from clustered regularly interspaced short palindromic repeats (CRISPR) targeting of *Cybb* exon 1 or exon 3. **(A)** *Cybb* exon 1 deletions >12 bp. Shown are a portion of the *Cybb* promoter and 5' untranslated region (UTR), all of exon 1, and a portion of intron 1. The target site of the exon 1 CRISPR is also shown, as are the locations of larger deletions identified in mice, including the 235 bp deletion of the NSG.Cybb[KO] strain. **(B)** *Cybb* exon 3 deletions >12 bp. Shown are a portion of the introns 2 and 3 and all of exon 3, with the target site of the exon 3 CRISPR and the locations of larger deletions identified in mice. **(C)** and **(D)** Small (≤ 12 bp) *Cybb* deletions identified in mice from CRISPR targeting of **(C)** exon 1 or **(D)** exon 3. Wild-type (wt) sequences for a portion of the target region are listed. Exon sequences are in uppercase, intron sequences are in lowercase, and CRISPR target sequences are underlined. For deletions matching those predicted to occur through the microhomology-mediated end joining (MMEJ) DNA repair pathway, the 2–3 bp microhomology sequences are listed.

sulted in germline transmission of the deletions, as confirmed by PCR analysis. Male offspring with the 235 bp deletion in *Cybb* exon 1 were confirmed to completely lack ROS activity in peripheral blood phagocytes by DHR assay (Fig. 2A) in contrast to wild-type NSG control mice (Fig. 2B). The NSG.Cybb[KO] mouse strain was established and maintained through breeding of females heterozygous for one 235 bp deleted allele and one wild-

type allele with wild-type NSG males, generating heterozygous or wild-type females and wild-type or knockout male offspring.

Despite maintenance of mice with Sulfatrim antibiotic, increased morbidity due to spontaneous bacterial and fungal infections was observed in *Cybb* knockout mice, along with a significant decrease in mouse survival compared with heterozygous or wild-type NSG mice (Fig. 2C; $p < 0.000001$ based on log-

Figure 2. NSG.Cybb[KO] mouse phenotype. (A) Dihydrorhodamine (DHR) assay of peripheral blood from NSG.Cybb[KO] male mouse, demonstrating no detectable reactive oxygen species (ROS) production. (B) DHR assay of wild-type NSG mouse control, demonstrating normal ROS production. (C) Mouse survival out to 180 days of age for *Cybb* knockout NSG (*Cybb* KO), *Cybb* heterozygous NSG (*Cybb*^{+/-}), or wild-type NSG mice. Survival analysis included only mice that were not used in transplants or other experiments; the number of mice in each group is listed.

rank statistical analysis²⁵ using the Kaplan–Meier estimator²⁶ demonstrating that NSG.Cybb[KO] mice exhibit the characteristic susceptibility to microbial infections of human CGD. Microorganisms identified in mouse infections included *Staphylococcus xylosus*, *Staphylococcus hominis*, *Klebsiella oxytoca*, *Escherichia coli*, *Aerococcus viridans*, *Enterobacter cloacae*, and *Candida albicans*, with multiple infectious species detected in some mice. Further analysis of bacterial cultures established from a *K. oxytoca* infection confirmed its antibiotic resistance to Sulfatrim. Infections in NSG.Cybb[KO] mice were typically systemic, occurring at multiple tissues in the same mouse. Infection sites included the mouth, esophagus, stomach, lymph node, lung, and liver. Granulomatous inflammation was also observed in knockout mice at sites of microbial infections (Fig. 3), consistent with the phenotype of human CGD. No other causes of morbidity were identified in NSG.Cybb[KO] mice.

Human HSPC transplants in NSG.Cybb[KO] mice

In order to test the utility of NSG.Cybb[KO] mice as a human xenograft model, CD34⁺ HSPCs from normal, healthy donors or X-CGD patients were transplanted into knockout mice preconditioned with

busulfan. As expected for the lymphocyte-deficient NSG mouse background, NSG.Cybb[KO] mice could be engrafted with human HSPCs to give rise to human CD45⁺ hematopoietic cells of multiple lineages, including CD13⁺ myeloid cells, CD14⁺ monocytes/macrophages, CD19⁺ B cells, CD56⁺ NK cells, and CD3⁺ T cells (Fig. 4). Normal donor HSPC transplants resulted in engraftment with phagocytes capable of ROS production in both bone marrow and peripheral blood (Fig. 5A and Supplementary Fig. S1A), while engrafted X-CGD patient cells lacked ROS activity in bone marrow and peripheral blood (Fig. 5B and Supplementary Fig. S1B). The lack of DHR activity by mouse phagocytes in the NSG.Cybb[KO] background allowed for unambiguous detection of even small numbers of human phagocytes that are functional for ROS production.

To test further the utility of the NSG.Cybb[KO] mouse strain for preclinical modeling of gene therapy approaches to CGD in human cell transplants, X-CGD patient HSPCs were transduced with a self-inactivating CL20i4r-EF1a-gp91OPT lentiviral vector expressing a codon-optimized human *CYBB* cDNA from the elongation factor-1a (EF1a) short promoter (Fig. 6A). Lentiviral transduction of X-CGD patient HSPCs was confirmed by flow cytometry analysis of gp91^{phox} antibody staining after 3 weeks of *in vitro* culture in medium containing G-CSF for granulocyte differentiation, which demonstrated the expected defect in gp91^{phox} expression in untransduced patient cells (Supplementary Fig. S2A), while lentivirus-transduced patient cells (Supplementary Fig. S2B) and untransduced normal donor cells (Supplementary Fig. S2C) expressed gp91^{phox}. Transplant of transduced X-CGD patient HSPCs or normal donor HSPCs into NSG.Cybb[KO] mice immediately following the final day of transduction resulted in bone-marrow engraftment and myeloid reconstitution with human hematopoietic cells (Fig. 6B), including transduced patient cells that constitutively expressed gp91^{phox} at levels approximately 70–80% of endogenous gp91^{phox} expression in engrafted normal donor HSPC-derived phagocytes (Fig. 6C) based on mean fluorescence intensities of the positive populations. Corrected human phagocytes in bone marrow produced ROS at mean fluorescence intensities comparable to normal human phagocytes by DHR assay (Fig. 6D), with similar levels observed in peripheral blood phagocytes (Supplementary Fig. S2D).

DISCUSSION

This study describes the CRISPR/Cas9-mediated knockout of mouse *Cybb* to generate

Figure 3. Histology of NSG.Cybb[KO] mouse infections. **(A)** Infected mass near salivary gland (bar = 5,000 μ m). **(B)** Infected mass around jaw of mouse skull (bar = 5,000 μ m). **(C)** Liver with infected mass showing pyogranuloma replacing normal tissue (bar = 500 μ m). **(D)** Spleen, also showing pyogranuloma distending parenchymal structure (bar = 500 μ m). Images in **(B)–(D)** are from the same mouse, demonstrating systemic infections with granulomatous inflammation. **(E)** Magnification of mass in **(B)**. Cells are arranged in ribbons of nested cords, which are separated by a fibrous stroma. Basophilic bacterial colonies (arrows) are present, surrounded by aggregated small granulomas with surrounding loose connective tissue, fibroblasts, and macrophages (bar = 100 μ m). **(F)** High magnification of mass in **(C)**. Present is a basophilic bacterial colony (arrow), surrounded by large numbers of viable and degenerate neutrophils mixed with necrotic cell debris and large numbers of epithelioid macrophages (bar = 50 μ m).

a phagocyte-defective model of X-CGD in the lymphocyte-deficient NSG mouse strain, enabling engraftment of human cells to test gene and cell therapies for all forms of human CGD without the background of mouse phagocytes capable of ROS production. Efficient gene targeting using CRISPR/Cas9 in lymphocyte-deficient mice was previously shown in NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG) mice by Li *et al.*,²⁷ who reported a 100% mutation rate at the *Fah* gene following CRISPR/Cas9 microinjection into mouse zygotes for the purposes of establishing a model for human hepatocyte and hematopoietic cell xenografts. The similar highly effi-

cient induction of *Cybb* mutations by CRISPR/Cas9 to generate the NSG.Cybb[KO] model of X-CGD in the current study further demonstrates the utility of CRISPR technology for establishing humanized disease models involving human cell transplants in immunodeficient mice.

The *Cybb* CRISPRs used in this study were designed without prior microhomology analysis of the target sites to predict deletions that could result from the MMEJ DNA repair pathway. Based on the microhomology deletion pattern scoring metric described by Bae *et al.*,²¹ approximately half of the mice in the current study exhibited in-frame mu-

Figure 4. Multi-lineage human hematopoietic cell engraftment in NSG.Cybb[KO] mice. Shown are bone marrow cells stained for antibodies to human CD45 (pan-leukocyte), CD13 (myeloid cells), CD14 (monocyte/macrophages), CD19 (B cells), and CD56 (NK cells), for **(A)** untransplanted NSG mouse control or **(B)** NSG.Cybb[KO] mouse at 6 weeks post transplant with normal donor CD34⁺ human hematopoietic stem/progenitor cells (HSPCs). Data shown are from a representative transplanted mouse; similar multi-lineage engraftment was observed with X-linked chronic granulomatous disease (X-CGD) patient CD34⁺ cell transplants. For analysis of human T-cell reconstitution in additional mice, cells were stained with antibodies to human CD3, CD4, and CD8, from **(C)** peripheral blood and **(D)** the spleen of an untransplanted NSG mouse control, or **(E)** peripheral blood and **(F)** the spleen of a transplanted NSG.Cybb[KO] mouse analyzed at 10 weeks post transplant with normal donor CD34⁺ human HSPCs.

Figure 5. ROS production from engrafted human cells in NSG.Cybb[KO] mice. Shown are DHR assays of ROS production in bone-marrow samples from NSG.Cybb[KO] mice at 6 weeks post transplant with **(A)** normal donor human HSPCs or **(B)** X-CGD patient human HSPCs. Data shown are from three representative mice from transplanted cohorts.

Figure 6. Transduction and transplant of X-CGD patient HSPCs with lentivirus expressing gp91^{phox}. **(A)** Schematic of integrated provirus of self-inactivating CL20i4r-EF1a-gp91OPT lentiviral vector with self-inactivating lentiviral long terminal repeats (SIN LTRs) containing HS4 chicken β -globin insulator elements, lentiviral packaging signal (Ψ), constitutive EF1a short promoter, and a codon-optimized human *CYBB* cDNA (gp91opt). **(B)** Flow cytometry analyses of antibody staining for human CD45 (pan-leukocyte) and CD13 (myeloid cells) in bone marrow of NSG.Cybb[KO] mice transplanted with transduced X-CGD patient HSPCs (left graph) or normal donor HSPCs (right graph). **(C)** Flow cytometry analyses of antibody staining for gp91^{phox} expression in bone marrow after transplant with transduced X-CGD patient HSPCs (left) or normal donor HSPCs (right). **(D)** DHR assay of ROS production in bone marrow after transplant with transduced X-CGD patient HSPCs (left) or normal donor HSPCs (right). Data shown in **(B)–(D)** are from one representative mouse each from cohorts transplanted with transduced X-CGD patient HSPCs or normal donor HSPCs, analyzed at 6 weeks post transplant; for the cohort transplanted with transduced X-CGD cells, $2.3 \pm 0.5\%$ of cells were gp91^{phox}-positive and $1.9 \pm 1.1\%$ were DHR-positive (mean $\pm$ standard deviation; $n=3$). Negative DHR controls from mice transplanted with untransduced X-CGD patient HSPCs can be found in Figure 5B. Mean fluorescence intensities (MFI) of the gp91^{phox}-positive and DHR-positive populations are shown.

tations that matched three of the top five predicted deletion patterns for the *Cybb* exon 1 site or the top predicted deletion pattern for the exon 3 site. This high incidence of predictable in-frame deletions lends support for the use of microhomology analysis in se-

lecting nuclease target sites that reduce the likelihood of in-frame deletions in favor of frameshift mutations for more effective knock out of a target gene.

The functional defect in ROS production of the NSG.Cybb[KO] mice resulted in increased sensi-

tivity to spontaneous microbial infections, including bacterial *Staphylococcus* and *Klebsiella* species and the fungus *C. albicans*, which are also common infections in CGD patients.¹ The infections that were identified in moribund animals were all from catalase-positive microorganisms, as expected for CGD infections, except for *A. viridans*, which is classified as catalase-negative or weakly catalase-positive, and which has previously been reported as a spontaneous pathogen in NOD.Cg-*Prkdc*^{scid} (NOD/SCID) mice.²⁸ Morbidity due to infection was also observed in a smaller number of *Cybb* heterozygous and wild-type NSG mice, including littermates and cage mates of *Cybb* knockout mice, which is consistent with a previous report of spontaneous bacterial infections, including *K. oxytoca*, that have been observed in NSG mice at the Jackson Laboratory and other independent breeding colonies.²⁹

To the authors' knowledge, the NSG.Cybb[KO] strain is the most immunodeficient mouse strain to be engineered to date, since it completely lacks lymphocyte immune functions and is defective in myeloid oxidative killing function. Consequently, this strain requires particular care in order to maintain mouse health and survival. Morbidity of NSG.Cybb[KO] mice due to spontaneous infections was reduced when routine changing of mouse cages was performed prior to handling of other mice in the facility, and use of microisolator cages or other barrier facilities is recommended.

The demonstrated susceptibility of NSG.Cybb[KO] mice to spontaneous microbial infections would also allow for experimental infection studies in

these mice, as were performed in the prior C57BL/6 mouse X-CGD model.^{10–14} This aspect of the NSG.Cybb[KO] model will enable the CGD research community to begin assessing the effectiveness of corrected CGD patient HSPCs or other therapeutic human cells for *in vivo* protection from new infections or clearing of existing infections, expanding the current assays available for pre-clinical testing of human CGD therapies in a transplant setting. Due to its functional defect in killing of select microorganisms by phagocytic cells, this mouse strain may also be useful for *in vivo* modeling of other human phagocyte deficiencies or for infection studies involving human cell transplants.

ACKNOWLEDGMENTS

This research was supported by the Intramural Research Program of NIAID under intramural project numbers Z01-AI-00644 and Z01-AI-00988. The authors thank the Diagnostic and Research Services Branch of the Division of Veterinary Resources of NIH for pathology and bacteriology analysis, the Department of Laboratory Medicine of the NIH Clinical Center for additional bacteriology analysis, and Crystal Thomas of the Laboratory of Animal Medicine Section of NIAID for advice and assistance in maintaining the health of NSG.Cybb[KO] mice.

AUTHOR DISCLOSURE

No competing financial interests exist.

REFERENCES

- Marciano BE, Spalding C, Fitzgerald A, et al. Common severe infections in chronic granulomatous disease. *Clin Infect Dis* 2015;60:1176–1183.
- Brenner S, Whiting-Theobald NL, Linton GF, et al. Concentrated RD114-pseudotyped MFGS-gp91^{phox} vector achieves high levels of functional correction of the chronic granulomatous disease oxidase defect in NOD/SCID/ β 2-microglobulin^{-/-} repopulating mobilized human peripheral blood CD34⁺ cells. *Blood* 2003;102:2789–2797.
- Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to *EV1* activation after gene therapy for chronic granulomatous disease. *Nat Med* 2010;16:198–204.
- Ulrich S, Anna P, Heidi H-G, et al. Successful combination of sequential gene therapy and rescue allo-HSCT in two children with X-CGD—importance of timing. *Curr Gene Ther* 2015;15:416–427.
- Naumann N, De Ravin SS, Choi U, et al. Simian immunodeficiency virus lentivector corrects human X-linked chronic granulomatous disease in the NOD/SCID mouse xenograft. *Gene Ther* 2007;14:1513–1524.
- Barde I, Laurenti E, Verp S, et al. Lineage- and stage-restricted lentiviral vectors for the gene therapy of chronic granulomatous disease. *Gene Ther* 2011;18:1087–1097.
- De Ravin SS, Reik A, Liu P-Q, et al. Targeted gene addition in human CD34⁺ hematopoietic cells for correction of X-linked chronic granulomatous disease. *Nat Biotechnol* 2016;34:424–429.
- Merling RK, Kuhns DB, Sweeney CL, et al. Gene-edited pseudogene resurrection corrects p47^{phox}-deficient chronic granulomatous disease. *Blood Adv* 2016;1:270.
- De Ravin SS, Li L, Wu X, et al. CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. *Sci Transl Med* 2017;9(372).
- Pollock JD, Williams DA, Gifford MAC, et al. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 1995;9:202–209.
- Dinauer MC, Li LL, Björngvinsdóttir H, et al. Long-term correction of phagocyte NADPH oxidase activity by retroviral-mediated gene transfer in murine X-linked chronic granulomatous disease. *Blood* 1999;94:914.
- Dinauer MC, Gifford MA, Pech N, et al. Variable correction of host defense following gene transfer and bone marrow transplantation in murine X-linked chronic granulomatous disease. *Blood* 2001; 97:3738.
- Sadat MA, Pech N, Saulnier S, et al. Long-term high-level reconstitution of NADPH oxidase activity in murine X-linked chronic granulomatous

- disease using a bicistronic vector expressing gp91^{phox} and a ΔLNGFR cell surface marker. *Hum Gene Ther* 2003;14:651–666.
14. Takeuchi Y, Takeuchi E, Ishida T, et al. Curative haploidentical BMT in a murine model of X-linked chronic granulomatous disease. *Int J Hematol* 2015;102:111–120.
 15. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-*scid* *IL2Rγ*^{null} mice engrafted with mobilized human hematopoietic stem cells. *J Immunol* 2005; 174:6477–6489.
 16. Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337:816–821.
 17. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013;339:819–823.
 18. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science* 2013;339:823–826.
 19. Wang H, Yang H, Shivalila Chikdu S, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013;153:910–918.
 20. Hwang WY, Fu Y, Reyon D, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013;31:227–229.
 21. Bae S, Kweon J, Kim HS, et al. Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods* 2014;11:705–706.
 22. Sweeney CL, Merling RK, Choi U, et al. Generation of functionally mature neutrophils from induced pluripotent stem cells. *Methods Mol Biol* 2014;189–206.
 23. Choi U, Thom RE, Kang EM, et al. Development of a pCL20c-backbone based HIV lentivirus vector that achieves efficient functional correction of neutrophils arising from transduced CD34⁺ hematopoietic progenitor cells of patients with X-linked chronic granulomatous disease. *Mol Ther* 2010;18 (Suppl 1):S137–138.
 24. Hanawa H, Kelly PF, Nathwani AC, et al. Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol Ther* 2002;5:242–251.
 25. Peto R, Peto J. Asymptotically efficient rank invariant test procedures. *J R Stat Soc Ser A* 1972; 135:185–207.
 26. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457–481.
 27. Li F, Cowley DO, Banner D, et al. Efficient genetic manipulation of the NOD-*Rag1*^{-/-}*IL2Rγ*^Δ mouse by combining *in vitro* fertilization and CRISPR/Cas9 technology. *Sci Rep* 2014;4:5290.
 28. Dagnæs-Hansen F, Kilian M, Fuursted K. Septicaemia associated with an *Aerococcus viridans* infection in immunodeficient mice. *Lab Anim* 2004; 38:321–325.
 29. Foreman O, Kavirayani AM, Griffey SM, et al. Opportunistic bacterial infections in breeding colonies of the NSG mouse strain. *Vet Pathol* 2011;48:495–499.

Received for publication January 6, 2017;
accepted after revision February 25, 2017.

Published online: March 6, 2017.