1   **Lentiviral gene therapy for X-linked chronic granulomatous disease**

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Introductory Paragraph

Chronic granulomatous disease (CGD) is a rare inherited disorder of phagocytic cells[1,2]. We report initial results of nine severely affected X-linked CGD (X-CGD) patients who received *ex vivo* autologous CD34+ hematopoietic stem and progenitor cell-based lentiviral gene therapy following myeloablative conditioning in first-in-man studies (Trial registry numbers: NCT02234934, NCT01855685). Primary objectives were to assess safety and evaluate efficacy and stability of biochemical and functional reconstitution in the progeny of engrafted cells at 12 months. The secondary objectives included the evaluation of augmented immunity against bacterial and fungal infection, and assessment of hematopoietic stem cell transduction and engraftment. Two enrolled patients died within 3 months of treatment from pre-existing comorbidities. At 12 months, six of the seven surviving patients demonstrated stable vector copy number (0.4–1.8 copies/ neutrophil) and persistence of 16–46% oxidase-positive neutrophils. There was no molecular evidence of clonal dysregulation or of transgene silencing. Surviving patients have had no new CGD-related infections, and six have been able to discontinue CGD-related antibiotic prophylaxis. The primary objective was met in 6 of the 9 patients at 12 months follow-up suggesting that autologous gene therapy is a promising approach for CGD patients.

Introduction

X-CGD is caused by mutations in *CYBB* encoding the gp91phox subunit of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX2). Patients are susceptible to recurrent life-threatening infections, impacting their quality of life and life-expectancy. Allogeneic hematopoietic stem cell transplantation (HSCT) can be curative, and recent advances have improved the results from this treatment considerably[3,4]. Clinical trials of hematopoietic stem and progenitor cell (HSPC) gene therapy were first initiated with gammaretroviral (γ-RV) vectors.[5,6]. However, clonal expansion of gene-corrected cells mediated by potent enhancer elements in the γ-RV long-terminal repeats (LTRs)[5,7], eventually lead to leukoproliferative complications. In addition, CpG dinucleotide promoter methylation lead to silencing of transgene expression[7]. To retain the efficacy of gene therapy for X-CGD, but minimize mutagenic risk, a self-inactivating lentiviral vector called G1XCGD was developed (Fig. 1a, full sequence shown in Extended Data 1). To enhance the safety of this vector, a novel chimeric internal promoter was used to preferentially drive gp91phox expression at high levels in phagocytes [8,9]. Complementary clinical studies were initiated, including a multicenter trial in the USA, a UK study and compassionate-use program with nearly identical clinical protocols, eligibility criteria, myeloablative conditioning, stem cell product manufacturing methods, vector batches, and post-transplant analyses.

Results

Recovery of functional oxidase activity in patients. Ongoing clinical studies of gene therapy with G1XCGD were initiated in the UK (*n* = 3, plus one compassionate-use patient) and in the USA (*n* = 5). Dates of therapy are provided in Table 1. Patients 1, 3, 5, and 9 were treated in the UK. Patients 2, 4, 6, 7, and 8 were treated in the USA. The patients were all male with severe deficiency of gp91phox and absent NADPH-oxidase activity. They ranged in age from 2 to 27 years, and six of the nine were >18

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71 years of age at entry. All patients had clinical histories of severe X-CGD-related infections, some active at

72 the time of treatment, and several had chronic inflammatory complications (Table 1). Patients were

73 followed until death or for a minimum of 12 months, with a maximum follow-up of 36 months. Drug

74 products were manufactured from granulocyte colony-stimulating factor (G-CSF) and Plerixafor-

75 mobilized leukaphereses, and infused after myeloablative conditioning (Table S1, see online methods).

76 Drug product infused for the nine patients achieved final $CD34^+$ cell doses of $6.5–32.6×10^6$/kg (Table S2,

77 Extended Data 2).

78 Within 1 month of gene therapy, corrected circulating neutrophils were detectable in peripheral blood

79 (Fig. 2a, 2b; assays conducted in a subset of patients, once at each time point for each patient).

80 Dihydrorhodamine (DHR) fluorescent assays were applied serially to follow the levels of corrected

81 neutrophils. DHR+ activity was observed in >15% of polymorphonuclear neutrophils in all patients within

82 1 month Fig. 2c and Extended Data 3a. Follow-up demonstrated sustained, stable persistence of

83 oxidase-positive neutrophils in six of seven surviving patients; the percentage of oxidase-positive

84 neutrophils at 12 months was 16–46% in these individuals, indicating that the primary objective of

85 evaluating the efficacy in the progeny of engrafted cells and stability at 12 months had been met.

86 Expression of transgene-derived gp91phox was confirmed by flow cytometry as occurring in a

87 percentage of circulating neutrophils that paralleled the DHR data (data not shown). Quantification of

88 NADPH-oxidase generation of superoxide was performed by measurement of neutrophil-stimulated

89 reduction of ferricytochrome c and corrected for the percentage of functional cells. These results

90 demonstrated that the level of activity was within, or just below, the expected normal range of

91 superoxide production per activated neutrophil (Fig. 2d; assays conducted in a subset of patients, once

92 at each time point for each patient), further strengthening the conclusion that the primary objective

93 relating to efficacy was met.

94   **Vector integration.** Following reinfusion, VCN remained stable in neutrophils in six patients over the

95   course of follow-up, suggestive of successful transduction and engraftment of HSPCs (Fig. 2e, Extended

96   Data 3b). In general, the percentage of DHR-positive neutrophils increased linearly with granulocyte VCN

97   (Fig. 2f). VCN also remained stable in other cell lineages (Extended Data 4). One patient (Patient 5, who

98   required three attempts to collect CD34+ cells) had a high initial VCN and a high percentage of DHR-

99   positive neutrophils, but a decrease in VCN and a parallel decrease in the percentage of oxidase-positive

100   neutrophils (<0.5%) over 18 months of follow-up. The patient remained clinically well at last follow-up

101   on prophylactic antimicrobials.

102   Longitudinal analysis of vector integration-site distributions was carried out for the nine patients

103   over 3 months–3 years of sampling. More than 106 million sequence reads identified 724,685 unique

104   integration sites in multiple cell types (Fig. 3a). Lentiviral vector integration was favored in transcription

105   units and transcription-associated features, as seen previously[10]. The analysis documented highly

106   polyclonal populations of gene-modified cells; after 1 year, an average of 9,482 unique integration sites

107   were detected in peripheral blood mononuclear cells (PBMCs; assayed in Patients 2, 4, 6 and 7).

108   Mathematical reconstructions of population sizes using Chao1 estimation[11] with PMBC samples from

109   these same four patients 12 months after treatment suggest an average of at least 69,034 progenitor

110   cells were delivering gene-corrected cells to peripheral blood at 1 year (Extended Data 5). In a previous

111   gene therapy trial to treat CGD, which used a γ-RV vector, clones with integration sites in MECOM

112   (MDS/EVI1) expanded by 1 year in peripheral blood to comprise more than 20% of cells in the first

113   patient and more than 80% of cells in the second[7], and were implicated in later adverse events. In this

114   study, with the lentiviral G1XCGD vector and assayed in neutrophils, the most abundant clones did not

115   harbor integration sites in or near these genes (Fig 3b), and no clone at genes of concern in the previous

116   trial expanded to comprise more than 0.3% of the total population in any cell type (Extended Data 6). In

117   addition, there was no evidence of significant gene silencing, nor of CpG dinucleotide methylation in

118   vector regulatory sequences (Fig. 3c)

119   **Clinical outcomes.** The infusion of the medicinal drug product containing genetically modified cells was

120   well tolerated and there were no infusion-related adverse events. All patients experienced typical

121   conditioning-related events, including transient neutropenia, thrombocytopenia and/or mucositis. There

122   were two deaths: one (Patient 1) due to hyperacute sterile pneumonitis 9 weeks after infusion of gene-

123   corrected cells on a background of prior extensive *Aspergillus* lung disease and pneumonectomy; and

124   the other (Patient 8) due to a fatal intracranial bleed post-transplant associated with refractory

125   autoimmune platelet destruction precipitated by previous alloimmunization to multiple granulocyte

126   transfusions. Pre-mortem magnetic resonance imaging showed the bleed to be centered at a site of

127   metastatic fungal infection. These deaths were not considered related to the drug product. There was

128   one serious adverse reaction, consistent with similar cases in other diseases treated with gene therapy

129   and transplant[12,13]: Patient 5 experienced immune reconstitution inflammatory syndrome at initial

130   engraftment of functional neutrophils, manifesting as a pericardial effusion and abdominal pain, which

131   was fully resolved with steroid cover. In the remaining patients, there were two adverse events

132   recorded after transplant: one patient (Patient 2) developed transient symptoms of gastric outflow

133   obstruction 3–4 months after gene therapy that had been a recurrent pre-transplant problem; there

134   was no evidence of outflow obstruction on endoscopy and symptoms did not recur. Another patient

135   (Patient 6) had several spontaneous pneumothoraces, related to pre-transplant bronchiectasis,

136   established pulmonary fibrosis and was markedly lymphopenic at enrollment, having received several

137   courses of corticosteroid for inflammatory pulmonary disease. Of the patients who had colitis at some

138   time prior to gene therapy, none have had clinical recurrences to date. In one patient (Patient 3) who

139   had active colitis with a perianal fistula and perineal ulceration at the time of gene therapy, there was

140   resolution of the lesion soon after gene therapy with no recurrence.

After 2,036 patient days (approximately 66 patient months), all seven surviving patients remained clinically well without new CGD-related infections. The six patients with stable DHR+ neutrophils >10% were no longer receiving CGD-related prophylactic antibiotic or antifungal treatment (some patients continued to receive penicillin V or equivalent prophylaxis per individual institution-specific protocols for patients who received myeloablative conditioning). Patient 6 remained on antibiotic prophylaxis due to pre-existing lung disease and lymphopenia but is currently off antifungal therapy (Table 1). The secondary objectives to assess immunologic reconstitution and patient health (US protocol) and immunity against bacterial and fungal infection (UK protocol) were therefore met.

Discussion

Following gene therapy, all patients with successful engraftment remain stable through to the last follow-up (up to 3 years in three patients). Seven patients remained free of new infectious complications, and six had sustained presence of neutrophils and restored NADPH-oxidase activity with no evidence of transcriptional silencing of the integrated vector genome or clonal expansion. Two patients died within 3 months of gene therapy likely due to pre-existing disease-related complications present at transplant and unrelated to the drug product itself. . The other seven patients have remained well, apart from persistent chronic fibrotic pulmonary problems in one individual (Patient 6).

One patient (Patient 5), a pediatric patient, initially achieved high levels of neutrophil recovery although the levels of activity declined over several months, suggesting that only a low frequency of long-term transduced HSCs had engrafted, possibly as a result of chronic inflammation[14]. This patient had also received long-term linezolid, an antimicrobial agent with known myelosuppressive activity[14], which may also have contributed to difficulty recovering sufficient CD34+ cells for transduction, requiring three separate attempts. Since this analysis was performed, four additional patients (aged 3, 8, 11, and 31 years) have been treated in the USA with cryopreserved cells, with no product-related complications. At the last follow-up (2–9 months post-treatment), all were well, with no new CGD-

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165   related infections. While the additional adult patient has sustained high levels of DHR+ neutrophils

166   (77.2% at 6 months), the response of the newly treated pediatric patients has been similar to that of

167   Patient 5 with initial high levels of neutrophil recovery followed by a decline. It is unclear at present why

168   the engraftment of gene-marked cells was poor in these pediatric patients and whether the mechanisms

169   were similar (due to pre-existing disease and concomitant drug therapies) or unrelated (immunological

170   or technical).

171   Early attempts at gene therapy for CGD with γ-RV vectors achieved some transient therapeutic

172   benefits, but in several cases were associated with clonal leukoproliferation[5,615]. Here, integration-site

173   analysis in blood cells revealed highly polyclonal engraftment of gene-corrected stem cells, with no

174   clonal expansion associated with enriched integration near cancer-associated genes[5,7] (Fig. 3a, 3b and

175   Extended Data 4). In a previous γ-RV trial, methylation of CpG dinucleotides within the vector promoter

176   sequence resulted in silencing of gene expression. The chimeric myeloid promoter in G1XCGD responds

177   to transcription factors present mostly in mature myeloid cells and drives sufficient expression of

178   gp91phox to reconstitute oxidase production in blood granulocytes and monocytes[8]. No silencing or

179   methylation was detected. In each patient, all blood cell lineages had similar levels of gene marking,

180   with the exception of T cells that had lower frequencies and lower diversity of vector integrants

181   (Extended Data 3). This latter finding may reflect a lack of lymphodepletion in the conditioning regimen.

182   The level of reconstitution of phagocyte oxidase function required to mediate a meaningful

183   clinical effect can be estimated from studies of female carriers of X-CGD, patients with X-CGD with small

184   amounts of residual NADPH-oxidase activity (who have been shown to exhibit a significant survival

185   advantage compared with patients with complete absence of oxidase activity), and from preclinical

186   vector assessments[6,16-18]. On that basis, we predict that long-term functional correction of more than

187   10% of circulating myeloid cells with oxidase activity per cell approaching the normal range will provide

188   lasting clinical benefit. The level of benefit may approach normal resistance to infection at or above 20%

189   of circulating myeloid cells corrected, although individual factors may be influential. In this study, a

190   target threshold of 10% was demonstrated in the six surviving adult patients, and above 20% in five of

191   these six surviving adult patients. Quantitative assessment of superoxide production in engrafted

192   neutrophils confirmed sustained activity. Clinical efficacy could in the future be improved with higher

193   titer vector preparations or the use of transduction enhancers, and by avoiding the loss of primitive

194   HSPC in the autologous graft, which may already be compromised by chronic inflammation[19,20].

195   The advantages of autologous HSC gene therapy over HSCT includes the avoidance of graft-

196   versus-host disease and other alloreactive complications, and reduced complexity of conditioning

197   regimen. These results demonstrate promising effective autologous gene therapy in severely affected

198   patients with X-CGD without evidence of genotoxicity. Further studies are warranted to formally assess

199   longer term clinical efficacy and safety of G1XCGD in patients with X-CGD.

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227   **Author contributions**

228 The manuscript was written primarily by D.B.K. (Kohn), A.J.T., D.A.W. and H.L.M. The VISA analyses were

229 performed by F.D.B., J.G., H.E.R. and J.K.E. M.G. and G.S. helped to construct the initial vector. G.H. and

230 A.G. provided key resources for the conduct of studies including clinical-grade vector, preclinical data

231 package for CTA, study monitoring and pharmacovigilance and review of the manuscript. A.G.

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233 C.Y.K., D.L.-R., C.R., N.I., K.G., K.S., J.X-B.D., J.D., E.C.M., D.T., L.D.W., C.A.B., T.P., D.B.K. (Kuhns), L.B.,

234 P.E.N., and H.B.G. were involved in manufacturing the drug product, delivering the drug to patients for

235 their respective clinics or performing analyses of patient samples. All authors had full access to all data,

236 statistical reports and tables in the study and can take responsibility for the integrity of the data and

237 accuracy of the data analysis. All authors approved the final manuscript.

238 **Competing interests**

239 D.B.K. (Kohn), H.L.M., D.A.W. & A.J.T. are Scientific Advisory Board members and H.B.G. is Chief

240 Scientific Officer for Orchard Therapeutics. H.B.G. is an employee and equity/stock holder for Orchard

241 Therapeutics. A.J.T. is an equity/stock holder for Orchard Therapeutics. Orchard Therapeutics has

242 obtained an exclusive option to license from Genethon for the rights and know-how related to the

243 lentiviral vector G1XCGD. C.A.B. and T.P. consult for a sequencing service provider, and C.A.B. consults

244 for Novimmune and SOBI. Eurofins Genomics Sequencing Europe (formerly GATC Biotech AG) is a for-

245 profit company (sequencing service provider). The work performed by Eurofins Genomics Sequencing

246 Europe included in the manuscript is provided to the greater scientific community as a fee for service

247 product. E.C.M. reports Advisory Board attendance for Orchard Therapeutics.

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Data availability

257   All relevant data are included in the paper. Additional supporting data areavailable from the

258   corresponding authors upon request. All requests for raw and analyzed data and materials will be

259   reviewed by the corresponding authors to verify if the request is subject to any intellectual property or

260   confidentiality obligations. Patient-related data not included in the paper were generated as part of

261   clinical trials and may be subject to patient confidentiality.

Statement on the use of human embryos, gametes and stem cells

263   All experiments were performed in accordance with relevant guidelines and regulations. Informed

264   consent was obtained from all recipients or their guardians. The protocol and informed consent

265   documents were reviewed and approved or accepted by the US National Institutes of Health

266   Recombinant DNA Advisory Committee, the UK Gene Therapy Advisory Committee, and the local

267   institutional ethical and biosafety review boards at all participating clinical sites. The studies were

268   performed in the US under Food and Drug Administration-approved IND BB#16141, Medicines and

269   Healthcare products Regulatory Agency (MHRA) approved EudraCT Number 2012-000242-35 in the UK

270   and are registered at Clinicaltrials.gov ([USA] NCT02234934, [UK] NCT01855685).

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323   **Table 1. Patient demographics, pre-transplant conditioning, and outcome results**

Pt. #	Center	Age (y)	Race (ethnicity)	X-CGD (CYBB) mutation	Prior medical history	Date of gene therapy (MM/YY)	Status at gene therapy	Busulfan conditioning AUC (ng/mL*h)	DHR+ granulocytes, %		Status at last follow-up
									At 12 months	At last follow-up	
1	GOSH	4	Caucasian	c.1027A>C (p.Ther343Pr)	Pneumonectomy, EBV, *Aspergillus*, organomegaly, lymphadenopathy	7/13	Ongoing fever, organomegaly and lymphadenopathy	75873	−	12 (3 mo)	Death (at 3 mo) due to hyper-acute idiopathic pneumonitis
2	BCH	22	Caucasian	c.664 C>T (p.His222Tyr)	Klebsiella lymphadenitis, MRSA liver abscess, lung abscess, Nocardia abscess, Serratia abscess, GI and urethral granulomas	12/15	No active infections at gene therapy	65117	16	13 (24 mo)	Clinically well, off antibiotic prophylaxis
3	GOSH	18	Mixed	c.1234_1257dup (p.Gly412_Ile419dup)	Osteomyelitis, cerebral hemorrhage colitis, perianal fistula, perineal ulceration	7/16	Suspected fungal chest infection	75668	29	31 (24 mo)	Clinically well, off CGD-related antibiotic prophylaxis (remains on azithromycin)
4	NIH	18	Caucasian (Hispanic)	c.1169 C>T (p.Pro390Leu)	Burkholderia cepacia Nocardia and	7/16	Stable	66,191	28	25 (24 mo)	Clinically well, off antibiotic prophylaxis

Pt. #	Center	Age (y)	Race (ethnicity)	X-CGD (CYBB) mutation	Prior medical history	Date of gene therapy (MM/YY)	Status at gene therapy	Busulfan conditioning AUC (ng/mL*h)	DHR+ granulocytes, %		Status at last follow-up
									At 12 months	At last follow-up	
					Aspergillus pneumonias						
5	GOSH	2	Asian (Korean)	c.469C>T (p.Arg157X)	Liver and splenic abscesses	4/17	Refractory spleen, liver abscesses, treatment with linezolid	73337	0.5	1 (24 mo)	Clinically well; receiving antimicrobial support (oral clindamycin and clarithromycin); weaned off steroids; gut improvement
6	UCLA	27	Asian	c.1314+2 T>A (Homozygous T>A at splice site exon 10 ([downstream 2 bases])	*Aspergillus*, Nocardia and Serratia pneumonia, IBD, Crohn's disease	4/17	Bronchiectasis and pulmonary fibrosis, lymphopenia	67666	36	63 (24 mo)	Infection-free, off CGD-related antibiotic prophylaxis (remains on TMP/SMX); has pneumothoraces requiring treatment (pre-existing condition); Crohn's and IBD improved (no active inflammation)

Pt. #	Center	Age (y)	Race (ethnicity)	X-CGD (CYBB) mutation	Prior medical history	Date of gene therapy (MM/YY)	Status at gene therapy	Busulfan conditioning AUC (ng/mL*h)	DHR+ granulocytes, %		Status at last follow-up
									At 12 months	At last follow-up	
7	NIH	24	Black/ African American	c.676 C>T (p.Arg226x)	Multiple bacterial and fungal lung infections, lobectomy (lung), granulomatous liver, GI, recurrent lymphadenitis	6/17	Chronic persistent culture-positive Phellinus fungal pneumonia	77378	46	49 (24 mo)	Clinically well, off antibiotic prophylaxis
8	NIH	3	Caucasian (Hispanic)	c.374 G>A (p.Trp125*Ter*)	BCGosis. >1yr hx of disseminated *Aspergillus* (bone, lungs, CNS)	8/17	Resistant *Aspergillus* at gene therapy. Status post: multiple granulocyte transfusions	62349	—	44 (1 mo)	Death (at 1 mo) from cerebral bleed into pre-existing fungal infection site (pre-existing antiplatelet antibodies impeding post-conditioning platelet transfusions)
9	GOSH	22	Caucasian	c.271 C>T (p.Arg91X)	Cerebral Aspergilloma, colitis, warts, granulomatous folliculitis; interstitia	9/17	Steroid-dependent colitis. Multifocal inflammatory changes on HRCT	75000	35	40 (24 mo)	Clinically well, off CGD-related antibiotic prophylaxis (remains on TMP/SMX); colitis resolved; being weaned

Pt. #	Center	Age (y)	Race (ethnicity)	X-CGD (CYBB) mutation	Prior medical history	Date of gene therapy (MM/YY)	Status at gene therapy	Busulfan conditioning AUC (ng/mL*h)	DHR+ granulocytes, %		Status at last follow-up
									At 12 months	At last follow-up	
					nephritis		chest				off oral steroid; HRCT chest findings resolved; intermittent skin rashes persist

AUC, area under the curve; BCH, Boston Children's Hospital, USA; CGD, chronic granulomatous disease; CNS, central nervous system; DHR+, dihydrorhodamine-positive granulocytes; EBV, Epstein-Barr virus; GI, gastrointestinal; GOSH, Great Ormond Street Hospital, UK; HRCT, high-resolution computed tomography; hx, history; IBD, inflammatory bowel disease; MRSA, Methicillin-resistant *Staphylococcus aureus*; NIH, National Institutes of Health, USA; TMP/SMX, trimethoprim-sulfamethoxazole UCLA, University of California, Los Angeles, USA; X-CGD, X-linked chronic granulomatous disease;

329 **Figure legends**

330 **Fig. 1. Materials and methods (vector map and procedures)**

331 **Panel a** displays the schematic representation of the G1XCGD lentiviral vector (LV) used to transduce

332 CD34+ peripheral blood and bone marrow stem and progenitor cells in which expression of a codon-

333 optimized human *CYBB* cDNA encoding for gp91phox is controlled by a chimeric regulatory element

334 containing the Cathepsin G and Cfes gene promoter/enhancers, with a downstream WPRE to boost

335 expression. **Panels b** and **c** show the schemas for the fresh cell and frozen/cryopreserved cell

336 procedures, respectively. Differences between the two procedures include the timing of the primary

337 harvest and back-up 'rescue' harvest of CD34$^+$ cells, the timing of G1XCGD vector addition, and that the

338 cryopreservation procedure allowed full cell product characterization and release criteria to be met

339 before cytoreductive conditioning was performed.

340 AUC, area under the curve; BM, bone marrow; CatG/Cfes, *CTSG* encoding Cathepsin G and the *FES* gene

341 encoding Cfes; COA, certificate of analysis; G-CSF, granulocyte colony-stimulating factor; LV, lentiviral

342 vector; mPB, mobilized peripheral blood; WPRE, Woodchuck hepatitis virus post-transcriptional

343 regulatory element

344 **Fig. 2. Biochemical and clinical evidence of successful engraftment**

345 **Panel a** shows the nitro-blue tetrazolium (NBT) test to detect functional circulating neutrophils from a

346 peripheral blood sample from Patient 2 pre-gene therapy (left) and at 3 months after receiving gene

347 therapy with G1XCGD (right). Generation of superoxide leads to reduction of NBT and formation of dark

348 blue formazan precipitates in the observed cells. The scale bar measures approximately 10 micrometres;

349 mature human neutrophils on a blood smear have an average diameter of 12–15 micrometres. **Panel b**

350 shows the results of DHR fluorescence flow cytometry assaying functional oxidase activity in neutrophils

351 over a 2-year period in Patient 3 post-gene therapy, and in a healthy control. After neutrophil

352 stimulation with PMA (right), the fraction of DHR+ neutrophils were quantified. The percentage of DHR+

353   neutrophils in all treated patients at each time point is shown in **Panel c.** NAPDH-oxidase activity was

354   quantified by measurement of neutrophil-stimulated reduction of ferricytochrome c, corrected for the

355   percentage of oxidase-positive cells. Data for Patients 2, 4 and 7 are shown in **Panel d** following 10

356   minutes and 60 minutes of stimulation with PMA. The lower limit of normal superoxide generation for

357   each timepoint is indicated by the dashed line. **Panel e** shows the neutrophil VCN for all patients

358   measured at each time point for which data were available. VCN remained stable for six of seven

359   surviving patients but decreased over time for one patient (Patient 5), who remains clinically well at

360   follow-up with antimicrobial support. **Panel f** shows the percentage of DHR+ neutrophils versus

361   granulocyte VCN for the seven surviving patients at 12 months. R^2=0.44; the dashed line represents 10%

362   DHR.

363   NBT, nitroblue tetrazolium; DHR, dihydrorhodamine; GT, gene therapy; NL, normal; PMA, phorbol

364   myristate acetate; VCN, vector copy number

365   **Fig. 3. Analysis of vector integration-site distributions and promotor methylation**

366   **Panel a** shows longitudinal analysis of unique cell clones contributing to each cell type, inferred from

367   counts of unique integration sites. The x-axis shows time since cell infusion. D0 indicates the pre-

368   infusion product. The y-axis shows the numbers of unique integration sites (log scale). Cell types are

369   color coded (bottom). For a few patients a reduced number of cell types were available for analysis.

370   **Panel b** is a heat map illustrating the most abundant clones in each patient and their longitudinal

371   behavior. Neutrophils were selected for this analysis because previous adverse events in CGD gene

372   therapy with γ-RV vectors involved outgrowth of myeloid cells. The x-axis shows the time post-

373   treatment. The rows show cell clones, named by the nearest human gene (labels on left of figure). The

374   relative abundance is shown by the heat map scale (bottom of figure). Quantification was carried out

375   using fragment lengths to estimate abundance [11]. In **Panel c**, methylation of CpG dinucleotides is shown

376   in Patients 2, 3 and 9 at 2.5 years, 18 months and 9 months post-gene therapy, respectively. The x-axis

377   shows the positions of CpG dinucleotides relative to the gp91 mini-gene. The y-axis shows the

378   percentage of methylation at each position. The methylation levels across the CpG islands are low for all

379   samples, indicating that the gp91 mini-gene is not transcriptionally repressed.

380   CGD, chronic granulomatous disease; MSP, myeloid-specific promoter; NK, natural killer; PBMC,

381   peripheral blood mononuclear cell; PCR, polymerase chain reaction; VISA, longitudinal vector

382   integration-site analysis.

Methods

384   **Study design and investigational therapy**. Inclusion and exclusion criteria for the UK and US studies are

385   detailed in Supplementary Table S1 In brief, male patients with X-linked chronic granulomatous disease

386   (X-CGD), aged 2 years and older (>6 months for UK protocol), with molecular diagnosis of X-CGD

387   confirmed by DNA sequencing and supported by laboratory evidence for absent or significantly reduced

388   biochemical activity of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, and absence

389   of 10/10 human leukocyte antigen (HLA)-matched donor (sibling or unrelated) were eligible.

390   The primary objective of the UK and US studies included evaluation of safety and the evaluation of

391   efficacy and stability by biochemical and function reconstitution in progeny of engrafted cells at 12

392   months. The secondary objectives differed slightly between the two trials, and included clinical efficacy

393   and evaluation of augmented immunity against bacterial and fungal infection (UK protocol), evaluation

394   of immunologic reconstitution and patient health (US protocol) and assessment of hematopoietic stem

395   cell transduction and engraftment (both protocols). Primary and secondary objectives are detailed more

396   fully in Supplementary Table S1.

397   The protocol and informed consent documents were reviewed and approved or accepted by the US

398   National Institutes of Health Recombinant DNA Advisory Committee, the UK Gene Therapy Advisory

399   Committee, and the local institutional ethical and biosafety review boards at all participating clinical

400   sites. The studies were performed in the USA under Food and Drug Administration-approved

401   investigational new drug (IND) BB#16141; Medicines and Healthcare Products Regulatory Agency

402   (MHRA) approved EudraCT Number 2012-000242-35 in the UK, and are registered at Clinicaltrials.gov

403   ([USA] NCT02234934, [UK] NCT01855685). Genethon sponsored the UK trial and acquired exclusive

404   rights for the commercial exploitation of the data generated under the US trial; during the trial, Orchard

405   Therapeutics obtained an exclusive license from Genethon to certain rights and intellectual property

406   related to the lentiviral vector G1XCGD, including clinical data, and named this program OTL-102.

407   The procedural schemas for the clinical trials are shown for the use of either fresh cells or

408   cryopreserved cells (Fig. 1b, 1c). Cryopreserved cells were used increasingly over fresh cells as the

409   studies progressed. The UK sites started using cryopreserved cells in 2016 and the US followed in 2018

410   in patients treated after those included in this manuscript. This was primarily for safety reasons as

411   cryopreserved cells gave an extended shelf-life, which allowed full cell product characterization to be

412   performed prior to initiation of conditioning. Following informed consent and eligibility confirmation,

413   CD34+ cells recovered by bone marrow or apheresis were transduced ex vivo by the G1XCGD lentiviral

414   vector. Patients received busulfan myeloablative conditioning with pharmacokinetic monitoring. Drug

415   products were infused intravenously through a central venous line over 30–45 minutes. Patient vital

416   signs and clinical condition were monitored closely during and after the infusion for adverse reactions.

417   **G1XCGD lentiviral vector production**. Clinical lots of G1XCGD lentiviral vector21 were produced at

418   Genethon and Yposkesi (Evry, France). 293T cells were transfected with plasmids encoding the G1XCGD

419   vector, HIV-1 gag/pol, HIV-1 rev, and the VSV-G glycoprotein. Culture supernatants were collected and

420   processed using clarification, ion exchange chromatography, tangential flow filtration, gel filtration,

421   formulation in X-Vivo 20 medium (Lonza), and aliquots were cryopreserved22. The titer of the vector

422   preparations ranged between 2.1 and 3.3 E+09 IG (average 2.5 ± 0.5 E+09 IG/mL n = 5) genomes/mL,

423   measured at Genethon using HCT116 colon carcinoma cells. Physical titers ranged between 1.3 and 3.8

424   E+04 ng P24/mL (average 2.4 ± 1.1 E+04 ng P24/mL n = 5) measured by enzyme-linked immunosorbent

425   assay (ELISA). All lots of vector tested negative for replication competent lentivirus and met GMP release

426   specifications.

427   *G1XCGD lentiviral-vector-modified CD34+ cell product manufacturing*. Cells were manufactured onsite at

428   study sites in the US and UK (University of California, Los Angeles, USA; Boston Children's Hospital, USA;

429   National Institutes of Health, USA; Great Ormond Street Hospital, UK). Harvested cells to use as back-up

430   were collected from bone marrow isolation or mobilized peripheral blood. Mobilization of peripheral

431   blood stem cells was accomplished by administration of granulocyte colony-stimulating factor (G-CSF)

432   10 μg/kg subcutaneously (SC) daily × 5 days, with leukapheresis performed on the fifth day. The back-up

433   leukapheresis units were cryopreserved as per each site's standard operating procedure for standard

434   hematopoietic progenitor cell products and stored in the vapor phase of liquid nitrogen (LN2) freezers.

435   At least 1 month later, a second mobilization was performed to collect stem cells to manufacture the

436   drug product. For some patients, cells for the back-up graft were obtained from the same mobilization

437   used for manufacture of the drug product. On the fourth or fifth day of G CSF, a dose of plerixafor

438   0.24 mg/kg was administered intravenously 8–12 hours prior to leukapheresis, with a fifth dose of G-CSF

439   given 2 hours prior to the apheresis. This collection was processed to isolate CD34+ cells using the

440   CliniMACS system (Miltenyi Biotec, Germany). For some patients, a second leukapheresis was performed

441   after a second dose of plerixafor on day 5 or 6, and a sixth dose of G-CSF was given on the sixth day of

442   mobilization protocol.

443   Selected CD34+ cells were placed into cell culture in serum-free medium with recombinant

444   human cytokines (stem cell factor, flt-3 ligand, thrombopoietin and interleukin-3). On the next 2

445   successive days, the G1XCGD vector was added to the cells in culture to a final concentration of 1E+08

446   IG/mL. The following day, the cells were removed from culture, washed twice, and formulated for

447   intravenous administration. Samples were retained at multiple steps during the processing and from the

448   final drug product (FDP). FDP was tested for sterility, gram-stain reactivity, endotoxin, mycoplasma,

449   viability, CD34, and gp91phox surface expression by flow cytometry, colony-forming unit (CFU) numbers,

450   and percentage vector-insert+ CFU by polymerase chain reaction (PCR). Aliquots of the drug product

451   were also grown in vitro for 7 days, followed by extraction of genomic DNA for vector copy number

452   (VCN) determination by quantitative PCR (qPCR) or using digital droplet PCR (ddPCR) to measure the

453   number of vector copies per human genome. In some patients, after regulatory approval of an amended

454   manufacturing protocol, the drug product was cryopreserved in Cryostor 5 (5% dimethyl sulfoxide

455   [DMSO], Sigma Aldrich) in Kryosure 20 bags (Saint-Gobain Performance Plastics), stored in vapor phase

456   of LN2, and thawed at bedside immediately prior to administration.

457   **Cell products and busulfan conditioning**. Drug products were manufactured from G-CSF and Plerixafor-

458   mobilized leukaphereses. A cryopreserved unmanipulated cell fraction was taken as back-up in the

459   event of non-engraftment, and a CD34-selected cell preparation taken for G1XCGD transduction. In one

460   case (Patient 8), freshly collected CD34+ cells were augmented with additional thawed autologous

461   selected CD34+ cells that had been collected and frozen previously, to enable the initial required cell

462   dose for transduction to be achieved. Collection of CD34+ cells was problematic in another patient

463   (Patient 5), requiring three separate attempts (one aborted due to a systemic inflammatory reaction

464   during administration of mobilizing agents). Drug product infused for the nine patients achieved final

465   CD34+ cell doses of 6.5–32.6×106/kg (Table S2 Fig. S2). Transduction efficiency met release criteria for

466   all cell products, with VCN ranging from 0.7 to 5.5 copies per cell (Table S2; Fig. S2b) in infused cells,

467   indicating the secondary objective relating to evaluation of CD34+ hematopoietic cell transduction was

468   met. For some products, the transduction efficiency of colony-forming units grown from the drug

469 product were assessed for the presence of the vector by PCR; between 49.2 and 79.2% of the colonies

470 were PCR-positive (Fig. S2c), indicating the primary objective to assess the efficacy in the progeny of

471 engrafted cells was met. Busulfan was administered with pharmacokinetic drug monitoring and dosage

472 adjustment leading to consistent dosing (Fig. S2d). Fresh cells post-transduction were administered

473 within a few hours from the completion of processing, and cryopreserved cells were thawed at bedside

474 and infused directly.

475 *Pre-transplant cytoreductive conditioning*. For cytoreductive conditioning, patients received intravenous

476 busulfan twice daily for 3 days, starting at a dose of 2.0 mg/kg, and dose-adjusted based on

477 pharmacokinetics to reach a total net area under the curve (AUC) for busulfan of 70,000–75,000

478 ng/mL*h. Serum busulfan levels were measured at intervals (immediately after infusion [time = 0] and

479 at 0.5, 1, 2, 4 and 6 h) following the completion of the first infusion. Subsequent busulfan dosages were

480 adjusted based on pharmacokinetic measurements to reach the target AUC on average over all doses,

481 with busulfan levels re-measured after the final (6th) or penultimate (5th) busulfan dose to allow

482 calculation of total busulfan exposure (Table 1; Fig. S2d).

483 **Follow-up clinical and laboratory assessments**. Upon recovery of neutrophils, follow-up evaluations

484 were performed per the clinical protocol at 1, 2, 3, 6, 9, 12, 18 and 24 months after gene therapy (and

485 continue to be monitored in long-term follow-up). Neutrophil NADPH-oxidase activity was measured by

486 standard methods16. CGD-related antibiotic therapy and antifungal therapies were discontinued when,

487 in the opinion of the clinical team, adequate production of corrected neutrophils was evident and when

488 pre-existing infections were deemed to have been cleared.

489 *Promoter methylation analysis*. Bisulfite conversion, PCR, sequencing, and bioinformatics analysis were

490 performed at Eurofins Genomics Sequencing Europe (Konstanz, Germany). Bisulfite conversion was

491 performed using the Zymo Research EZ 96 DNA Methylation Lightning Mag Prep kit (Zymo Research)

492   according to manufacturer instructions. PCR was performed using a nested primer set to generate a

493   654bp fragment of the promoter region for sequencing. The PCR fragments were prepared for

494   sequencing using the Eurofins Genomics Sequencing Europe standard protocols. Sequencing was

495   performed on an Illumina HiSeq 4000 (Illumina, Inc.) to generate approximately 5 million 150bp read

496   pairs for each sample. The sequencing reads were mapped to the promoter reference sequence using

497   Bismark and Bowtie[23,24]. Methylation analysis was performed using the Bis-SNP adopted modules of

498   GATK[25-27].

499   **Analysis of integration target sites**. For patients 2, 4, 6, 7 and 8, analysis of integration site distributions

500   was carried out as described previously[28,29]. DNA was purified from transduction products prior to

501   infusion or from blood cells from patients sampled longitudinally. DNA was cleaved by sonication, then

502   DNA adaptors were ligated to the broken DNA ends. Two rounds of PCR were carried out to isolate host-

503   vector DNA junction fragments. Each sample was analyzed four times independently to suppress PCR

504   jackpotting. Samples were sequenced using the Illumina MiSeq. Analysis was carried out using the

505   INSPIIRED pipeline[29]. Clonal structure was assessed using the sonic abundance method[30], which uses

506   information on the numbers of linker positions recovered per integration sites to count the numbers of

507   cells sampled.

508   For patients 3, 5, and 9, integration sites were collected from Ficoll-purified granulocytes and

509   fluorescence-sorted lymphocytes and monocytes through linear-amplification mediated (LAM)-PCR and

510   high-throughput Illumina sequencing, as described previously[31,32]. Briefly, genomic DNA from isolated

511   cell types was extracted (QIAamp DNA Blood Mini kit or Micro kit, QIAGEN), and whole-genome

512   amplification was performed (Repli-G Mini Kit, QIAGEN) only on FACS-sorted lymphocytes as described

513   previously[33]. A total of 300 ng of genomic DNA underwent two rounds of linear amplification (100

514   cycles in total) to enrich for vector long terminal repeat (LTR)-genome junctions, which were then

515   captured using 5' biotinylated LTR specific primers and streptavidin magnetic beads. To follow,

516　complementary strand synthesis was performed, then parallel digestion with three different restriction

517　enzymes (MluCI, HpyCH4 IV and Aci I), and ligation to a linker cassette. The resulting fragments were

518　then amplified by two additional exponential PCR steps. These LAM-PCR products were separated by gel

519　electrophoresis on Spreadex high resolution gels (Elchrom Scientific) for visual inspection, and were

520　pooled using barcoded adaptors in a library suitable for Illumina sequencing.

521　　　　Available samples that passed our quality control of a minimum of 100 cells (by break-point

522　analysis) were included in the analysis. The maximum relative clonal abundances in neutrophils was

523　2.3% and PBMC was 1.0% in the samples included.

524

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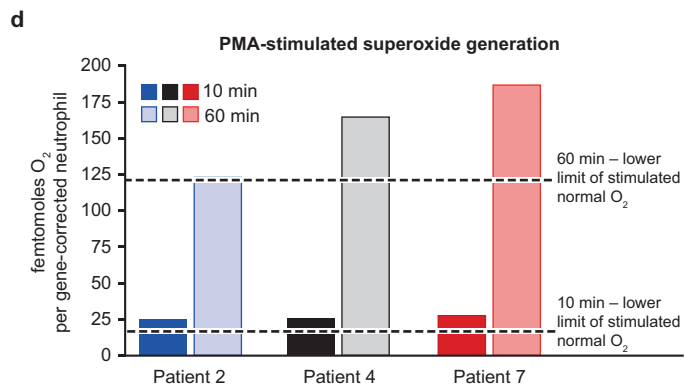556   doi:10.1016/j.stem.2016.04.016 (2016).
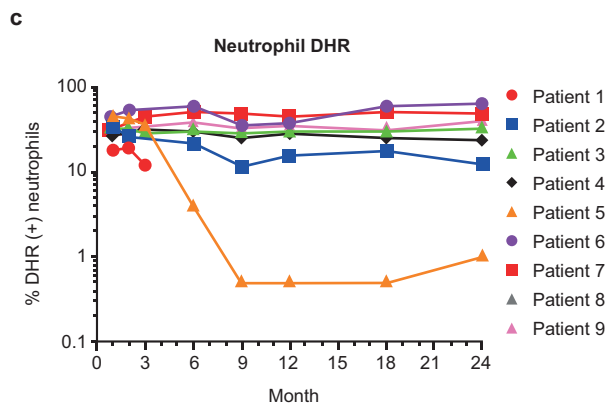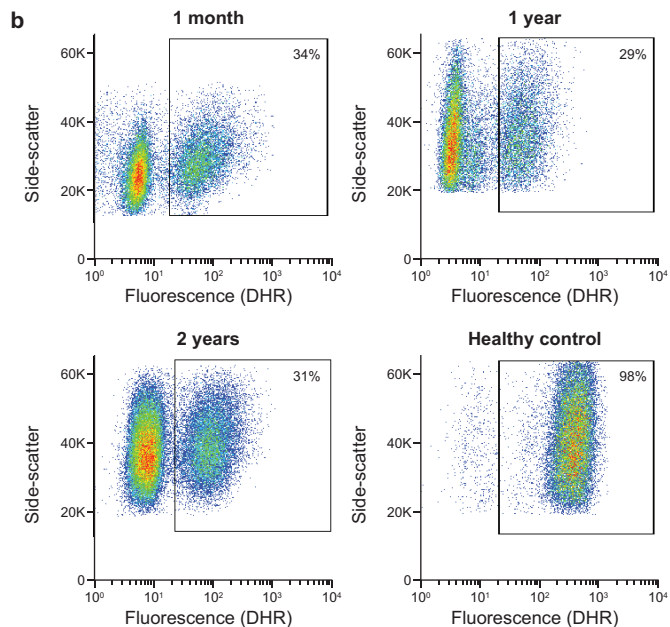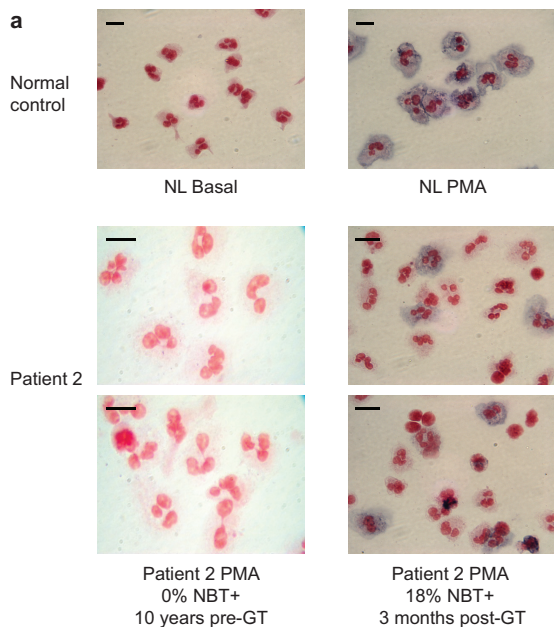
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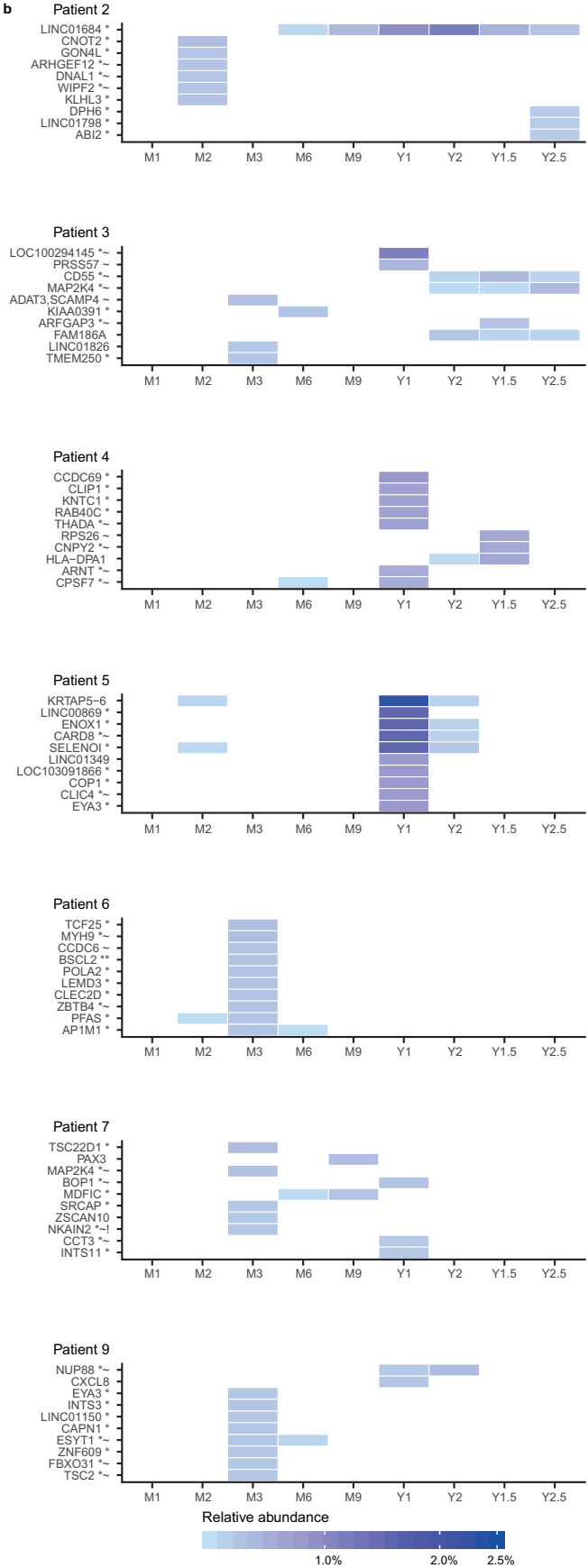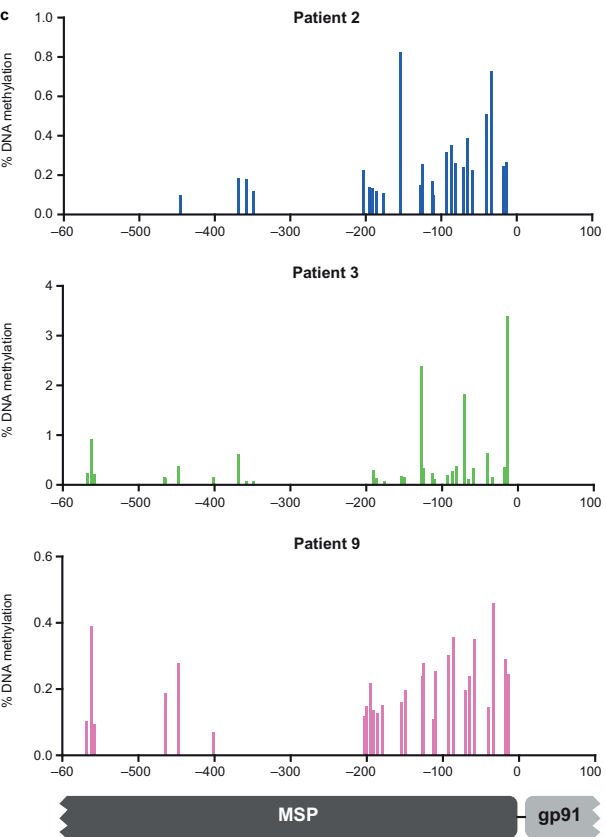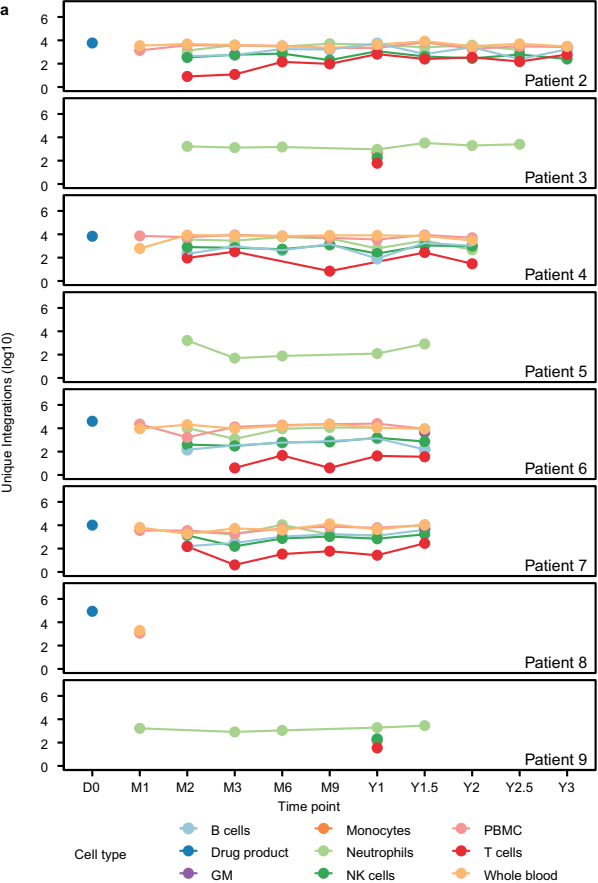559   3 (2018).

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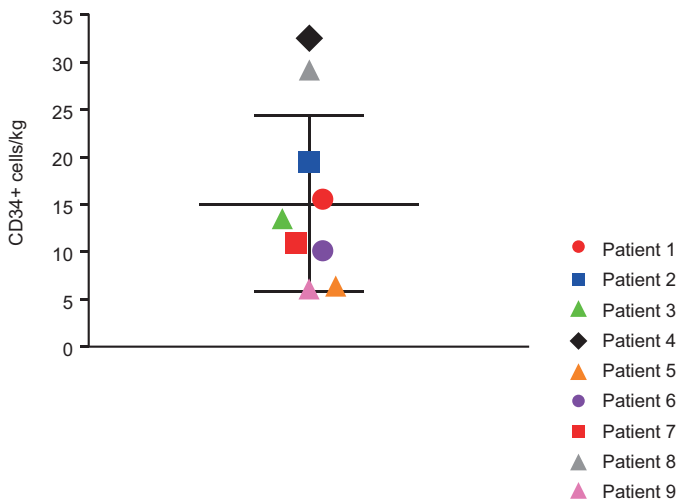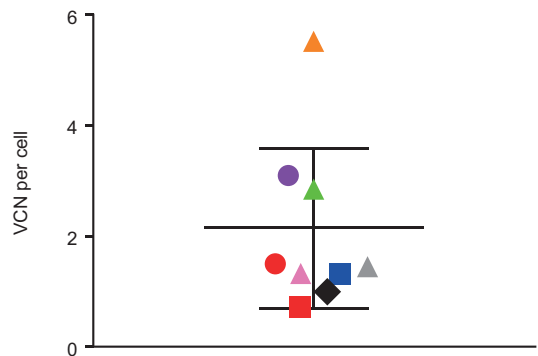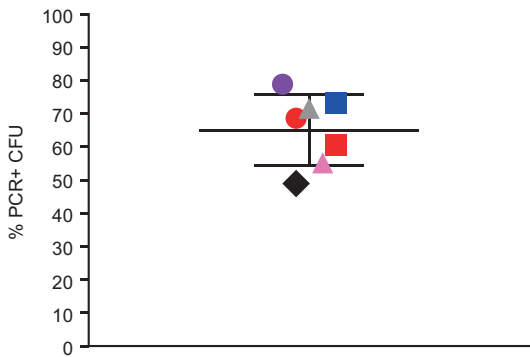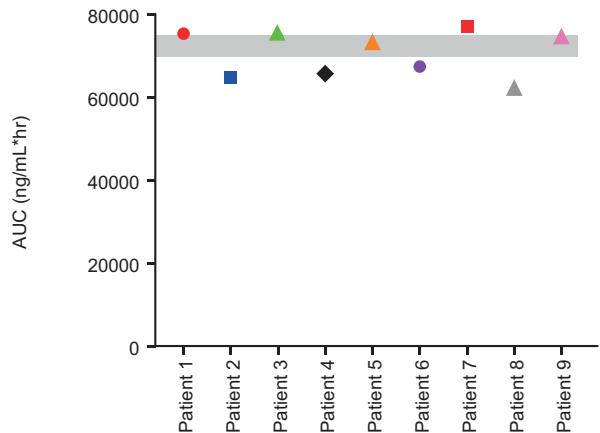562   doi:10.1126/scitranslmed.3010314 (2015).

563

564

a

ΔU3 | R | U5 — ψ RRE — cPPT — CatG/Cfes | hGP91-PHOX — WPRE — ΔU3 | R | U5

CatG/Cfes = Cathepsin G/cfes promoter

b

Admit → Consent Screening Enrolment

G-CSF mPB or BM back-up harvest (≥1x10⁶ CD34+ cells/kg)

G-CSF Pheresis (1x)

(1 month)

BM isolation or G/Plerixafor mPB for genetic modification (≥3x10⁶ CD34+ cells/kg)

G-CSF Plerixafor Pheresis (1–2x)

Busulfan i.v given according to institutional dosing schedule (approx. 3·2–5 mg/kg/day) Dose adjusted to achieve an AUC of 70,000–75,000 ng/mL*h

Infuse drug product

Day –4 (CD34+ isolation) · Day –3 (Pre-stim) · Day –2 (Round 1 vector transduction) · Day –1 (Round 2 vector transduction) · Day 0

c

1st admit → Consent Screening Enrolment

BM isolation or G/Plerixafor mPB for genetic modification (≥3x10⁶ CD34+ cells/kg)

G-CSF (daily) Plerixafor (Day 5) Pheresis (1–2x)

Second collection back-up harvest (≥1x10⁶ CD34+ cells/kg)

Day –4 (CD34+ isolation) · Day –3 (Pre-stim) · Day –2 (Round 1 vector transduction) · Day –1 (Round 2 vector transduction) · Cryopreserve drug product

(4–6 weeks) Issue COA

2nd admit

Busulfan i.v given according to institutional dosing schedule (approx. 3·2–5 mg/kg/day) Dose adjusted to achieve an AUC of 70,000–75,000 ng/mL*h

Day –4 · Day –3 · Day –2 · Day –1 · Day 0

Thaw and infuse drug product

a

Normal control

NL Basal — NL PMA

Patient 2

Patient 2 PMA
0% NBT+
10 years pre-GT

Patient 2 PMA
18% NBT+
3 months post-GT

b

1 month — 34%

1 year — 29%

2 years — 31%

Healthy control — 98%

Side-scatter vs Fluorescence (DHR)

c Neutrophil DHR

% DHR (+) neutrophils vs Month

- Patient 1
- Patient 2
- Patient 3
- Patient 4
- Patient 5
- Patient 6
- Patient 7
- Patient 8
- Patient 9

d PMA-stimulated superoxide generation

femtomoles O_2 per gene-corrected neutrophil

10 min / 60 min

Patient 2, Patient 4, Patient 7

60 min – lower limit of stimulated normal O_2

10 min – lower limit of stimulated normal O_2

e Neutrophil VCN

Neutrophil VCN vs Month

- Patient 1
- Patient 2
- Patient 3
- Patient 4
- Patient 5
- Patient 6
- Patient 7
- Patient 8
- Patient 9

f % DHR (+) vs VCN (1 year)

% DHR (+) vs VCN

- Patient 2
- Patient 3
- Patient 4
- Patient 5
- Patient 6
- Patient 7
- Patient 9

G1XCGD lentiviral vector
pCCL_chimGP91_WPRE_Kana
(9362 bp)

Map labels: junction marker, CMV, R, U5, PSI, RRE, cPPT, CTSG, Chimeric Promoter, FES, coGP91, WPRE, 3'PPT, dU3, R, U5, SV40 ori, KANr, COLE1
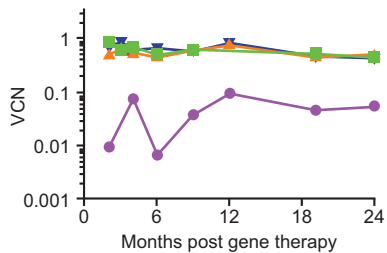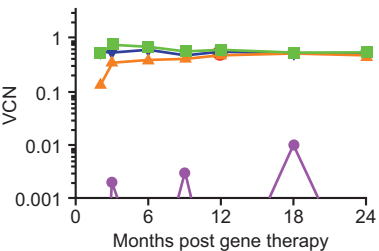
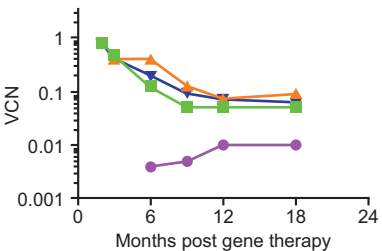**a** CD34+ cells

b VCN

c % PCR+ CFU

d Busulfan AUC

Patient 1
Patient 2
Patient 3
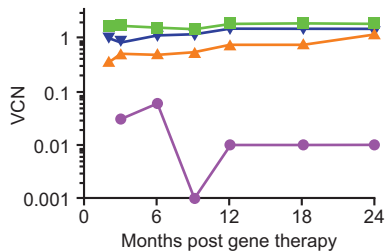Patient 4
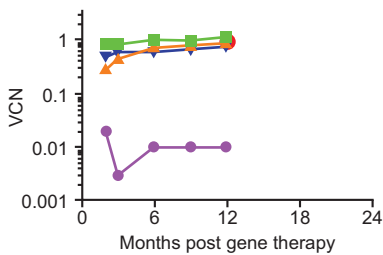Patient 5
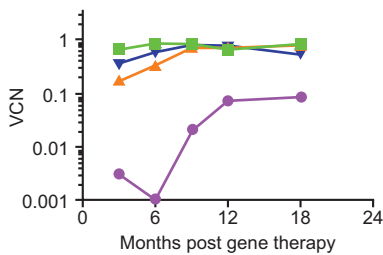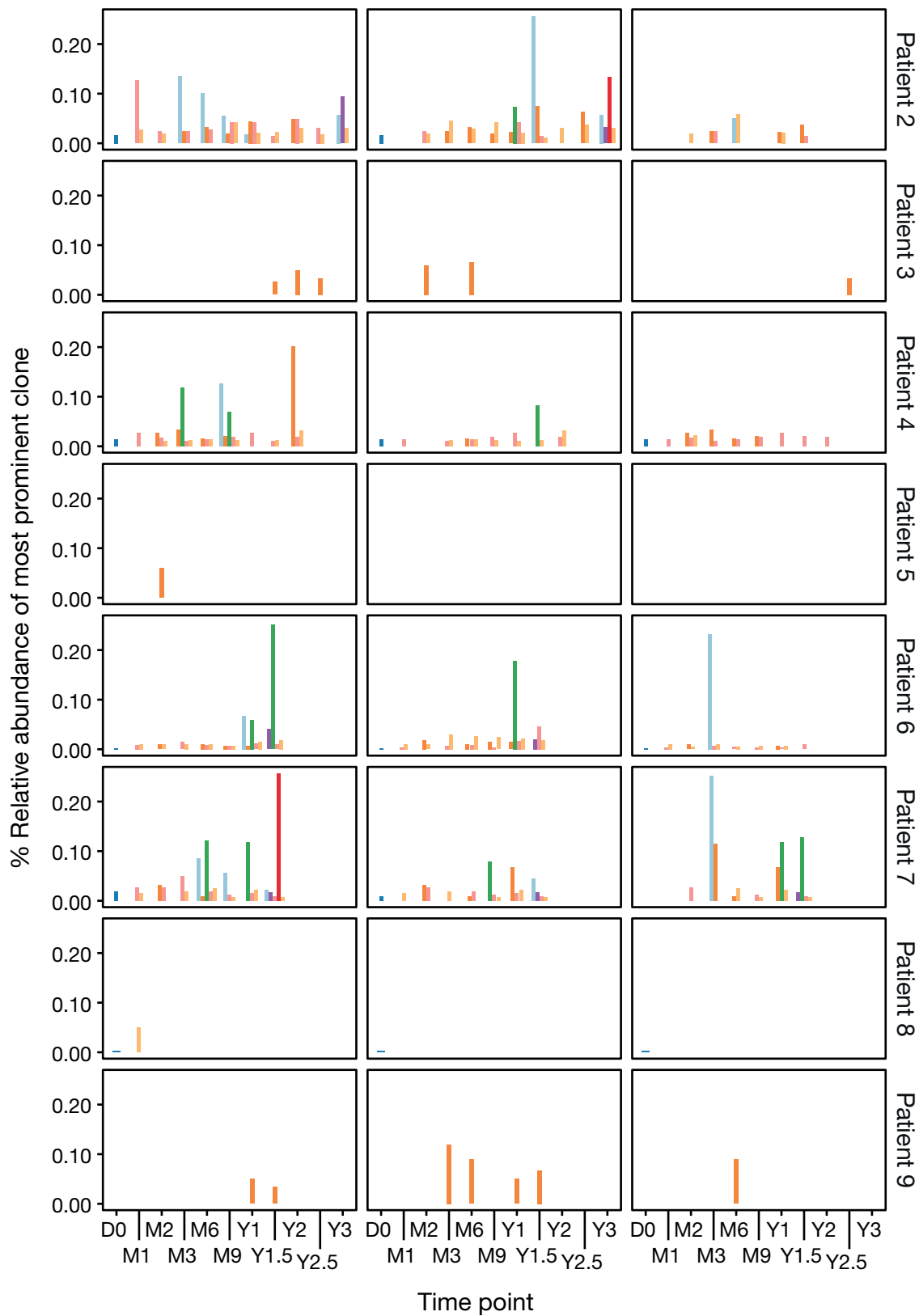Patient 6
Patient 7
Patient 8
Patient 9

a

Neutrophil DHR

%DHR neutrophils

Month after gene therapy

n = 5 9 8 6   7   7   7   5   2

b

Neutrophil VCN

Neutrophil VCN

Month after gene therapy

n = 8 6 8 8   7   7   7   6   4

Legend: CD3 (T), CD15 (My), CD19 (B), CD56 (NK), BM CD34+