SUPPLEMENTARY INFORMATION

PHARMACODYNAMICS AND BIOSAFETY

Overview of the procedures

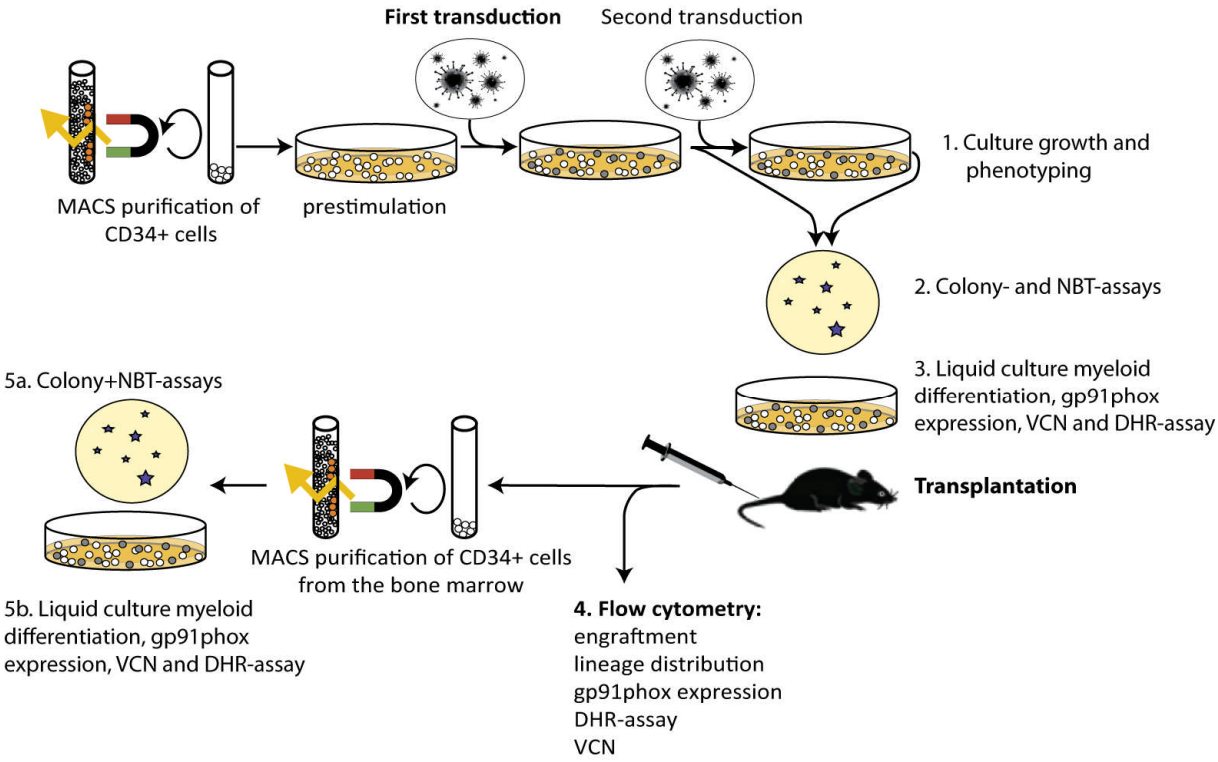

Figure 1. Overview of the experimental procedures followed during this study. Individual steps at which data was obtained are numbered. Two separate experiments were performed, each along with the two control groups (untransduced X-CGD cells and healthy donor cells).

Isolation of XCGD CD34+ cells

Exp. A-B. CD34+ cells were purified from frozen G-MCSF mobilized peripheral blood cells obtained from Patient MD (Exp. A) or taken from frozen aliquots after purification of mobilized peripheral blood cells obtained from Patient MR (Exp. B). In both cases $CD34^+$ cells were purified using the MACS $CD34^+$ selection kit (Miltenyi Biotec) as suggested by the provider. For Exp. C frozen human CD34+ cells isolated from the peripheral blood of an X-CGD patient were provided by H. Malech (NIH, Bethesda, USA, Group 4), while for the experiments in group 5 human CD34+ cells 5 were freshly

isolated from G-CSF and plerixafor mobilised peripheral blood of a 15 years old X-CGD patient.

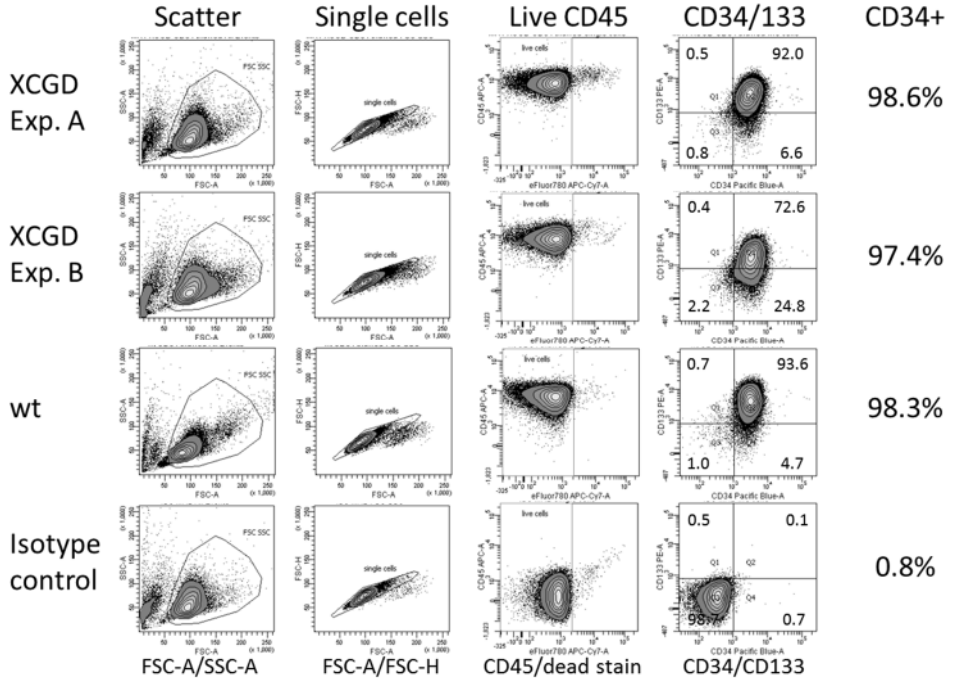

Figure 2. Phenotyping of purified CD34+ cells after one-day culture.

Transduction of XCGD CD34+ cells

Cells were prestimulated at a density of 1-2x10^6/ml for 24h (Exp. A) or 36h (Exp. B) in X-VIVO10 medium supplemented with 300ng/ml hSCF, 300ng/ml hFlt3L, 100ng/ml hTPO and 20ng/ml hIL-3 (all Peprotech) in T25 flasks. Cells were transduced in Retronectin coated 24 well plates at an MOI of 100 (Groups 1 and 3) or 150 (Group 2) for 24h (Groups 1 and 2) or 12h (Group 3) at densities of 1x10^6 (Groups 1 and 2) or 2x10^6 (Group 3) cells per ml. For Exp. C the CD34+ cells were pre-stimulated for 24 hours in the above-mentioned cytokine cocktail with the exception that X-VIVO 20 was used and HSA1% was added to the cell culture.

Myeloid differentiation

For Exp. A and B myeloid differentiation was initiated on day 4 by changing the media to IMDM supplemented with 20% FCS and 50ng/ml hGCSF, 1%Pen/Strep and 2mM Glutamine. In Exp. C granulocytes (CD11B +ve cells) were obtained after culturing untransduced and G1XCGD transduced CD34+ cells at a concentration of

2.5x10^5 cells/ml in differentiation medium [IMDM, 20% FCS, IL3 (20ng/ml), SCF (20ng/ml) and human recombinant G-CSF (100ng/ml)] over a period of 10-20 days.

Determination of vector copy number

Exp A-B. The mean vector copy number per cell (VCN) was determined by qPCR. Samples were measured in triplicates using 50 to 100 ng of genomic DNA. Primers and probe specific for the codon optimized gp91phox coding region and the human EpoR as endogenous standard were used to determine the amount of viral sequences per genome in a duplex reaction. A serial dilution of gDNA from a PLB985 clone harbouring a single lentiviral provirus was used for quantification of vector copy numbers.

@ Christian: qPCR Primers and conditions?

In Exp C, vector copy number in transduced cells were determined after genomic DNA extraction using the DNAeasy extraction Kit (QiaGen). The quantitative PCR was performed in an ABI 7000 Sequence Detection System (ABI, Applied Biosystems, Warrington, United Kingdom) using the primers shown below.

Gene name	Primer name	Sequence
hALB	hALB-probe	5'CCTGTCATGCCCACACAAATCTCTCC3'
hALB	hALB-foward	5'GCTGTCATCTCTTGTGGGCTGT3'
hALB	hALB-reverse	5'ACTCATGGGAGCTGCTGGTTC3'
HIV-Psi	255LentiP.P	5'CGCACGGCAAGAGGCGAGG3'
HIV-Psi	233LentiP.F	5'CAGGACTCGGCTTGCTGAAG-3'
HIV-Psi	363LentiP.R	5'TCCCCCGCTTAATACTGACG-3'

Antibody-staining and flow cytometry

1 x10^5 cells (from culture) or up to 1x 10^6 cells (total BM) were resuspended in 200 µl of FACS-buffer (1xPBS, 1mM EDTA, 0.5% BSA), antibodies and dead cell stain were added and the mix was incubated for 20min. Thereafter the FACS-tubes were filled to the top with staining buffer, pelleted at 300g for 5min at RT, the supernatant was discarded and the pellet resuspended for analysis on a FACS-Canto II device.

List and sources of antibodies used in this study.

Antibodies	Clone	Manufacturer	Cat Nr	Lot#
Anti-gp91-FITC	7D5	MBL	D162-4	042
CD11b-APC	M1/70	eBiosciences	17-0112-83	E07073-1631
CD11b-PC7	M1/70	eBiosciences	25011282	E07514-1630
CD34-APC	4H11	eBiosciences	17-0349-42	E1136-1631
CD34-Vioblue	AC136	Miltenyi	130095393	5110105005
CD133-PE	AC133	Miltenyi	130080801	5110902146
hFc-block	-	Miltenyi	120000464	5070214090
mFC-block	-	Miltenyi	120003855	5111007114
eFluor780	-	eBiosciences	65086514	E11447-1635
hCD45-APC	2D1	eBiosciences	340910	E13612-102
CD38-PC7	HIT2	eBiosciences	25038973	25-0389-73
CD14-APC	61D3	eBiosciences	17014942	11622-1631
CD15-eF450	MMA	eBiosciences	48-0158-42	E11306-1630
CD33-PE	P67.7	BD	345799	38800
CD33-APC	P67.7	eBiosciences	90170337025	E13231-102
mCD45-AC7	30-F11	Biolegend	103116	B142583
hCD45-PC5.5	2D1	eBiosciences	45945942	E10071-1633
CD19-PC7	HIB19	Biolegend	302216	B135004
mCD45-eF450	30-F11	eBiosciences	48-0451-82	E10032-1631
hCD-45-PE	2D1	eBiosciences	12-9459-42	E13572-102
hCD133APC	AC133	Miltenyi	130-090-826	5101102218

Colony assay and NBT-assay

2000 and 6000 cells were plated in Methocult H4433 (StemCell Technologies) in 3.5cm plates in duplicates according to the manufacturer's instructions. 14 to 16 days later an NBT-assay was performed to detect ROS-producing colonies. For this 750µl of freshly prepared NBT-solution (1mg/ml NBT in PBS + 2µg/ml PMA) was applied to each plate and incubated for 45min at 37°C. The reaction was stopped via transfer of the plates to 4°C. The colonies were counted immediately and scored for NBT+ve and

NBT-ve. Only non-erythroid colonies were taken into account, as only those are capable of producing ROS.

Gp91phox expression by FACS

The product of the CYBB gene, a glycoprotein of 91kD (Gp91phox) localizes to internal membranes as well as to the cellular membrane and can be detected by the mouse anti-human monoclonal antibody 7D5 (MBL). The epitope recognized by the 7D5 antibody localizes to the extracellular peptide portion of primate gp91phox (Nakamua et al., 1987; Yamauchi et al., 2001).

DihydroRhodamine 123 (DHR) Assay

Exp A-B. 10^5 cells (*in vitro* cultured) or 10^6 (total BM) were pelleted at 300g for 5min at RT and resuspended in 200µl of room temperature reaction buffer (HBSS+Ca^{2+}+Mg^{2+} supplemented with 7.5mM Glucose, 0.5% BSA, 5000U Catalase per ml) supplemented with Fc-block, antibodies, dead cell stain and DHR (Dihydrorhodamine123 at 1µg/ml). The suspension was transferred onto a 96well plate and incubated at 37°C under interval shaking (12s intervals). After 5 min preincubation 10µl of a 5µg/ml PMA solution was added to start the reaction. 20min later the reaction was stopped by placing onto ice and recorded immediately on a FACS Canto II flow cytometer.

Exp. C. To carry out the DHR test, $5x10^5$ cells were co-stained with CD11B-APC (BD) or CD16 -APC (BD). After the staining cells were washed with PBS-gg (PBS with 0.055 of gelatine and 0.09% of were D-glucose) and resuspended in 1ml of the same buffer. Then 6µl of Catalase (Sigma, C9322. 25U/µl) and 2µl of 2.9mM DHR123 were added to the cells. After 15 minutes of incubation at 37°C the cells were splitted into two tubes, adding only to one of them PMA 1mg/ml to a final concentration of 1µg/ml (Sigma, P8139). The tubes were then incubated another 15 minutes at 37°C, set on ice and the number of oxidative burst positive granulocytes were analyzed using a 525nm band pass filter in a Cyan ADP FACS (Beckman Coulter).

Transplant experiments using NSG mice

Exp A-B. After transduction between 0.5 and 5 million cells were transplanted i.v. in irradiated NSG animals (2.5 Gy) as described in Supplementary Table 1). A total of 30 mice were transplanted. Eight weeks after transplantation animals were sacrificed and bone marrow cells were analyzed for human cell engraftment, lineage distribution and gp91phox expression. Engraftment of human cells in the bone marrow of transplanted mice was measured by estimating the proportion of CD45+ve cells using the gating strategy illustrated in Fig. 7. The presence of human myeloid and B-lymphoid cells was detected using the antibodies CD33, CD14, CD15 and CD19 along with an anti-gp91phox (7D5), respectively after gating for human cells (Fig. 3)

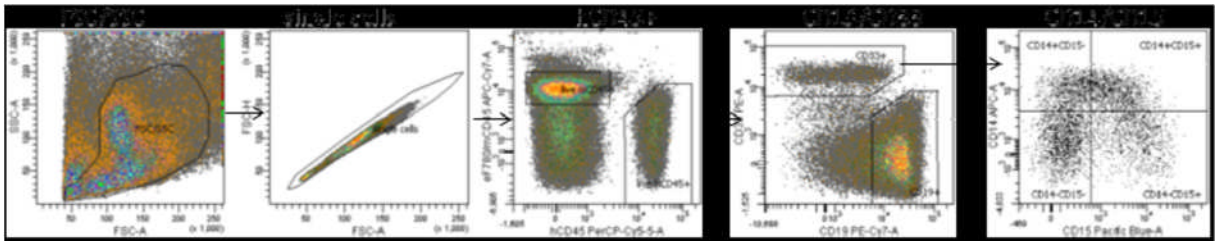

Figure 3. Representative FACS analysis of the bone marrow of transplanted animals showing the gating strategy followed for the analysis of human cells.

Exp. C. The NSG mice [8] were obtained from Jackson Laboratories (USA). All experimental procedures were approved by the Institutional Research Ethics Committee (Institute of Child Health, University College London, UK) and European convention ETS-123 for the use and protection of vertebrate mammals used for experimentation and other scientific purposes. At day 3 after transduction CD34 cells (ranging from 0.4-1 x 10e6) were injected intravenously into the tail vein of 6- to 7-week old sublethally irradiated (325cGy) NSG mice. Mice were sacrificed 13-15 weeks after injection and bone marrow cells were harvested for flow cytometric analysis, presence of Gp91phox protein using the 7D5 antibody and reconstitution of superoxide production by DHR assay.