

Non-Clinical Efficacy and Safety Studies on G1XCGD, a lentiviral vector for *ex vivo* gene therapy of Chronic Granulomatous Disease

Christian Brendel^{1,2,3*}, Michael Rothe^{4*}, Giorgia Santilli^{5*}, Sabine Charrier⁶, Stefan Stein¹, Hana Kunkel¹, Daniela Abriss¹, Uta Müller-Kuller¹, Bobby Gaspar⁵, Ute Modlich^{4,7}, Manuel Grez¹, Axel Schambach^{2,4,8}, Christopher Baum⁴, Adrian Thrasher⁵, Anne Galy⁶

¹Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt, Germany

²Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts, USA.

³Harvard Medical School, Boston, Massachusetts, USA

⁴Hannover Medical School, Institute of Experimental Hematology, Hannover, Germany

⁵Centre for Immunodeficiency, UCL Institute of Child Health, and Great Ormond Street Hospital for Children NHS Trust London, UK

⁶Genethon, Evry, France

⁷LOEWE-Research Group, Paul-Ehrlich-Institute, Langen, Germany

⁸Hannover Medical School, Cluster of Excellence REBIRTH, Hannover, Germany

*Equal contribution

Corresponding author:

SUMMARY

Chronic Granulomatous Disease (CGD) is a debilitating primary immunodeficiency affecting phagocyte function due to the lack of gp91^{phox}, the redox center of the phagocytic nicotinamide dinucleotide phosphate (NADPH) oxidase. Current treatments of X-CGD are not entirely satisfactory and prior attempts at autologous gene therapy using gammaretroviral vectors did not provide long-term curative effects. We developed a new strategy based on the use of the lentiviral vector G1XCGD expressing high levels of the gp91^{phox} transgene in myeloid cells. As a requisite for the submission of a clinical trial dossier on the clinical application of G1XCGD gene modified cells to the competent authorities in Europe, we conducted GLP-conform non-clinical studies with G1XCGD transduced cells. Pharmacodynamics and biosafety studies were performed in NSG mice. G1XCGD transduced CD34⁺ cells derived from XCGD patients did not differ in migration, engraftment, lineage distribution and myeloid differentiation *in vivo* from their non-transduced counterparts. Moreover, we found therapeutic relevant levels of NADPH activity in gp91^{phox} expressing human myeloid cells *in vivo*. In addition, biodistribution studies in mice demonstrated normal distribution of hematopoietic cells expressing functional gp91^{phox} protein in the organs of the animals. Lastly, in extensive *in vitro* and *in vivo* genotoxicity studies, we found no evidence for adverse events related to the IMP. Thus, the use of X1CGD transduced cells represents a valid therapeutic option for the treatment of XCGD patients without any other alternatives. These studies paved the way for the approval of clinical trials (EudraCT: 2012-000242-35 and EudraCT: 2014-002222-12) in the UK, Germany, Switzerland and France on G1XCGD gene modified autologous hematopoietic cells for the treatment of XCGD.

Word count: 263 / max. 300

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare (1:200,000) inherited disorder of the innate immune system characterized by the inability of phagocytes to produce reactive oxygen species (ROS) due to the absence of NADPH oxidase activity (Roos, 1994; Segal, 1996; Segal, 2000; Holland, 2010; Segal, 2005; van den Berg, 2009). Affected patients present an elevated susceptibility to bacterial and fungal infections, as well as an excessive inflammatory response that leads to granuloma formation. The vast majority of CGD patients (>65%) contains mutations in the *CYBB* gene encoding for gp91^{phox} (NOX2), one of subunits of the NADPH oxidase complex. Despite the recent progress in preventing and treating infections, CGD remains characterized by a high prevalence of complications with the risk of a fatal outcome (Seger, 2008). To date, hematopoietic stem cell transplantation (HSCT) is the only established option for permanent cure and has been particularly successful in patients with an HLA-matched donor (Seger, 2008; Gungor, 2013). Gene therapy may represent a definitive cure for patients for whom conventional hematopoietic stem cell transplantation (HSCT) is not possible (Seger, 2008; Gungor, 2013). Clinical evidence in support of a gene therapy approach was obtained from the first studies using gammaretroviral vectors (summarized in Grez et al., 2011). Despite low engraftment levels in some studies and the occurrence of severe adverse effects in combination with gene silencing in others, those studies provided first evidence that gene therapy can be an effective strategy for the long-term correction of CGD (summarized in Grez, 2011). To achieve this goal we developed a lentiviral vector containing a myeloid-specific promoter driving the expression of gp91^{phox}. We have shown that this vector directs transgene expression primarily in myeloid cells *in vitro* and *in vivo*, generates high levels of gp91^{phox} in committed myeloid cells and granulocytes, and restores normal NADPH-oxidase activity and function in murine and human XCGD cells (Santilli, 2011). Based on these findings, we developed a clinically applicable lentiviral vector (G1XCGD) and conducted GLP-conform pre-clinical studies to evaluate the biosafety and efficacy of G1XCGD gene transfer into patient derived hematopoietic stem cells *in vitro* and after transplantation into immunodeficient mice. Moreover, we performed extensive toxicity assays *in vitro* and *in vivo* in support of a Phase I/II clinical trial with G1XCGD gene modified hematopoietic CD34⁺ cells.

RESULTS and DISCUSSION

1. Biopotency, Pharmacodynamics and Biosafety of G1XCGD-transduced XCGD CD34⁺ cells

1.1. Aim and experimental strategy.

We evaluated the biosafety and efficacy of gene transfer using the G1XCGD vector in patient derived XCGD-CD34⁺ cells. We assessed NADPH activity after *in vitro* culture of transduced cells and after engraftment of transduced cells into NSG-mice. We compared these results to data obtained with untransduced XCGD-cells and CD34⁺-cells from healthy donor as control groups. We conducted three separate experiments (A-C) with a total of five experimental groups (G1-G5). We used different multiplicities of infection to estimate the minimal mean vector copy number (VCN) required for a clinically relevant functional reconstitution of NADPH activity in transduced cells. The experimental groups, including transduction and transplantation details are summarized in [Suppl. Table 1](#). In experiments B and C, where material was not limiting, FACS analysis of gp91^{phox} expressing CD34⁺ cells was performed in duplicate at day 3-4 post-transduction. Although the G1XCGD vector contains a myeloid promoter, low levels of gp91^{phox} were always measurable in CD34⁺ cells allowing for a first estimation of transduction efficiency. Between 25.7 % and 52.0 % of the CD34⁺ cells expressed low levels of gp91^{phox} ([Suppl Table 2](#)). After the first transduction round, cells were washed in X-VIVO10 w/o cytokines (Group 1) or reseeded in cytokine supplemented growth media and subjected to a second round of transduction (Groups 2-5). Thereafter cells were transplanted into irradiated NSG-mice via tail vein injection. In parallel to all transplantation groups, 10⁶ cells were kept in culture for *in vitro* experiments.

1.2. Colony numbers and NBT assays of transduced CD34⁺ cells

We tested functional correction of NADPH-oxidase activity on individual hematopoietic colonies using the NBT assay. The percentage of NBT positive colonies ranged from 38% to 80% ([Fig. 1A](#)). In comparison, the percentage of NBT positive colonies derived from healthy CD34⁺ cells was 78% - 85%, while colonies derived from XCGD progenitors did not reduce NBT. The total number of colonies derived from transduced and non-transduced progenitors was similar indicating that the cultivation and

transduction conditions as well as the vector supernatant used in this assay did not negatively affect the ability and frequency of colony formation in semi-solid media (Fig. 1B). Likewise, trypan blue counting at the end of the gene therapy procedure for G4 and G5 revealed that 88% to 96 % stained negatively in this assay indicating again lack of toxicity of the vector preparation and the transduction protocol (data not shown).

1.3. Superoxide production after myeloid differentiation in liquid culture

We kept a fraction of the transduced and control cells in culture. Myeloid differentiation was induced in the presence of 50-100 ng/ml hGCSF for 15 - 25 days. The proportion of CD11b⁺ cells was 50%-80% after 21 to 24 days in all cultures. We measured superoxide production in the CD11b positive fraction by the dihydrorhodamine (DHR) 123 assay. Between 11.0% and 25.5% of the CD11b⁺ cells expressed gp91^{phox} at the cell surface, while 20% to 60.5% of the CD11b⁺ cells were found to produce superoxide radicals in this assay (Fig. 1C and Suppl Table 2). Only in Experiment B the number of available cells was sufficient to perform a Cytochrome C assay in parallel to the DHR-assay, as a minimum of one million cells is required for this assay. In this experiment, the G1XCGD transduced population produces slightly less than 50% superoxide compared to healthy donor cells (Figure 1D), which closely matches the vector copy number of 0.45 ± 0.09 determined for this cell population. This suggests that most of the transduced cells contained a single vector integration, sufficient to produce close to wild type levels of ROS per cell in this assay.

1.4. *In vivo* detection of gp91^{phox}, oxidase activity, engraftment and lineage distribution in NSG mice

After transduction between 0.4 and 5 million cells were transplanted intravenously into sublethally irradiated NSG animals. Human cell engraftment, lineage distribution and gp91^{phox} expression were analyzed at 8 (groups 1-3) or 13/15 (groups 4 and 5) weeks after transplantation. Engraftment of donor cells was determined by human CD45 expression in flow cytometry. It ranged from 3% to 15% in experiment A and between 40% and 65% in experiment B , while in experiment C human cell engraftment ranged between 3% and 75% (Suppl. Fig. 1A and Supplementary Table 3). We found no major differences between the engraftment capabilities of transduced vs. non-transduced cells, demonstrating once again that the transduction process and the vector preparation were not detrimental to the migration or engraftment of the cells. Likewise

lineage distribution (myeloid vs. lymphoid) did not differ between transduced and non-transduced cells. Subfractions of myeloid cells in bone marrow of animals reconstituted with transduced cells were not different from those observed in animals transplanted with non-transduced cells (Suppl. Fig.1B, C).

We analyzed expression of gp91^{phox} in myeloid and lymphoid cells obtained from the bone marrow of transplanted animals. As shown in Fig. 2, robust gp91^{phox} expression was detected in myeloid cells derived from animals transplanted with healthy CD34⁺ cells, while no expression was detected in bone marrow cells of animals transplanted with XCGD cells. In all animals transplanted with G1XCGD transduced cells gp91^{phox} expression was clearly detectable in myeloid cells (CD33⁺, CD13⁺). Gp91^{phox} protein was detected in 44.5 ± 11.6% of the CD45/CD33⁺ cells and in 47 ± 9.5% of the CD45/CD13⁺ cells, while expression in CD19⁺ lymphoid cells was significantly reduced and difficult to distinguish from background levels (Fig. 2, Suppl. Fig. 2A and Suppl. Table 3). Gp91^{phox} expression in myeloid cells was robust with levels of expression (MFI) half of that observed in myeloid cells derived from healthy CD34⁺ progenitors (Fig. 2 and Suppl. Fig. 2B).

The detection of NADPH-oxidase activity directly from the blood or bone marrow of transplanted NSG mice is hampered by the low frequency of mature neutrophil granulocytes, which make up only a minor fraction of the anyway rare myeloid cells (mostly <10% of hCD45+) in the xenograft NSG-mouse model. Consequently, high engraftment rates, as achieved in Experiment B, are essential for a reliable DHR-assay. In this group, mice treated with gene-modified cells had in average 40% functional corrected cells within the CD11b⁺ fraction at a mean vector copy number of 0.48 ± 0.43 and a mean fluorescence intensity around 50% of that found in healthy donor cells (Figure 3).

1.5. Reconstitution of superoxide production in G1XCGD transduced cells derived from transplanted animals.

Reconstitution of superoxide production after transplantation of X1CGD gene modified cells was also assayed after *in vitro* differentiation of hCD34⁺ cells recovered from the bone marrow of transplanted animals. Purified cells were plated on methylcellulose for colony formation and NBT assays or differentiated towards myeloid cells in liquid culture. NADPH-oxidase activity in differentiated cells was monitored using the DHR-assay. As shown in Suppl. Fig 3, the percentage of NBT⁺ colonies derived from

G1XCGD transduced cells ranged between 20% and 40%. For comparison the percentage of NBT+ colonies obtained from healthy CD34⁺ cells was in the mean 48% in experiment A and 94% in experiment B. Thus, superoxide production in G1XCGD transduced cells is about half of that observed in wild type cells. However considering transduction efficiencies between 40% - 50%, as estimated from the gp91^{phox} expression observed in myeloid cells (Suppl. Fig. 2), we can conclude that almost every single gp91^{phox} expressing cell leads to substantial superoxide production.

Likewise, the percentage of DHR positive CD11⁺ cells differentiated from engrafted CD34⁺ cells fluctuated between 24.4% and 45%, depending on the vector concentration used in the different experiments (Suppl. Fig.3 and Suppl. Table 4). These values correlate well with the values obtained in the NBT assays and more importantly with the gp91^{phox} and DHR values found *in vivo* (Suppl. Fig. 2 and Fig. 3A, B). In Exp. C between 16 ± 8.5 and 36 ± 15% of the CD16⁺ cells derived from engrafted G1XCGD-transduced CD34⁺ cells were positive for superoxide production as estimated by the DHR assay (Suppl. Table 4).

In Experiment B the recovery of hCD34⁺ was sufficient to perform a quantitative cytochrome C assay in parallel to the DHR-assay. Transduced cells produced roughly 23% of ROS compared to healthy donor cells, which is slightly less than expected from the average vector copy number of 0.39 (Suppl. Fig. 4).

2. Biodistribution of G1XCGD gene modified cells in irradiated animals

2.1. Aim and experimental strategy.

Transduction of hematopoietic stem cells in the context of gene therapy studies may result in the expression of the therapeutic gene in stem or progenitor cells and may alter their innate homing and engraftment properties. Therefore, a careful study on the biodistribution of gene modified cells *in vivo* is essential to disclose any potential accumulation of gene transduced cells in non-hematopoietic organs. The study was carried out using primary lineage negative (Lin⁻) murine bone marrow cells isolated from male X-CGD mice. Cells were transduced with G1XCGD and transplanted into lethally irradiated female X-CGD mice. After hematopoietic reconstitution (6 weeks), organs (lung, liver, spleen, heart, brain, thymus, gonads, kidney, bone marrow and peripheral blood) of half of the treated animals was analyzed for the presence of *CYBB*

sequences and engraftment by PCR. In addition, cells from bone marrow, spleen and peripheral blood were analyzed by FACS for gp91^{phox} expression. The rest of the transplanted animals was observed for a total of 3 months and analyzed as described above.

2.2. Engraftment of gp91^{phox}-transduced cells in mice.

Engraftment of male cells into organs of female recipients was determined by qPCR specific for the male Y-linked gene SRY, while a specific CYBB qPCR was used to determine vector copy numbers. As expected, engraftment of male donor cells into the hematopoietic organs (blood, bone marrow, spleen, thymus) of female recipients was high ranging from 80% to 100%, while engraftment of male donor cells in all other organs analyzed was equal to or below 20%. Vector copy numbers ranged between 0.35 and 1.5 in cells of the hematopoietic organs, and was lower than 0.35 copies per cell in all other organs except the lung. Overall, a clear correlation was found between engraftment levels and vector copy numbers. As shown in **Figure 4A, B** mean vector copy numbers were low in non-hematopoietic organs and high in hematopoietic organs. This implies that the gp91^{phox} expression does not alter the homing of hematopoietic cells into non-hematopoietic organs of transplanted animals. In two animals strong PCR signals for gp91^{phox} were observed in the lung of the animals. The lungs of these animals showed signs of granuloma formation, a typical clinical manifestation of X-CGD. Thus, it is reasonable to assume that macrophages and granulocytes preferentially accumulated at the site of inflammation. As neutrophils from CGD animals show impaired *in vivo* recruitment to sites of inflammation (Hattori et al., 2010), our results suggest an enhanced migration rate of gene corrected myeloid cells over non-corrected cells.

2.3. Lineage distribution and gp91^{phox} expression in blood cells from transplanted animals at weeks 6 and 12 post transplantation.

Gp91^{phox} expression and hematopoietic lineage distribution was analyzed in peripheral blood, bone marrow, thymus and spleen of sacrificed animals. Hematopoietic lineage distribution in G1XCGD transplanted animals did not differ from the lineage distribution observed in animals transplanted with non-modified cells supporting again the notion the gp91^{phox} expression does not alter lineage differentiation or proliferation. (**Figure 4C and Suppl Figure 5, 6**). Gp91^{phox} expression was detected preferentially in

granulocytes and monocytes obtained from peripheral blood, bone marrow and spleen of transplanted animals (Figure 4D, E and Suppl Figure 5, 6).

3. Genotoxicity Studies

3.1 G1XCGD shows a beneficial safety profile *in vitro*

We used the *in vitro* immortalization (IVIM) assay to assess the genotoxic potential of the G1XCGD vector. The IVIM assay relies on the induction of a survival advantage by insertional activation of cellular proto-oncogenes and thus determines the risk of transformation. It measures the replating frequency (RF) of primary murine hematopoietic stem/progenitor cells as a consequence of insertional upregulation of growth promoting genes (Modlich *et al.*, 2006; Modlich *et al.*, 2009; Zychlinski *et al.*, 2008). We compared the genotoxicity of G1XCGD to that of RV.SF and LV.SF, with known *in vivo* and *in vitro* mutagenic potential. The gammaretroviral vector RV.SF contains strong spleen focus forming (SFFV) promoter/enhancer sequences in the intact long terminal repeat (LTR) regions driving the enhanced green fluorescent protein (eGFP) transgene expression. The lentiviral vector LV.SF is similar to the G1XCGD vector with the exception that LV.SF uses the SFFV sequence as an internal promoter and contains the eGFP as transgene (Suppl. Figure 7). The mutagenic vectors served as a positive control for the IVIM assay. Additionally, we used the IVIM assay to determine potential unspecific toxic effects of vector preparations or encoded proteins, by documenting the growth rate of the cells during the transduction procedure and the subsequent expansion phase (first 8 days).

Murine Lin⁻ cells were pre-stimulated prior to two rounds of consecutive transductions. After two weeks of expansion, cells were replated at low density on 96-well plates. Under these conditions, non-transformed cells show a proliferation disadvantage. Plates are scored for actively growing cells, before replating frequencies of insertional mutants are estimated by Poisson distribution. We performed three independent experiments at MOIs between 5 and 20. Vector copy numbers ranged between 0.3 and 23.5 copies per cell (Tables 1&2 in Suppl. Information).

For a better statistical comparison, we combined data from a meta-analysis of RV.SF (n=145), LV.SF (n=34) and MOCK (n=88) transductions with the actual control samples of the assays in which G1XCGD was analysed. The gammaretroviral control vector RV.SF scored positive in 78.4% of the assay, whereas the lentiviral vector with

the internal SFFV sequence showed positive assays only in 35% of the cases. In contrast, none of the assays conducted with the G1XCGD scored positive in this assay (n=16) (Figure 5). Furthermore, cells transduced with the test vector G1XCGD at high MOI showed normal proliferation behaviour and was statistically not different from the mock treated control or the positive control vectors (Figure 1, Supplementary Information).

Thus, under the conditions used in this study, the IVIM assay shows the absence of mutagenic potential for the G1XCGD vector compared to the positive control vectors. Since immortalization of bone marrow cells is caused by retroviral activation of proto-oncogenes, the absence of genotoxicity observed for the G1XCD vector argues in favor of a clinical application of this vector. Furthermore, there was no significant cytotoxicity associated to high titer virus transduction with the test vector.

3.2 Study design of the *in vivo* genotoxicity assessment.

The cytokine conditions of the IVIM assay supports a myeloid differentiation of cells. Hence, also the repertoire of immortalizing mutations in the assay is subject to a myeloid bias selecting preferentially for immortalized clones with Mecom and Prdm16 integrations (Modlich et al., 2006). To extend the genotoxicity analysis to the complete hematologic compartment, we performed *in vivo* experiments. The experimental setup and conditions are shown in Supplementary Figure 8. Briefly, we isolated Lin⁻ negative cells from CD45.2 mice. After prestimulation for 27 hours, cells were transduced with G1XCGD, LV.SF and RV.SF at different MOIs, before being transplanted into lethally irradiated CD45.1 recipients. Additionally, we transplanted prestimulated, non-transduced cells (MOCK) to yield an age-matched control for blood parameters and histopathology. To include a valid positive control for clonal dominance and leukaemia associated symptoms, we transplanted three mice with 1 x10⁶ whole bone marrow cells of a previously characterized, RV.SF triggered B-cell leukaemia (Maetzig et al., 2011). The list of animals in the different groups is presented in Supplementary Information Table 3. Mice were monitored for 26 weeks with regular weighing and bleeding intervals, before being subject to thorough end analysis. We analysed general health parameters (weight, blood count), performed a blinded GLP-grade histopathology and performed insertion site analysis for the bone marrow samples of all animals.

3.3. Chimerism, Vector Copy Number and Blood parameters

Three animals had to be killed shortly after transplantation due to engraftment failure. Apart from that, all other 44 mice receiving transduced or MOCK treated cells survived with normal blood parameters, normal spleen size and either stable or increased body weight over time. A detailed description of blood parameters, animal weight and histopathology is presented in [Supplementary Information](#). We analyzed engraftment levels of donor cells in the bone marrow of animals at week 26 after transplantation. For the majority of animals (n=43) the contribution of donor derived cells was very high (96.9% \pm 3.1%) ([Supplementary Figure 9A](#)). We determined the gene expression for groups with eGFP marker genes ([Supplementary Figure 9B](#) and copy number in the bone marrow for all groups ([Supplementary Figure 9C](#)). For the lentiviral vector groups, we used a lower and a higher MOI for gene transfer. Gene marking ranged from 1.1 to 6.3 copies (mean 3.4 \pm 1.5) in the bone marrow of the G1XCGD animals. For the LV.SF group we observed a higher variability with 0.2 to 10.7 copies (mean 4.0 \pm 3.3). RV.SF received only one MOI and VCN ranged from 0.1 to 2.5 copies (mean 1.0 \pm 0.7).

3.6. Clonality Analysis

We retrieved a total of 298061 sequence reads ([see Supplementary Material and Methods](#)). For quality reasons, only sequences with a full LTR were included in downstream analysis (85.7%). After removing residual vector parts and internal control sequences, we clustered 115044 (38.6%) reads to 2597 individual sequences (0.9%), which were uploaded to the MAVRIC integration site analysis tool (Huston et al., Hum Gene Ther. 2012), resulting in 1212 analyzable insertions. The distance to the transcription start site (TSS) of the nearest genes was analyzed in a window of 250 kb up- and downstream of the integration sites ([Supplementary Figure 10a-c](#)). The insertion profile of gammaretroviral vector RV-SF recapitulated the known preference of MLV based vectors to insert close to the transcription start site of genes and near promoter regions, while both lentiviral vectors showed a tendency to insert downstream of the TSS into actively transcribed genes, as expected (Wu et al., 2003; Lewinski et al., 2006; Mitchell et al., 2004). We found significantly more integration sites listed in the retroviral tagged cancer gene database (RTCGD) for RV.SF than for both lentiviral vectors ([Supplementary Information Table 5](#)).

We determined the amount of sequences (= reads) for each individual integration as a semi-quantitative surrogate measure of clonal abundance. We compared the

contribution of the specific insertions relative to all reads found in one sample (Figure 6). We regarded the insertions with a contribution of <10% to the total sequence pool as the polyclonal background of a sample. Insertions with >10% contribution were defined as prominent insertions. In Figure 6, we show the read contribution as abundance in percent for each animal. In case the gene closest to the prominent insertion was listed in either the RTCGD, the network of cancer genes (NCG) or the allOnco cancer gene list, the gene symbol was highlighted in red. Alternatively, should a neighboring gene within 50kb around the TSS overlap with one of the databases, the symbol was marked in purple. We found a significantly higher background of low read insertions for the lentiviral vectors compared to the RV.SF samples, suggesting a lower genotoxic risk. One concerning finding in the G1XCGD group (animal #14) was an intronic insertion into the *Setbp1* gene with > 80% contribution to the total reads. The *SETBP1* gene has been reported in association with leukemia and was found in the Frankfurt gene therapy trial as one of the transiently amplified clones preceding the monoclonal adverse event, without being directly involved in the myelodysplastic outcome (Ott et al., 2006; Piazza et al., 2013; Albano et al., 2012; Oakley et al., 2012; Cristobal et al., 2010). Thus, the high read insertions could possibly originate from clonally amplified cells. However, as the amount of retrieved sequences can be influenced by the nested PCR steps during LAM-PCR (Brugman et al., 2013), we validate our results with a locus specific quantitative PCR (ls-qPCR). In total, we performed ls-qPCRs with 75 different primer combinations for 69 unique insertions. The ls-qPCR was able to positively identify 36 of 53 high read insertions (69%) and validated the absences of all background insertions. For *Setbp1* we performed several ls-qPCRs with four different reverse primers to exclude suboptimal oligonucleotide design. We found similar or identical contributions, which all were at the limit of detection. The highest contribution with 5.3% was found for the 10 weeks PB sample. The level of *Setbp1* carrying cells decreased to 4.0% after 15 weeks and stabilized at 20 weeks at 0.6%. The latest time point after 26 weeks showed levels of 0.9% contribution in the PB sample and only 0.5% in the bone marrow. In summary, we only found background levels of cells with this insertion and by that we exclude a major contribution of a *Setbp1* clone in that animal (Supplementary Figure 11).

In conclusion, we found no adverse events associated with the G1XCD vector for any of the mice in this study. Blood parameters were normal and the body weight stable over time. Histopathology revealed no signs of hematopoietic malignancies. Among

the vectors analyzed in this study, the G1XCGD vector gave the most consistent polyclonal marking pattern. For the remaining indicators of clonal skewing observed with the use of this vector, it remains to be determined whether they were a consequence of insertional mutagenesis, normal oligoclonal hematopoiesis in mice or spontaneous clonal skewing in association with a neutral marking event. Gene expression analysis revealed that none of the genes in proximity to vector insertions in the G1XCGD group showed a relevant up- or downregulation (data not shown).

CONCLUSIONS

The G1XCGD vector showed no evidence of toxicity on human CD34⁺ from X-CGD patients as these cells were able to normally engraft in NSG mice and further generate a normal distribution of lymphoid and myeloid cells in the peripheral blood as well as in lymphoid organs as compared to normal CD34⁺. In addition, the transduced cells expressed a clinically relevant amount of functional gp91^{phox} as assessed by the level of NADPH oxidase activity measured, for up to 15 weeks after transplantation. No significant adverse events related to the IMP were observed in toxicity studies. G1XCGD shows a markedly improved safety profile compared to LTR-driven gammaretroviral vectors such as those used in the previous gene therapy trial for CGD. Based on this information a European consortium (NET4CGD, FP7-HEALTH-2012, contract agreement 305011) was established focused on the clinical application of G1XCGD gene modified cells. The purpose of the NET4CGD consortium is to conduct a European multi-centric gene therapy trial for X-CGD in 4 European centers. A unique Clinical Trial Application (CTA) was prepared and submitted to the different competent national regulatory authorities. The clinical trial has been registered at the EMA under EudraCT: 2012-000242-35 and was approved in the UK, Germany and Switzerland. In France, the clinical trial with slight modifications was approved in Dec 2015 (EudraCT: 2014-002222-12).

ACKNOWLEDGMENTS

We are very grateful to Jennifer Buttler (Georg-Speyer-Haus) for her valuable contributions during the early steps of this work. The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreement n°305011 (Net4CGD). Work at the Georg-Speyer Haus was partially supported by grants from the LOEWE Center for Cell and Gene

Therapy Frankfurt funded by the Hessische Ministerium für Wissenschaft und Kunst (HMWK III L 5-518/17.004 [2013]). The Georg-Speyer-Haus is funded jointly by the German Federal Ministry of Health (BMG) and the Hessische Ministerium für Wissenschaft und Kunst.

REFERENCES

FIGURE LEGENDS

Figure 1. Functional reconstitution of NADPH oxidase activity in G1XCGD transduced cells. A-B. Percentage of NBT-positive colonies and total colony numbers in experiments A and B. Experiment A: n=4 plates per bar, Experiment B: n=8 plates per bar except for controls (wt and XCGD, n=4 plates per bar). Error bars: SD. Vector copy numbers at day 10 post transduction: Group 1: 0.35, Group 2: 0.96, Group 3: 0.57. **C.** Fraction of ROS-producing mature myeloid cells as assessed by DHR-assay and flow cytometric analysis. Sample sizes: XCGD n=1-2, wt n=2, experimental groups n=3-5. **D.** Cytochrome C assay to assess superoxide production in bulk cultures relative to healthy donor cells (wt) after 3 weeks of *in vitro* differentiation (n=3). Vector copy number of group 2 on day 25 post transduction: 0.45 ± 0.09 .

Figure 2. Gp91phox expression in human hematopoietic cells obtained from NSG-transplanted animals. FACS-blots showing expression of gp91phox in B-lymphocytes (CD19⁺) and myeloid cells (CD33⁺) from the bone marrow of all transplanted mice. XCGD: NSG mice were transplanted with untransduced XCGD-CD34⁺ cells; wt: mice were transplanted with untransduced CD34⁺ cells from a healthy donor; transduced: mice were transplanted with X1CGD transduced XCGD-CD34⁺ cells

Figure 3. Detection of NADPH-oxidase activity in freshly isolated cells from the bone marrow of NSG mice. A. Histograms showing DHR-activities within the human

CD45⁺CD11b⁺ fraction of bone marrow cells. **B.** Summary of the mean fraction of ROS-producing cells. **C.** Mean fluorescence activity (MFI) of the DHR positive cells shown in B. Vector copy number: 0.48 ± 0.43 .

Figure 4. Biodistribution of gp91^{phox} expressing hematopoietic cells in transplanted NSG mice. **A-B.** Comparative analysis of Gp91^{phox} expression in hematopoietic organs at week 6 (A) and week 12 (B) post transplantation. Hematopoietic organs are encircled. **C.** Summary of lineage distribution of G1XCGD transduced cells in peripheral blood (PB), bone marrow (BM), spleen (Spl) and thymus (Thy) of transplanted animals at week 12. **D-E.** Engraftment of human gene modified cells in various hematopoietic organs of transplanted NSG animals at week 6 (D) and week 12 (E) after transplantation.

Figure 5. Results of the IVIM assay determining the risk of insertional mutagenesis. Replating frequencies (RF) of non-transduced samples (MOCK) and those transduced with RV.SF, LV.SF or G1XCGD are shown. We included meta-data for MOCK, RV.SF and LV.SF. Each dot represents one sample in an assay. Samples which were measured in the same assay as G1XCGD, are labelled as 1 (assay #100818), 2 (#101222) and 3 (#110727). The bar indicates the mean RF. The first quantile (Q1) of RF-values of mutagenic vector RV.SF demarcates the threshold to count positive assays. Assays below the limit of detection (LOD) and the Q1 level were counted as negative assays. The incidence of positive and negative (pos:neg) assay is shown below the graph. The number of positive assays for RV.SF was significantly higher compare to all other groups (* $p < 0.05$; *** $p < 0.001$; Fisher's exact with Bonferroni multiple comparison correction). There was no statistical difference (NS) between G1XCGD and the MOCK control.

Figure 6. Relative abundance of sequencing reads. We defined sequencing reads of insertion sites as a semi-quantitative surrogate measurement of clone size in the animal groups transplanted with RV.SF, G1XCD and LV-SF vector. We show the gene symbols for prominent insertions with a read count contribution of more than 10% to the total sequencing pool (relative abundance in %). In case the gene closest to a prominent insertion was listed in the retroviral tagged cancer gene database, the network of cancer genes or the allOnco cancer gene list, the respective symbol was

highlighted in red. If the closest gene was not listed as a proto-oncogene but any other gene within 50 kb, the gene name is shown in purple. Gene symbols occurring more than once are highlighted in pink.