Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long term correction of oxidase activity in peripheral blood neutrophils

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Running head: X-CGD gene therapy corrects neutrophil oxidase

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Abstract

Chronic granulomatous disease (CGD) is associated with significant morbidity and mortality from infection. The first CGD gene therapy trial\(^1\) resulted in only short term marking of 0.01-0.1\% of neutrophils. Recently, Ott \textit{et al.}\(^2\), using busulfan conditioning and an SFFV retrovirus vector achieved $>20\%$ marking in two patients with X-linked CGD. However, oxidase correction per marked neutrophil was less than normal and not sustained. Despite this, patients clearly benefited in that severe infections resolved. As such, we initiated a gene therapy trial for X-CGD to treat severe infections unresponsive to conventional therapy. We treated three adult patients using busulfan conditioning and an MFGS retroviral vector encoding $\text{gp91}^{\text{phox}}$, achieving early marking of 26\%, 5\% and 4\% of neutrophils, respectively, with sustained long term marking of 1.1\% and 0.03\% of neutrophils in two of the patients. Gene marked neutrophils have sustained full correction of oxidase activity for 34 and 11 months respectively, with full or partial resolution of infection in those two patients. Gene marking is polyclonal with no clonal dominance. We conclude that busulfan conditioning together with an MFGS vector is capable of achieving long term correction of neutrophil oxidase function sufficient to provide benefit in management of severe infection. This study is registered at http://clinicaltrials.gov as NCT00394316.
Introduction

X-linked chronic granulomatous disease (X-CGD) results from mutations of the CYBB gene encoding gp91phox required by neutrophils to produce microbicidal oxidants. Patients develop recurrent life-threatening bacterial and fungal infections\(^3\). Management involves prophylactic antibiotics, interferon gamma, and aggressive diagnosis and treatment of infection. Allogeneic transplantation (BMT) is curative, but consensus is lacking regarding indications to use what is perceived to be a high risk modality for a non-fatal disorder. Further, finding a sibling matched donor in the setting of a genetic disease is a limiting factor. However, outcomes improve when patients are transplanted earlier, prior to numerous infections and end organ damage, and when infection free at the time of transplant.

Gene therapy may be capable of providing protection from or treatment of infection without the risks of BMT\(^4\). Several publications reported protection from infection challenge following gene therapy in gp91 and p47phox deficient mouse models\(^5-9\). Most studies used myeloablative conditioning to achieve the engraftment levels required for phenotypic correction. Gene marking studies with myeloablative conditioning in non-human primates have achieved levels of long term engraftment at levels that theoretically would cure patients with CGD\(^10,11\). Full engraftment is unnecessary as demonstrated by X-CGD female carriers who vary in their ratio of oxidase normal versus deficient neutrophils due to the stochastic nature of X chromosome inactivation\(^12\). Carriers with \(\geq 10\%\) oxidase normal neutrophils are generally infection free. However in the non-human primate model, gene marking has been significantly lower with non-myeloablative conditioning\(^13,14\) particularly where there is no selective advantage conferred by the transgene.

Still, an early gene therapy trial for X-CGD in humans resulted in detectable marking even without any conditioning. In that trial, gene corrected neutrophils occurred at 0.01-0.1\% of total neutrophils measured at various time points, but none persisted more than a year despite multiple infusions of modified CD34s\(^15\).

In disorders where selective advantage is conferred by the corrective transgene, such as X-linked severe combined immunodeficiency (SCID), there has been greater success\(^16,17\). However, even in adenosine deaminase deficiency SCID, use of low dose busulfan (4mg/kg) was required to obtain clinical benefit\(^18\). In 2002, Ott et al. initiated a clinical trial for X-CGD using a Spleen focus forming virus (SFFV) based vector and busulfan at 8 mg/kg for conditioning. The initial
marking was greater than 20% in the two patients, though oxidase correction per gene marked
neutrophil was significantly less than normal. The level of marked neutrophils rose due to
oligoclonal outgrowth of transduced cells with vector inserted in the EVII–MDS1 proto-
oncogene. Unfortunately, after two years both patients developed myelodysplastic syndromes
with one requiring BMT and the other dying of sepsis. Notably both patients’ infection at the
time of gene therapy resolved, despite the use of busulfan². Based upon their observation of early
benefit of gene therapy for infection control, we initiated a gene therapy trial to treat X-CGD
patients with a severe infection not responsive to conventional management. We used 10mg/kg
of busulfan followed by autologous CD34s transduced ex vivo with an amphotrophic
pseudotyped vector encoding gp91phox. We describe here results in three patients treated to date.

Methods

Clinical protocol regulatory review:
All procedures and treatments were conducted under NIH protocol 07-I-0017 (the current
protocol) or under protocol 95-I-0134 (closed to accrual, but results from this protocol are
discussed in this paper). Both protocols were approved by the NIAID Institutional Review
Board, the NIH Institutional Biosafety Committee, the Food and Drug Administration, and the
members of the Recombinant DNA Advisory Committee of the NIH Office of Biotechnology
Activities. Patients were eligible for protocol 07-I-0017 if they had an infection not responsive
to standard therapy, proven gp91phox deficient CGD, and no eligible HLA-matched sibling donor.
Availability of an HLA-matched unrelated donor was not determined given its more
experimental nature and high risk in CGD patients with an ongoing infection. Informed consent
was obtained from all patients in accordance with the Declaration of Helsinki.

Vector production:
The amphotropic pseudotyped murine Moloney leukemia retrovirus derived vector encoding
gp91phox cDNA (MFGS-gp91phox, see supplemental information (SI)), was produced from a 293
cell producer line under GMP conditions. Three production lots of the same vector were used
with the first two made by Magenta, (Rockville, MD) for an earlier gene therapy trial and
revalidated for titer and potency. The 3rd lot (Bioreliance-Invitrogen, Glasgow, Scotland) was
made to supplement the current trial.
Autologous CD34+ cells:
Patients were enrolled on a separate IRB approved protocol (94-I-0073) for collection of CD34+ hematopoietic stem cells. They underwent standard mobilization and apheresis using filgrastim (Amgen, Thousand Oaks, CA) at 10-16 mcg/kg daily for 5 to 6 days (see Figure 1). On day 5 and/or 6 a 15 to 25 liter apheresis collection was performed. CD34+ cells were selected using either the Isolex 300i (Baxter Healthcare; distributed by Miltenyi Biotec, Auburn, CA) or CliniMACS® Cell Selection System (Miltenyi Biotec) cryopreserved and held in the NIH Department of Transfusion Medicine (NIH-DTM) until needed.

Transduction:
Transduction was performed in the Cell Processing Section of NIH-DTM. CD34s were cultured and transduced in X-Vivo 10® (BioWhittaker Media, Cambrex Bio Science, Walkersville, MD) serum free medium supplemented with 1% human serum albumin and growth factors: IL-3 (5ng/ml), SCF (50ng/ml), TPO (50ng/ml), and Flt-3 ligand (50ng/ml) (all R&D Systems, Minneapolis, MN) in Retronectin® (recombinant fibronectin fragment; Takara Bio Inc., Otsu, Japan) coated X-FOLD® bags (Baxter Healthcare, Deerfield, IL). For each transduction, cells were resuspended in 90% neat vector supernatant at varying cell concentrations to maintain an MOI≈2. Cells were transduced daily x4 days for 6 hours/day beginning at 16-18 hours of culture, and at 96 hours washed and resuspended in human albumin supplemented PBS for infusion.

Study Design and Conditioning (Figure 1):
The extent of each patient’s infection was assessed prior to busulfan administration. Using a central venous line busulfan (Busulfex®; Otsuka America Pharmaceutical, Inc, Rockville, MD) 5mg/kg was infused over 2 hours daily for two days and levels measured at 0, 30, 60, 180, 240, 360, and 480 minutes after first infusion (Quest Diagnostics Inc). AUC and t1/2 were assessed by Scott Penzak, PharmD of the NIH Clinical Center Pharmacy using analytical software. The cells were given a minimum of 24-48 hours after the busulfan was completed (Day 0) which, based on the calculated AUC and t1/2, was sufficient to ensure full clearance of drug. Patients 2 and 3 were treated with palifermin (recombinant keratinocyte growth factor; Biovitrum AB Stockholm, Sweden) 60 mcg/kg/day, for three days before and after the busulfan. Patient 3 also received rapamycin (Wyeth Pharmaceuticals, Inc, Philadelphia, PA) beginning on day-1 with a loading dose of 5mg given TID then dosed to maintain a level of 10-15mg/dl.
Polymerase chain reaction (PCR) quantification of vector marking:
Semi-quantitative real time PCR primers were designed to anneal to the 5’ and 3’ LTRs of the MFGS vector19. Forward primer sequence: CGC AAC CCT GGG AGA CGT CC; Reverse Primer: CGT CTC CTA CCA GAA CCA CAT ATC C; FAM labeled probe: CCG TTT TTG TGG CCC GAC CTG A. PCR was 40 cycles, melting temperature 95°C 15 sec, and annealing temperature 60°C 60 sec (PE Biosystems Realtime PCR 9600, Foster City, CA).

Detection of gp91phox expression:
Intracellular gp91phox expression was quantified by flow cytometry. Briefly, 200ul whole blood was lysed (ACK buffer), washed with PBS/BSA in 10mM EDTA buffer and permeabilized using a kit (BD Biosciences, San Jose, CA). Permeabilized leukocytes were resuspended in Solution B along with anti-gp91phox monoclonal antibody (antibody 7D5, a generous gift from Dr. Michio Nakamura) for 30 minutes, washed and exposed to rat anti-mouse FITC conjugated antibody (BD Biosciences) 30 minutes and then analyzed using a FACS Caliber (BD Biosciences).

Dihydrorhodamine (DHR) flow cytometry assay of phagocyte respiratory burst:
Analysis for DHR oxidation was performed and analyzed on a FACS Calibur using forward and side scatter characteristics to define neutrophil and monocyte gates1.

Superoxide measurement:
A quantitative ferricytochrome C reduction assay was used to measure superoxide production at 10 and 60 minutes post stimulation1.

Linear Amplified Mediated (LAM) PCR analysis of vector insert sites:
LAM PCR was performed20 on samples from patients on this trial as well as archived samples from the 1998 trial (95-I-0134) using a primer pair set designed for the MFGS vector. Linear PCR product using 5’ biotinylated primer was magnetic selected and purified product mixed with Klenow polymerase and random hexamers. Products were enzymatically cut with TASI (Fermentas, Glen Burnie, MD) (and PUVI in some cases to remove the internal control band) and then ligated to a linker cassette using a Fast-link DNA ligation kit (Epicentre Technologies, Madison, WI). Product was denatured to remove single stranded DNA and beads. Finally, exponential PCR was performed using linker and 5’ end specific primers (LCI and LTR-R1 and LCIII and LTR-R2) and analysed on spreadex gels for visualization. Individual bands were sequenced using primers LCIII and LTR IV and TA cloning. Shotgun cloning was also
performed for integration site analysis using pooled LAM PCR products and the TOPO TA cloning kit. (See SI for primer sequences.) Clonal tracking was performed by designing primers specific for LAM-detected insertion sites. Samples from various time points were then reanalyzed to assess for presence of the clone with the specific insert using semi-nested PCR.

**Results**

*Patient Characteristics (Table 1):*

Three adult males with X-CGD (total deficiency of gp91phox protein) were enrolled in this trial. In 1995 Malech *et al.* initiated a clinical gene therapy trial for patients with p47phox deficient CGD without using conditioning. This was followed in 1998 by a trial for X-CGD, also without conditioning, the results of which were reported in only preliminary detail. There, six X-CGD patients were treated with the same MFGS-gp91phox vector as used in the current trial. Those patients were given multiple infusions of genetically modified cells but without conditioning (except for patient 6 where it was used in conjunction with an allogeneic transplant). Patient 1 from the current trial was also the third patient treated on that previous study, allowing comparison of gene transfer in the same individual; first without conditioning (Figure 2), then 7 years later with busulfan, using the same vector lot (results presented below). In the earlier study he, along with three others (4 of 6 patient in that study) produced very low, but detectable numbers of oxidase positive neutrophils, which rose transiently in the circulation after each infusion, but did not persist longer than one year. However, 2 of 6 patients in that previous study had no detectable marking.

For the current study, Patient 1 presented with fevers caused by *Staphylococcus aureus* liver abscesses not amenable to surgical resection due to their size, location, and his previous complications from 5 prior liver resections. He was enrolled and treated in November 2006. Patient 2 had a *Paecilomyces* fungus pneumonia extending into the chest wall and rib with recurrent accumulations of pus and spontaneous drainage despite two years of combination antifungal treatment and surgical resections. Patient 3 had *Aspergillus* fungus lung infection extending to ribs and vertebrae persisting and expanding despite multi-agent antifungal therapy for over one year.

*Effects of Busulfan:*

All patients developed neutropenia and thrombocytopenia (Figure 3). Time to neutrophil nadir varied from Day 11 to 14 but all patients had ≥500k/uL by day 28. Patient 3 required G-CSF for
prolonged neutropenia. All patients required platelet transfusions with Patient 3 requiring them to Day 48. Patient 1 developed moderate mucositis. With the addition of palifermin Patients 2 and 3 had little mucositis. Overall the patients tolerated the busulfan well.

Patient 2 received intravenous nutrition supplementation due to inanition from infection. All patients were maintained on their infection specific antimicrobials. The protocol was amended to include rapamycin treatment for Patient 3 because of early loss of marking in Patient 2. This was tapered off after 4 weeks and there was no evidence of toxicity from rapamycin.

**Graft characteristics:**
The characteristics of the autologous mobilized transduced CD34+ cell graft and busulfan AUC is shown in Table 2. Note that significantly fewer cells were available for transduction for Patient 3 compared to Patients 1 and 2, as he mobilized poorly.

Functional correction of oxidase activity in circulating neutrophils and monocytes:
At 2-3 weeks post infusion, DHR analysis of peripheral blood circulating neutrophils and monocytes from Patients 1, 2 and 3 demonstrated that 26%, 5% and 4%, respectively, had become oxidase normal. Figure 4 shows the results for neutrophils in each patient over time, but monocytes had similar percent correction at each time point. In Patient 1 the percent of oxidase normal cells declined slowly, reaching a plateau of 1% at 7 months; was still 1.1% at 2 years after gene therapy (Figure 5); and remains stable at 1.1% at 34 months (not shown). After initially producing 5% oxidase normal neutrophils, Patient 2 had no DHR positive neutrophils and no PCR detectable inserts at 4 weeks or longer. Like Patient 1, Patient 3 has persistence of oxidase normal neutrophils in the circulation, declining from a high of 4% to a steady state level of 0.03% (Figure 4) that has persisted at this level now with 11 months of follow up (not shown).

**Superoxide production:**
Before gene therapy, DHR assays of oxidase function in PMA stimulated neutrophils from all three patients were uniformly negative with low mean fluorescence intensity (MFI) similar to unstimulated cells. After gene therapy, at all time points at which marking was detectable, the MFI of all three patients’ PMA stimulated neutrophils was not merely increased, but at each assay was identical to the MFI of the PMA stimulated normal neutrophils from a healthy volunteer (example of this comparison shown in Figure 5). The ferricytochrome C reduction assay was performed on patient neutrophils to provide precise quantitative measurement of
superoxide production. These assays confirmed that the amount of superoxide produced by patient gene corrected neutrophils was the same as that from healthy volunteer neutrophils, when the data is corrected for the number of DHR positive neutrophils detected in the same sample (data not shown). This demonstrates that the MFGS-gp91phox vector achieves sufficient transgene protein expression in the cell to fully reconstitute phagocyte oxidase activity.

**Vector copy number and clonal tracking:**
PCR analysis of Patient 1 total neutrophils (CD15 cells) at the earliest time point, when 26% of neutrophils were DHR positive, showed a vector insert copy number of 1.335 per transduced cell. The overall marking has declined to and stabilized at about 0.7% consistent with the decline in percent DHR positive cells. Marking was also detectable in the B-cell fraction of Patient 1 (0.6% currently). As expected, peripheral T-cell marking in Patient 1 was lower, starting at 0.057 and now persisting at 0.002%. In addition, clonal tracking in this patient shown in Figure 6 demonstrates the presence of the same clones at both early (6 months), and late (2 years) time points in different lineages, suggesting transduction and engraftment of long term repopulating progenitors (see Figure 4). Patient 2 had early loss of any marking detectable by PCR, correlating with the DHR analysis. Although PCR detectable marking in Patient 3 has persisted, correlating with a low level persistence of DHR positive cells, the very low marking precludes accurate PCR quantification or clonal tracking.

**Infection Course:**
As shown in Figure 7, Patient 1 demonstrated resolution of his liver abscesses after gene therapy. Near the end of his prolonged course of antibiotic therapy for liver abscess (8 months post gene therapy), the patient developed an infection of the central venous catheter that resolved quickly after removal of the catheter plus a short course of antibiotics. For the next two years (now 34 months post-gene therapy), this patient has been free of any infection and remains well on standard CGD antimicrobial prophylaxis.

Patient 2, who had early gene marking that quickly disappeared, succumbed to his invasive Paecilomyces fungus infection, dying almost six months after his gene therapy despite continuous intensive antifungal therapy and allogeneic irradiated granulocyte transfusions. A donor search was initiated, but the best possible matches were only 5/6 cord blood products. Unfortunately the patient’s infection continued to worsen precluding even the possibility of a double cord transplant.
Patient 3 demonstrated significant radiologic evidence of regression of his *Aspergillus* infection followed by stabilization at 6 months post gene therapy with residual scarring and chronic pulmonary changes. Given the extent of disease and the development of neutropenic fevers, this patient was treated with allogeneic irradiated granulocyte infusions until recovery of an absolute neutrophil count >500 cells per μl, complicating interpretation of the role of gene therapy in resolution of his fungus infection.

Patients were not treated with granulocytes prior to the gene therapy to avoid the development of alloimmunization. However as in both patient 2 and 3, they were used once gene therapy had been performed. For patient 3, they were given for only the first few weeks, a time that overlapped in part with the period of rapamycin administration, and alloimmunization did not occur.

*Insertional mutagenesis monitoring:*

All patients were closely monitored for evidence of clonality of gene marked cells. At no point has there been any evidence of oligoclonality or clonal dominance. In particular, for Patient 1 who is now almost 3 years from treatment, the marking levels have remained steady and monitoring by LAM PCR shows a diverse polyclonal population with multiple bands seen in all marked lineages. A bone marrow biopsy performed at 1 year and repeated at approximately 3 years following gene therapy shows normal marrow histology and karyotype. Of note we have also performed an analysis on archived samples from our 1998 X-CGD gene therapy trial. Despite the very low marking, we cloned and characterized 42 unique sites from the first trial and none involved EV11/MDS, PRDM16 or Set BP1 nor were any sites found within 100kb of those genes. Additionally, none of the original participants have developed any evidence of MDS with follow up more than 10 years.

*Discussion:*

Gene therapy has achieved lasting clinical benefit and restoration of immunity for some primary immune deficiencies. However in disorders such as CGD where there is no selective advantage provided by the corrective therapeutic gene, conditioning appears necessary for long term persistence of marked cells. In our 1998 gene transfer trial for X-CGD without conditioning, 4 of the 6 patients achieved only very low levels of gene marking, and that marking did not persist. Busulfan conditioning in this current protocol greatly improved initial marking.
and resulted in persistence of marking in two of the three patients. Patient 1 had early marking peaking at 26% and has continued stable production of oxidase normal cells comprising ≥1% of circulating neutrophils and monocytes from 7 months to almost 3 years at last follow up. Since this patient was also treated on the previous gene therapy study without conditioning but using the same vector lot, it is noteworthy that marking in the same patient in the previous study never exceeded 0.055%, and did not have any evidence of genetically marked cells 9 months after the last infusion. Although there are some differences in the transduction methods used in these two studies the initial presence of marked cells during the first trial with their subsequent disappearance versus continued presence of marked cells after busulfan supports the need for bone marrow suppression to promote long-term engraftment.

Clinical benefit from ADA-SCID gene therapy required some busulfan conditioning (4mg/kg) despite selective growth advantage conferred on lymphocytes. In that setting marrow engraftment of gene marked CD34+ cell compartment was 5.1%, versus the 88% seen for the peripheral blood T cells. Since no selective growth advance was anticipated for correction of X-CGD, we used a higher but still not ablative dose of busulfan (10mg/kg). The source of CD34s, i.e. bone marrow versus mobilized peripheral blood (PB) may also play a role; however cell dose, which is also a factor, is usually larger with PB grafts. Ott et al. chose busulfan at 8mg/kg for their trial as they also assumed a greater degree of conditioning was necessary for long-term engraftment in the CGD setting. We chose 10mg/kg based on our own experience of using this dose in an allogeneic transplant for CGD. The difference between 8 and 10mg/kg is unknown although the increased effect may be more than linear. Patient 1, who had the highest AUC perhaps due to altered metabolism from his liver abscesses, had the highest degree of marking. Despite the elevated AUC and his underlying abscesses, he had no evidence of liver toxicity. Moreover his duration of neutropenia, thrombocytopenia, and other side effect profile were similar to the other two patients.

Our second patient had initial marking of 5% but lost that marking extremely rapidly. Gene silencing occurs often in murine gene transfer studies; it can also occur, though much less often in human cells and human clinical trials; and there was evidence for silencing of transgene protein production in the Ott et al. CGD gene therapy trial. New vectors may incorporate transcriptional insulators to reduce silencing. However, vector marking by PCR analysis also became undetectable in Patient 2 blood in parallel with loss of oxidase activity, confirming
that loss of graft rather than gene silencing had occurred. We looked for development of an immune response as an etiology, but did not find evidence for this.

None the less, development of an immune response to the transgene, vector components, or other immunogens has clearly been shown to occur in a large animal model of gene therapy\textsuperscript{19}. Thus, despite our inability to detect an immune cause of the rapid loss of graft in Patient 2, we could not ignore this possibility as we planned the treatment of our third patient in this study. Since the purpose of this gene therapy treatment trial is to provide salvage therapy for an infection not controlled by standard therapy, there was concern that we not interpret our failure to detect immune mediated graft loss as definitive evidence that immune elimination of graft did not occur. We therefore sought to incorporate a way of preventing immunity to the graft and enhancing tolerance without significantly increasing risk to the patient. A consensus settled on short-term treatment with rapamycin, a well tolerated and widely used immune suppressant with few side effects, a short half-life, and a proven track record of inducing tolerance. For this reason, Patient 3 was treated with rapamycin starting two days before gene therapy until 30 days after the gene therapy. The graft that Patient 3 received had much fewer cells at lower transduction than Patient 2, although initial marking of Patient 3 was similar to that of Patient 2, 4.1\% versus 5.0\% respectively. However, 0.03\% of circulating neutrophils and monocytes remain oxidase normal in Patient 3 at last assessment at 13 months. Although we cannot be conclusive about any benefits from rapamycin, it was well tolerated and a persistence of corrected neutrophils was achieved that did not occur in Patient 2.

Regardless of the several mechanisms that may lead to cell clearance, it is clear from our results that both the early and late in vivo marking is directly affected by the ex vivo transduction efficiency and total number of transduced cells infused. This effect of transduction efficiency and cell dose has also been noted in the NOD/SCID mouse xenograft model and in non-human primate gene marking studies\textsuperscript{28-30}. Although profound effects on outcome may also occur from vector insert effects such as observed in the study using the SFFV vector, where gene marking of neutrophils was very high in vivo despite only a 30\% ex vivo transduction efficiency, there were undesired side effects from this outgrowth. We did not observe any evidence of insertional mutagenesis or clonal expansion in our patients, but do not believe that vector insertion driven expansion is a desirable way to enhance gene marking. While the degree of conditioning we achieved with 10mg/kg of busulfan significantly improved both the short term and long term marking, it is unlikely that higher doses will achieve significantly higher levels of marking.
without increasing the toxicity beyond acceptable levels. In order to achieve clinically beneficial levels of marking, we believe that maneuvers enhancing ex vivo gene targeting of true hematopoietic stem cells such as the use of lentivirus vectors, may be one means of reaching this goal.

The MFGS-gp91^{phox} vector mediates sufficient production of gp91phox transgene to fully correct oxidase function in an X-CGD neutrophil. This was clearly demonstrated in our study by the DHR and supporting quantitative assays of superoxide production. The SFFV vector used in the Ott et al. study mediated oxidase function correction on a per neutrophil basis that was estimated by the authors as only 15-30% of the amount produced by a normal neutrophil. The level of oxidase production on a per neutrophil basis may be as crucial for infection management as overall transduction efficiency, since microbial killing occurs within an individual cell’s phagosome. Although there are safety and other advantages to lentiviral vectors that continue to drive an interest in their development, our experience with production of gp91^{phox} proteins from lentivectors is that production levels to date have been significantly much lower than what we can achieve with the MFGS vector. Even if fewer cells are transduced, cells with normal function can act synergistically with residing abnormal cells to improve infection response.

Patient 1 had complete resolution of his massive liver infection. This is a setting where experience has demonstrated that antibiotics alone, without the major surgery that was contraindicated in this patient, would not have cured this infection. While this does not constitute proof that gene therapy cured his infection, a very strong case can be made for significant clinical benefit to this patient from the gene therapy. Furthermore, this patient has been infection free for almost 23 months, the longest infection free period he has experienced in the last 10 years despite being only partially compliant with his maintenance antimicrobial prophylaxis.

Despite significantly less efficient gene correction than in Patient 1, Patient 3 may also have obtained clinical benefit from the gene therapy in that the progression of his aspergillus infection that was occurring before gene therapy stopped; significant resolution of infection was documented; and the infection may be cured. Patient 3 did receive a short course of allogeneic granulocyte transfusions during the neutropenic period following busulfan conditioning, complicating any firm conclusion regarding the exclusive role of the gene therapy in helping to control and resolve his fungus infection.
The two X-SCID trials and the Ott et al. trial demonstrate that retroviral vectors pose a risk of insertional mutagenesis. There does appear to be a predisposition of the X-SCID phenotype to oncogenesis with gene therapy and possibly a cooperation of the common gamma chain therapeutic gene itself to tumorigenesis. By contrast there has been no leukemia or myelodysplasia seen in any ADA-SCID gene therapy patients. The CGD trial by Ott et al., demonstrated a predisposition to outgrowth of clones with vector insertions seen in EVI1, SetBP1 and PRDM16, but this may be in part an effect of the strong myeloid enhancing element of the SFFV vector LTR. Interestingly these insertions were not seen in an ADA-SCID patient treated with an SFFV-based vector. EVI1 has been shown to be a target in other large animal studies using non-SFFV gammaretrovirus vectors, although there was no progression to clonal hematopoiesis or leukemia. Still, an ex vivo mouse marrow myeloid immortalization study demonstrated that SFFV transduced clones had a higher rate of transformation, as compared to MSCV transduced cells (C. Baum, personal communication) possibly explaining the difference seen in our study as compared to the CGD trial using the SFFV2. In our current study we seen no evidence of any clonal outgrowth in our two patients that we have followed to date to 34 and 11 months of follow up. Analysis of more than 60 insertions characterized from Patient 1 to date did find one in EVI1 from two separate time points but not in all analyses, nor does it predominate, and overall pattern of inserts remains polyclonal with no evidence of clonal outgrowth and with continued evidence for normal hematopoiesis (see SI).

A brief report appeared in electronic print version very recently describing the outcome of gene therapy in an 8½ year old X-CGD child who had severe Aspergillus pneumonia. The patient received busulfan conditioning of 8.8 mg/kg and CD34+ cells gene-marked ex vivo at 31% to 34% (using the Ott et al. vector2) were administered intravenously and intra-osseously. The level of early gene marking of neutrophils reported at 26-29% was similar to that seen with our Patient 1, and as with him, their patient appeared to have significant clinical benefit with control of his infection. Although their report focused upon neutrophil extracellular DNA nets as a possible mechanism for benefit, less than three months of follow up from gene therapy is provided in the report, and level of oxidase function per neutrophil cannot be determined from the paper. However, their report supports the notion that gene therapy for X-CGD can help control a life-threatening infection.

We show here the results of three patients treated with busulfan and ex vivo genetically modified cells. Patient 1 had unequivocal clinical benefit with continued production of 1% oxidase
normal cells in his peripheral blood now almost 3 years after treatment. This represents the highest level of sustained marking with production of functional gene product in neutrophils seen in any patient to date beyond one year of follow up with any disease that does not have a selective advantage conferred by the corrective gene or as a result of unintended clonal outgrowth. Patient 3 may also have benefited in the control of his infection despite the substantially lower level of long-term gene marking. All patients tolerated the busulfan, which appears to be a critical element to achieve engraftment of gene corrected long-term repopulating cells. Additional progress in the field will require higher rates of gene transfer into pluripotent stem cells. Work to achieve this goal has focused on new vectors such as lentivectors\textsuperscript{44}, or novel pseudotyping such as RD114\textsuperscript{44-46}. Although gene therapy has not yet cured patients with CGD, the field has made significant progress with the demonstration that gene therapy can provide clinical benefit to patients.

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**Author Contributions:**
EMK - designed and implemented the study, wrote the paper.
UC - performed analyses, created figures, helped with vector design and production, reviewed the paper.
NT, GL, DLP, and DK - performed the majority of the assays in the study.
HLM – helped design and support the study as well as write the paper.

The authors have no conflicts of interest to disclose.
References:


Three patients with confirmed X-linked Chronic Granulomatous disease were enrolled in the study using genetically modified cells to treat an unresolving infection. Patient’s infections, specific genetic mutation and infection history as well as the medical therapy used concurrently are listed here.

Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Indication for Gene therapy</th>
<th>Mutation</th>
<th>Infection history</th>
<th>Medical treatment pre and during gene therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>Staphylococcal liver abscess</td>
<td>461delA; N154fsX160 (frameshift)</td>
<td>Recurrent infections including previous bacterial abscesses of the liver</td>
<td>IV meropenem, vancomycin and linezolid</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>Paecilomyces chest wall infection</td>
<td>4 nucleotide deletion that spans splice site-1150_E9(+2)de IAA/gt, deletion of Exon 9 in mRNA</td>
<td>Recurrent pneumonias requiring thoracotomies</td>
<td>IV ambisome then IV voriconazole - switched to posaconazole and 5FC, then caspofungin added</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>Aspergillus lung infection</td>
<td>Nonsense mutation: C217A; R73X</td>
<td>Prior aspergillus lung infection, burkholderia and bartonella infections as well as osteomyelitis</td>
<td>IV ambisome, then treated with voriconazole and micafungin</td>
</tr>
</tbody>
</table>

Table 2: Graft description

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD34/kg dose starting</th>
<th>CD34/kg dose after transduction</th>
<th>CD34%</th>
<th>GP91%</th>
<th>Vector copy number</th>
<th>Vector Lot number</th>
<th>Busulfan AUC (uMol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>43</td>
<td>85%</td>
<td>73%</td>
<td>4.05</td>
<td>1</td>
<td>11324</td>
</tr>
<tr>
<td>2</td>
<td>13.7</td>
<td>71</td>
<td>&gt;90%</td>
<td>41%</td>
<td>.8</td>
<td>2</td>
<td>7638</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>18.9</td>
<td>70%</td>
<td>25%</td>
<td>.005</td>
<td>3</td>
<td>7560</td>
</tr>
</tbody>
</table>

The CD34 doses as well as the marking of the graft as measured by either flow or GP91 expression or PCR for copy number, along with the Busulfan levels for each patient are given.
Figure Legends

Figure 1: Study Design
Patient’s cells were collected and stored on a separate protocol. Day 0 indicates the day of cell infusion. Cells were thawed on Day -5, and transduction was begun on Day -4. The busulfan was infused on Days -3 and -2. Patients 2 and 3 received palifermin given on Days -6 to -4 and -1 to +1. Patient 3 also started rapamycin on Day -1 and continued it for 30 days post transplant.

Figure 2: Results from Patient 1 during his first gene therapy trial in 1998.
Patient 1 from our trial was enrolled previously on a gene therapy trial that used the same vector, however did not include conditioning. The patients received 2 separate infusions of cells as marked by the arrows and the number of cells given x10^6. Marking was detectable early, and increased after each infusion, but eventually was undetectable after one year.

Figure 3: Effect of Busulfan on Neutrophil and Platelet counts.
A) The absolute neutrophil count for each patient is plotted beginning prior to transplant (baseline) to Day 50 post transplant. Cell infusion was Day 0 and the busulfan was given Days -3 and -2.
B) The platelet counts for each patient is plotted beginning prior to the busulfan and until all patients no longer required any transfusions, Day 48.

Figure 4: Percentage of DHR positive cells post cell infusion (Day 0).
Flow cytometric analysis was performed at various time points for each patient to assess for oxidase positive cells in the peripheral blood. Each patient had undetectable DHR prior to beginning the therapy. The percentage is plotted on a logarithmic scale with each patient having a different range starting at 0.1 for Patient 1, 0.001 for Patient 2 and 0.01 for Patient 3.

Figure 5: Flow Cytometry Panel showing DHR analysis for Patient 1 at 2 years post gene therapy.
Patient 1 who had the best results continues to have approximately 1% DHR positive cells in the peripheral blood. The MFI for this patients cells are consistently in the same range as the normal control run concurrently, indicating a close to normal level of oxidase production on a per cell basis.
Figure 6: Patient 1 Clonal Tracking PCR
Primers were designed based on inserts identified from the LAM –PCR from Patient 1 and a semi-nested PCR was performed on samples obtained at various time points to determine their presence in the various lineages.

Figure 7: Clinical Results for Patient 1
Shown here are the CT scans obtained pre gene therapy and 6 months post gene therapy for Patient 1 who had biopsy proven *Staphylococcal aureus* liver abscesses as indicated by the arrow. These disappeared leaving only some scarring and regenerating liver as shown in the corresponding post films.
Figure 1: Study Design

- Days of GCSF administration (minimum two weeks prior to gene therapy)
- Palifermin (patients 2 and 3 only)
- Cells thawed (collected previously and stored)
- Busulfan infusion
- Rapamycin begins (patient 3 only)
- Cell infusion
- Apheresis

Figure 2: Results from Patient 1 during his first gene therapy trial in 1998.
**Figure 3:** Effect Of Busulfan on Neutrophil and Platelet counts

Days Post Transplantation

- **A**
  - Absolute Neutrophil Count (K/μl)
  - Days Post Transplantation
  - Clones: #1 Inserted at Chr.1, #3 Inserted at Chr. 11

- **B**
  - Platelet count (K/μl)
  - Days Post Transplantation
  - Clones: #1-3

**Figure 4:** Patient 1 Clonal Tracking PCR

- DNA size marker
- CD3+ cells at 6 months after transplantation
- CD3+ cells at 1.5 year after transplantation
- CD3+ cells at 2 years after transplantation
- CD19+ cells at 6 months after transplantation
- CD19+ cells at 1.5 year after transplantation
- CD19+ cells at 2 years after transplantation
- CD14+ cells at 6 months after transplantation
- CD14+ cells at 1.5 year after transplantation
- CD14+ cells at 2 years after transplantation
- CD15+ cells at 6 months after transplantation
- CD15+ cells at 1.5 year after transplantation
- CD15+ cells at 2 years after transplantation

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Figure 5: Percentage of DHR positive cells post cell infusion (Day 0) in all three patients.

Figure 6: Flow Cytometry Panel showing DHR analysis for Patient 1 at 2 years post gene therapy.

Figure 7: Clinical Results for Patient 1

- Image 1 pre gene therapy
- Image 2 pre gene therapy
- Image 1 post gene therapy
- Image 2 post gene therapy

Indicates location of abscess
Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long term correction of oxidase activity in peripheral blood neutrophils

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