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HIGH LEVEL OF PERFORIN EXPRESSION ~~PER CELL~~ IS REQUIRED FOR EFFECTIVE CORRECTION OF HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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ABSTRACT

Perforin-1 mutations result in a potentially fatal hemophagocytic lymphohistiocytosis (HLH) with heightened immune activation, hypercytokinemia, pancytopenia and end-organ damage. Currently, hematopoietic stem cell (HSC) transplant is curative, but limited by donor availability and associated mortality, making gene therapy an attractive alternative approach for HLH. We recently reported that perforin expression driven by cellular promoters in lentiviral vectors (LV) resulted in significant, albeit partial, correction of the inflammatory features in a murine model of HLH. We hypothesized that the level of perforin expression achieved/cell from ectopic moderate strength cellular promoters (PGK/PRF) are inadequate and thus engineered a LV utilizing a viral promoter (MND) containing microRNA126 target sequences to restrict perforin expression in HSC. We show here that MND-LV restored perforin expression to normal levels in a perforin-deficient human NK cell-line and perforin gene corrected Perforin1-/- transplant recipients, while cellular promoters drove only partial correction. Upon lymphocytic choriomeningitis virus challenge, the clinical scores and survival improved only with the MND-LV but inflammatory markers and cytotoxicity were improved with all LVs. Our studies suggest that although moderate levels of expression can result in partial amelioration of the HLH phenotype, high levels of perforin expression *per cell* are required for complete correction of HLH.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal immune regulatory disorder principally caused by impaired lymphocyte cytotoxicity. Patients experience episodes of extreme immune activation triggered by a variety of infectious organisms, but most commonly viruses. HLH is marked by an exaggerated release of cytokines, such as IFN-γ, IL-6, IL-10 and IL-1; hemophagocytosis of hematopoietic cells; lymphoproliferation; and end-organ damage[1-4]. Patients with HLH present with signs and symptoms that include high-grade fever, hepatitis, seizures, cytopenias, lymphadenopathy, splenomegaly, and may ultimately progress to death from respiratory and/or cardiovascular collapse.

Familial HLH (FHLH) results from an underlying genetic defect in the cytotoxic or degranulation pathways. The known mutations causing FHLH are either in the degranulation/granule trafficking pathway genes, such as Munc13-4, Stx11, Rab27a, StxBP2 or the cytolytic pathway gene, Perforin-1 (PRF1)[5, 6]. The most common cause of FHLH (in 20-40%) is perforin gene mutations designated as FHLH2[7]. Perforin is a critical component of lymphocyte cytotoxicity and is expressed in CD8+ T cells and natural killer (NK) cells[8-10].

The short-term treatment of HLH is suppression of the overwhelming immune activation combined with identification and treatment of potential triggering infections. The long-term curative option for FHLH is allogeneic hematopoietic stem cell transplantation (HCT). HCT, however, is limited by availability of donors, can have potential serious adverse effects such as graft versus host disease; and is associated with 20-50% mortality. HCT-related mortality has improved by using reduced intensity conditioning; however, mixed chimerism or graft rejection still poses challenges in 30-50% of transplanted patients[11, 12].

We have previously shown that 10-20% of wild type (WT) chimerism corrects HLH symptoms in a $Prf1^{-/-}$ mouse model[13, 14]. We recently reported significant, but partial, correction of cytopenias

and cytotoxicity in Prf1$^{-/-}$ mice using lentivirus vectors (LV) expressing perforin from cellular promoters of the phosphoglycero kinase gene (PGK) or the perforin-1 gene (PRF)[15]. We theorized that this incomplete rescue may have been due to an insufficient level perforin expression driven by these promoters. It has been previously shown that cellular promoters from ubiquitously expressed or tissue-restricted genes were inadequate for correcting the defect in leukocyte adhesion deficiency, where a strong viral promoter was required for phenotypic correction of this defect[16]. Similar results have been reported in chronic granulomatous disease [17, 18] and Wiskott-Aldrich syndrome (WAS) [19, 20].

We hypothesized that a high level of perforin expression from a viral promoter would be necessary for complete correction of the HLH phenotype. Perforin expression is restricted to cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells[10]. In order to permit high perforin expression in cytotoxic lymphocytes and to prevent potential toxicity due to perforin expression in HSCs, we placed miR126 target sequences in the 3' untranslated region of the perforin cDNA in LVs driven by the MND promoter/enhancer. MND driven perforin expression restored cytotoxicity in perforin deficient human NK and murine CTL. Perforin deficient mice that received gene-corrected HSC with the MND vector exhibited a dose responsive increase in survival and viral clearance following LCMV infection.

MATERIALS AND METHODS

Lentivirus vectors: The sequence for hsa-miR126-3p was obtained from the miRbase directory. To create a vector with 4 target sites of the miR126-3p, we designed an oligonucleotide to include 4 repeats of the miR126-3p sequence (5'-CTAGAATGCATCGCATTATTACTCACGGTACGACTAGCGCATTATTACTCACGGTACGAACCGGTCGCATTATTACTCACGGTACGATCACCGCATTATTACTCACGGTACGAGTTTAAACCCC-3'. The underlined region of ~~is~~ the sequence is complementary to the hsa-miR126-3p sequence. This oligonucleotide was cloned using XbaI/SmaI into LV.NGFR.CMV.PGK.GFP.WPRE (kindly provided by Dr. Luigi Naldini, San Rafaelle Institute, Milan, Italy) to derive LV.NGFR.CMV.PGK.GFP.4TmiR126 (GFP4T).

For the construction of LV.NGFR.CMV.PGK.Perforin1.WPRE.4TmiR126 (PGK4T), an EcoRI/XhoI PGK.Perforin1 fragment was isolated from LV.PGK.PRF.IRES.GFP (PGK) that we previously published[15]. The EcoRI end was blunted and the PGK.Perforin1 ligated into the EcoRV/SalI digested GFP4T. The same strategy was used to clone the LV.NGFR.CMV.PRF.Perforin1.WPRE.4TmiR126 (PRF4T), with the fragment PRF.Perforin1 from the LV.PRF.Perforin1.IRES.GFP (PRF)[15] into GFP4T. The miR126 target sites were then removed from PRF4T with XbaI and relegation to create vector PRF0T.

The LV.NGFR.CMV.MND.Perforin1.WPRE.4TmiR126 (MND4T) was generated by ligating the EcoRI/XbaI PRF.Perforin1 fragment into an intermediate p-Blue script (pBS) vector from the LV.PRF.Perforin.I.GFP[15]. The EcoRI/AgeI MND promoter was isolated from MND-eGFP-WPRE vector and ligated into Xho1/Age1 pBS-PRF.Perforin1 to create the intermediate plasmid pBS-MND-Perforin1 from which, the SpeI (blunt)/XhoI fragment of MND.Perforin1 was ligated into the EcoRV/SalI sites of the GFP4T vector. LV was produced using transient transfection of 293T cells

and virus titers determined by transduction of mouse erythroleukemia cells after serial dilutions, followed by flow cytometry (all previously described)[15].

Mice Perforin-1 deficient mice (C57BL/6-Prf1tm1Sdz/J; Prf1-/-) were obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred in the animal facility at Cincinnati Children's Hospital Medical Center CCHMC. For HCT, 6-8 week old recipient Prf1-/- mice were treated with busulfan (20mg/kg from days -4 to -1) and cyclophosphamide (100mg/kg on days -2 and -1) and transplanted on day 0 (24 hours after chemotherapy conditioning) with either Prf1-/- or C57/Bl6 (WT) LSK cells or Lin-Sca-1+cKit+ (LSK) cells transduced with one of the three LV. Transplanted mice were challenged with 200 plaque forming units of lymphocytic choriomeningitis virus (LCMV) intraperitoneally 16 weeks post-HCT. Clinical scoring of the mice was done as previously described[21]. Eye infections, dehydration, and ascites were graded on a 0-2 scale while weight, coordination, and hunched posture on 0-3 scale. All experimental procedures in mice were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee at CCHMC.

Isolation and transduction of HSPC and NK cell line Bone marrow LSK cells and Lin-Sca-1-cKit+ (LK) cells from femurs and tibias of Prf1-/- or WT mice were isolated using a combination of magnetic labelling and FACS sorting as previously published[15]. LSK and LK cells were both cultured in StemSpan SFEM serum free medium (Stem Cell Technologies) supplemented with 2%FBS, 0.5% Lipoprotein Low Density (Sigma), 0.1% 10mM dNTP (New England Biolabs), 1% Penicillin/Streptomycin (Fisher Scientific), 10ng/ml mouse IL-3 and 50ng/ml mouse SCF (R&D Biosystems). LSK cells from Prf1-/- mice were transduced as described below; LK cells were irradiated with 3000 rads before co-injection into recipient mice.

LSK cells were pre-stimulated over-night and transduced twice at 8 hour intervals. The multiplicity of infection (MOI) of transduction was maintained at 25-30 per transduction. After 36 hours of

culture, the cells were harvested and 4-5x10^4 LSK cells along with 6-8x10^4 irradiated LK cells were injected IV into recipient mice. A portion of the LSK cells were kept in culture for 6-7 days to measure initial transduction efficiency.

KHYG1 cells (from Japanese Collection of Research Bioresources Cell Bank, JCRB0156) were transduced with retrovirus to overexpress miR30-based shRNAs targeting the 3'UTR of *PRF1* with mCherry expressed in *cis*[22] (a kind gift from I. Voskoboinik, Peter MacCallum Cancer Centre, Melbourne, Australia). Transduced KHYG1 cells were sorted for mCherry expression 7 days later to obtain the KHYG1 KD line. KHYG1 were maintained in complete RPMI with 100U/mL rIL-2 (Roche). LV transduction was performed at MOI of 5. 72 hours post-transduction, the cells were sorted for tNGFR after staining with Alexa-647 (BD Pharmingen anti CD271 antibody catalog number 560326) and then magnetic column sorted for alexa-647 positive cells using Miltenyi micro beads (catalog number 130-091-395).

Human umbilical cord blood (CB) cells were used to isolate CD34+ cells using the Miltenyi CD34 microbead kit (catalog number 130-046-702) as per manufacturer's protocol. CD34+ cells were pre-stimulated overnight in 1 ml α-MEM media (Gibco) containing 10% human serum (Sigma) and 5% FCS (Sigma) supplemented with 10 mM Hepes (Gibco), 10mM sodium pyruvate (Gibco), penicillin/streptomycin (Gibco), 20ng/ml recombinant human SCF (rhSCF) (PeproTech)and 20ng/ml rhIL-15 (PeproTech) and transduced with LV at an MOI of 10 twice at 12 hour intervals. After 36 hours of culture, a portion of cells were analyzed for initial transduction efficiency and the rest transferred for NK differentiation, as previously described with minor modifications[23]. Cells were seeded on a pre-established confluent layer of murine MS-5 stromal cells in the presence of rhSCF and rhIL-15 for three weeks. MS-5 cells were routinely expanded in α-MEM containing 20% FCS and passaged in 24-well plates at a concentration of 6 x 10^4 cells/ml/well 24 hours before initiation of the co-culture. Cultures were maintained at 37 degree, 5% CO_2, in an air atmosphere saturated with humidity. Half of media was renewed twice a week and cells were

tested for CD56+ cell percentage once a week. Eight days after the LCMV challenge, a subset of transplanted mice was sacrificed and spleens were harvested as single cell suspensions for CTL assays. Splenocytes were analyzed for tNGFR expression and stained for CD8 biotin and sorted for RNA isolation.

Flow Cytometry Chimerism in the transplanted mice was measured at 10-12 weeks post-transplant by staining for tNGFR (CD271) PE/APC (BD Pharmingen) in different lineages (CD8, CD4, Nk1.1 from BD Pharmingen) in peripheral blood. KHYG1 cells were stained for tNGFR PE/Alexa 647 and perforin stained with APC (BD Pharmingen). FACS analysis was done on BD FACSCANTO II (BD Biosciences).

Quantitative PCR and Quantitative Reverse Transcription PCR Genomic DNA was isolated with Qiagen Cell lysis solution using the manufacturer's protocol from bone marrow, spleen and peripheral blood. The VCN was determined by qPCR using WPRE primers for vector sequences and Flk-1 primers for endogenous control. Itaq Superprobes (Biorad) was used in accordance with the manufacturer's protocol on the BioRad CFX96 Thermocycler. The sequence of the primers and probes used were:

Flk-1 FP: AAGACCTTGAAGTTGGCAACGCAG, RP: AGGAAGAATGGGCAGATGGTCACA, Probe: HEX/TGCCGTGAATTGCAGAGCTGTTGTGT.

WPRE FP: TGTATAAATCCTGGTTGCTGTC, RP: GCACACCACGCCACGTTG, Probe: FAM/ATGAGGAGTTGTGGCCCGTTGT

RNA was isolated from KHYG1, spleen CD8+ and bone marrow cells with Qiazol Lysis Reagent using the manufacturer's protocol. Perforin mRNA expression was measured using primers that span exons 2-3 (20x assay mix from Life technologies - catalog number 4331182). The endogenous control used was human GAPDH with the primers specific to exon 3 (20x assay mix from Life Technologies – catalog number 4333764) or mouse Beta Actin (20x assay mix from Life

Technologies – catalog number 4352341E). For LCMV mRNA, iTaq™ Universal SYBR® Green Supermix was used to amplify the following primers:

LCMV FP: GAGTCCAGAAGCTTTCTGATGTCAT

LCMV RP: CAAGTATTCACACGGCATGGAT

Itaq Superprobes (Biorad) were used in accordance with the manufacturer's protocol on the Applied Biosystems 7900HT fast real time PCR system.

IFN-γ ELISA IFN-γ ELSIA was performed on plasma, as previously described [15, 21].

LCMV Quantification LCMV quantification was performed on lysates from bone marrow as previously described[24].

Chromium release assay ^{51}Chromium (^{51}Cr) release assay was performed as previously described[15]. Briefly, EL4 target cells were pulsed with 100ng/mL of LCMV gp33-41 for one hour prior to ^{51}Cr labelling. Effector and EL4 target cells (5,000 cells/well) were co-incubated for 4 hours at 37˚C. ^{51}Cr-release into supernatant was counted on a 96 well Lumaplate (PerkinElmer) by TopCount•NXT. Cytotoxicity was reported as the percent of ^{51}Cr released into the supernatant according to the following calculation: (test–spontaneous)/(max lysis – spontaneous) *100. Maximum lysis was determined by addition of 1% Triton X-100 (Sigma) to 5,000 EL4 target cells.

Western blot analysis Cell were lysed with lysis buffer (2% NP-40; 150 mM NaCl; 50 mM TrisCl [tris(hydroxymethyl)aminomethane chloride]; 1 mM MgCl2, pH 8; 2.5 mM EDTA; 2 mg/mL leupeptin; 2 mg/mL aprotinin; 50 mg/mL phenyl- methylsulfonyl fluoride; 1 mg/mL pepstatin). 15ug of lysate was prepared using Laemmli sample buffer without 2-mercaptoethanol and ran on a 10% Bis-Tris gel. Protein was transferred to a nitrocellulose membrane, blocked with Odyssey blocking buffer, probed by perforin antibody clone P1-8 1:500 (Kamiya Biomedical, Seattle, WA),

and visualized with a LICOR Odyssey CLx scanner. Equal loading was monitored by the detection of actin clone AC-15 1:5,000 (Sigma-Aldrich).

Statistical Analysis Statistical analysis was done using the GraphPad prism software.

RESULTS

Restriction of Expression in HSPC but not in the Cytotoxic Cell Progeny MicroRNA126 (miR126) target sequences placed 3' to transgenes expressed from LV have been reported to restrict transgene expression in HSC, but allow robust expression in its myeloid progeny[25, 26]. Reports on miR126 detection in NK cells are conflicting[26-28]. We evaluated whether miR126 target sequences would permit transgene expression in CD8 and NK cell progeny. We thus engineered LVs with a PGK promoter, GFP transgene, and either 4 (GFP-4T) or 0 (GFP-0T) repeats of the miR126 target sequences downstream of GFP cDNA. Both LV also included the truncated nerve growth factor (tNGFR) driven by a minimal CMV promoter in the opposite orientation, so that tNGFR expression would be unaffected by miR126 target sequences and permit determination of gene-modified chimerism **(Figure 1a)**.

We transduced human umbilical cord blood CD34+ hematopoietic stem and progenitor cells (HSPC) with the GFP-4T and GFP-0T LVs within the first 24 hours of isolation, and at 36 hours subjected a portion of the HSPC to flow cytometry and differentiated the rest to NK cells **(Figure 1b and c)**. Nearly 50% and 30% of HSPC showed transduction (tNGFR expression) with the GFP-0T and GFP-4T vectors, which corresponded with the vector copy number/cell (VCN) of 1.3 and 2, respectively. GFP expression was minimal in the CD34+ cells transduced with the GFP-4T vector, but robust in those transduced with the GFP-0T vector, confirming the previously reported restriction in expression in HSPC[25, 26]. However, upon differentiation into NK cells, GFP was robustly expressed from the GFP-4T vector in the same proportion of NK cells that expressed tNGFR. Collectively, we found that miR126 target sequences, while effectively restricting transgene expression in HSPC, allowed expression in their differentiated NK cell progeny at levels comparable to the vector without the miR126 target sequences.

Regulated Perforin LV and their relative expression and CTL activity We designed a new LV

using ubiquitously expressing MNDU3 promoter/enhancer derived from a modified murine myeloproliferative sarcoma retrovirus long terminal repeat (LTR)[29, 30], for comparison to the LV driven by the PGK promoter. Four repeats of the miR126 target sequences were placed 3' to the perforin cDNA to restrict expression from both these ubiquitously expressing promoters in HSC (termed MND4T and PGK4T vectors hereafter)[25, 26]. A third LV had perforin expression driven by the previously described PRF promoter (a -1430 bp 5' UTR of the perforin-1 gene) to achieve CTL/NK-specific expression of perforin[15]; no miR126 target sequences were placed in this LV since expression from the PRF promoter is naturally restricted in HSC (termed PRF0T). All LV also contained the miniCMV:tNGFR placed in the opposite orientation for unrestricted monitoring of gene transfer in HSC **(Figure 2a)**.

We determined the relative perforin expression from the PGK4T, MND4T and PRF0T LV in the KHYG1 cell line, a human NK cell line where we have suppressed perforin expression by approximately 90% stably with shRNA specific to the 3'UTR of perforin (KHYG1-KD)[22],[31]. The perforin cDNA in LV vectors lacks the 3'UTR and is hence unaffected by the shRNA. KHYG1-KD cells were transduced with all 3 LV at a low multiplicity of infection and sorted for tNGFR expressing cells to enrich for transduced cells with single or low VCN and tested for perforin expression in 3 independent transductions. The mean VCN were comparable: 1.22, 1.25 and 1.48 VCN in MND4T, PGK4T and PRF0T KHYG1-KD cells, respectively.

The relative perforin mRNA expression in the MND4T KHYG1-KD cells was about two-fold higher than that in the PGK4T and PRF0T cells and nearly comparable to WT perforin expression in KHYG1 **(Figure 2b)**. Flow cytometry analysis showed that both the percent cells and the mean fluorescence intensity of perforin was 2-fold higher in MND4T KYHG1-KD cells as compared to PGK4T and PRF0T KYHG1-KD cells **(Figure 2c)**. Results from Western blot revealed a similar trend in perforin protein levels from the MND4T LV compared to PGK4T and PRF0T LV, and that was comparable to WT perforin expression (**Figure 2d-e**; original western blot showed as Supplemental **Figure S1**). Taken together, expression of perforin from the MND4T vector in a human NK cell line was the highest

compared to other vectors and was equivalent to near normal levels.

Comparison of HLH phenotype between transplanted and untransplanted Prf1-/- mice following LCMV challenge: The murine transplantation model we have previously utilized included lethal radiation to ablate the marrow in the recipient mice[15]. In the current study, we gave busulfan and cyclophosphamide for myeloablation in order to better approximate clinical strategies used in human HCT. Following stable engraftment at 16 weeks, perforin deficient (Prf1-/-) mice were infected with LCMV to induce the HLH phenotype (experimental schema in supplemental **Figure S2**). Eight days following LCMV challenge, evaluation of cytotoxic T lymphocyte (CTL) function in splenocytes from a subgroup of mice showed similar strong CTL activity from WT and transplanted WT (TxWT) mice **(Figure 3a)**, and similarly poor CTL function in the $Prf1^{-/-}$ or the transplanted ($TxPrf1^{-/-}$) mice. A careful analysis showed distinct immunopathology in the transplanted mice compared to non-transplanted animals. We have previously shown that serum IFN-γ increases transiently after LCMV infection in WT mice, whereas in $Prf1^{-/-}$ mice, the increase in IFN-γ is highly exaggerated and persistent.[14] The IFN-γ response was substantially blunted in the $TxPrf1^{-/-}$ compared to $Prf1^{-/-}$ mice, although still significantly higher than in TxWT mice **(Figure 3b)**. Cytopenias from the hemophagocytosis and inflammation, another characteristic feature of HLH, develop around 2 weeks following LCMV challenge in $Prf1^{-/-}$ mice and these are normally minimal in WT mice. These differences were also blunted in $TxPrf1^{-/-}$ versus TxWT mice (Supplemental **Figures S3a, b**).

The clinical features of HLH were also compared. For clinical assessments, mice were graded for weight loss, activity, eye infections, skin turgor, and features of distress 2-3 times a week for 50 days after LCMV infection, after which the surviving mice were sacrificed. Parameters were graded on a 0-2/0-3 scale, with higher grades given for more severe signs or symptoms. In WT mice, clinical scores peaked at 15 days following LCMV infection and then came down and stayed low for the remainder of the experiment. In TxWT mice, peak scores were higher than untransplanted WT mice and there was

a delay before they returned to baseline **(Figure 3c)**. In the Prf1$^{-/-}$ and TxPrf1$^{-/-}$ mice, the clinical scores peaked and did not subside and mice succumbed to HLH, with the TxPrf1$^{-/-}$ mice dying earlier than Prf1$^{-/-}$ mice. Finally, survival in TxWT mice was lower than the WT mice **(Figure 3d)**. Hence, differences in clinical scores and survival reflected transplant associated mortality, with earlier death in TxPrf1-/- mice and some mortality even in TxWT mice, compared to untransplanted counterparts.

These findings suggested that the HLH phenotype in LSK transplanted mice with busulfan/cytoxan conditioning was distinct. Due to transplant-related mortality noted in WT mice, we reasoned that large numbers of animals and multiple experiments would be necessary to distinguish between HCT and HLH related mortality in experiments evaluating gene correction of Prf1-/- animals.

High levels of stable transgene chimerism corrects cytotoxicity and serum IFN-γ levels. We next evaluated the therapeutic efficacy of the MND4T, PGK4T and PRF0T LV in the Prf1-/- mouse model of HLH using the same myeloablation protocol. Prf1$^{-/-}$ LSK cells transduced with the 3 LV were transplanted into Prf1$^{-/-}$ mice (Supplemental **Figure S2**). Expression of tNGFR from the miniCMV promoter (**Figure 2a**) provided a measure of transduction efficiency in the PGK4T and MND4T LV transduced HSCs (Supplemental **Figure S4a**). Unexpectedly, however, the HSC transduced with PRF0T vector had reduced expression of tNGFR in HSC, but not in NK cells in the peripheral blood 12 weeks post-transplant (Supplemental **Figure S4a**). This suggested that the repressive elements that bind the PRF promoter in cells not meant to naturally express perforin[32], such as HSPC, also repressed the CMV minimal promoter immediately adjacent to the perforin promoter in the PRF0T vector (**Figure 2a**), Hence tNGFR expression was also co-repressed along with the perforin promoter in those cells. Just like the natural regulation of perforin, where NK cells express perforin constitutively[32], the repression of the miniCMV promoter driven tNGFR was not observed in NK cells (**Figure 4a**). Naive CD8 cells do not constitutively express perforin, and only express it when activated[32], and here, the repression of tNGFR expression was seen in naive CD8 cells (Supplemental **Figure S4b**), which was removed in activated CD8 cells following LCMV infection (**Figure 4d**, inset).

Hence, the perforin promoter co-regulated the adjacent miniCMV promoter and conferred it the same lineage specificity, since tNGFR expression paralleled natural expression of perforin. The chimerism of genetically-modified cells was therefore determined by tNGFR expression in resting NK cells which express perforin constitutively.

The median (and mean±SEM) gene-modified NK cell chimerism was 44% (52±3%), 51% (50±4%) and 70% (70±2%) in the MND4T, PRF0T and PGK4T mice, respectively, 12 weeks following HCT, with significantly higher chimerism in the PGK4T mice (**Figure 4a**). Notably, the median VCN in blood leukocytes were comparable between the PGK4T and PRF0T groups of mice (1.8 and 1.6 VCN respectively), but was significantly lower in MND4T mice at 0.98 **(Figure 4b)**. WT and $Prf1^{-/-}$ HSPC were also transplanted into $Prf1^{-/-}$ mice (TxWT and $TxPrf1^{-/-}$) as controls for the vector-modified $Prf1^{-/-}$ groups. We then induced HLH with LCMV infection 4 months following HCT and studied restoration of cytotoxicity.

Eight days following LCMV infection, a subset of mice in each of these groups was sacrificed to determine CTL activity of splenocytes against target EL4 cells *ex-vivo*; tNGFR expression was also determined on CD8 T splenocytes. In this subset, the median (mean±SEM) gene-modified CD8 T cells were the lowest in the MND4T splenocytes [25% (29±3%)], versus those in the PRF0T and PGK4T splenocytes [48% (49±6%) and 50% (54±6%), respectively (inset, **Figure 4c**)]. Despite half the chimerism, the CTL activity tended to be higher in the MND4T mice, closely followed by the PRF0T and PGK4T mice, albeit lower than that seen in TxWT mice, where all CTLs express perforin (**Figure 4c**). The IFN-γ levels in the MND4T and PRF0T groups were significantly lower than the $TxPrf1^{-/-}$ mice, although IFN-γ levels remained high in the PGK4T mice **(Figure 4d)**. However, there were minimal differences in peripheral blood cytopenias within the 3 groups compared to TxPrf1-/- mice (Supplemental **Figures S3a,b**).

Improved clinical score and survival are observed with the MND4T LV. The remaining mice were followed for clinical symptoms of HLH for up to 50 days following the LCMV challenge. The

median (and mean±SEM) gene-modified chimerism in these mice was 44% (51±3%), 55% (54±5%) and 67% (70+2%); and VCN was 1 (1.4±0.2), 1.6 (1.8±0.2) and 1.9 (2±0.2) in the MND4T, PRF0T and PGK4T groups of animals, respectively, (Supplemental **Figure S5a,b**) similar to the chimerism and VCN in the total mice in the 3 groups (**Figure 4a,b**)

As expected, all animals became clinically sick within 2 weeks of LCMV infection, with the TxPrf1-/- and PGK4T animals with worse (higher) clinical scores than the PRF0T, MND4T and TxWT animals. The TxWT and MND4T mice started improving thereafter, but the PRF0T mice continued to clinically worsen to clinical scores similar to the PGK4T and TxPrf1-/- animals. Overall, the MND4T group of mice had the lowest clinical scores that correlated with the highest survival of the 3 vector groups **(Figure 5a and b)**. The PGK4T and PRF0T animals remained at similar high clinical scores with almost all mice progressively succumbing to the challenge, even though the PRF0T mice had significantly lower IFN-γ , were alive much longer and had lower gene-modified cell chimerism than PGK4T mice.

Since the MND4T group had some mice with very high chimerism (>60%) and those with moderate to low chimerism (**Figure 4a**), we analyzed if higher gene-modified cytotoxic cells conferred a longer survival. Mice with 60% or greater chimerism in NK cells in the MND4T and PGK4T groups were alive for significantly more days than the mice with lower chimerism (below 60%; **Figure 5c**). However, in the PRF0T group, there was no correlation of mortality with chimerism (or IFN-γ levels); and although they were alive longer than PGK4T mice, they had similar eventual mortality.

We then determined LCMV viral RNA in bone marrow from the limited number of surviving mice or those that were sacrificed when moribund (**Figure 5d**). LCMV mRNA was high in the PRF0T and the PGK4T groups, and was at low levels in the MND4T mice. All surviving mice post LCMV challenge (shown in the rectangular shaded box, **Figure 5d**) had extremely low levels of LCMV mRNA, consistent with viral clearance or low grade chronic infection. This data was also confirmed with the LCMV plaque forming assay (Supplemental **Figure S5c**). Hence, our data suggests that low perforin expression improves some of the inflammatory parameters, but there is little to no LCMV clearance,

which is associated with mortality from HLH in this model.

DISCUSSION

An autologous transplant with gene therapy provides a potentially attractive treatment option that is not limited by donor availability and the immunological adverse consequences associated with allogeneic HCT. Herein, we show that FHLH2 correction requires high (normal) levels of perforin expression per cell for complete correction.

Strong enhancers in the γ-retroviral LTR were shown to cause oncogenesis by activating proto-oncogenes surrounding insertion sites. Since then, LV with self-inactivating (SIN) LTR design and internal tissue-specific/cellular promoters were developed to reduce genotoxicity. However, with the perforin and PGK promoter driven LV, despite high gene-modified chimerism, there was partial correction of HLH. We observed that the MND promoter/enhancer was necessary for normal levels of perforin expression from LV in a human NK cell-line, whereas the PGK/PRF promoters had significantly lower expression.

Similar phenomena are now being discovered in other diseases: In leukocyte adhesion deficiency (LAD), CD18 expression from PGK promoter-driven vector was insufficient to correct the LAD phenotype in dogs, and necessitated a strong promoter/enhancer from MSCV-LTR U3 region for disease correction[16]; and in gene therapy for Wiscott Aldrich syndrome (WAS) where the WAS cellular promoter significantly corrected the phenotype, but only partially corrected the thrombocytopenia[19, 20]. MND(U3) region of a modified γ-retroviral LTR is a strong promoter/enhancer, and has been previously successfully used internally in LV in a clinical trial of gene therapy for adreno-leukodystrophy, without evidence of genotoxicity after 10 years of follow up; it has also been shown to adequately correct the WAS phenotype in preclinical studies[33-35].

To avoid high expression of a pore forming protein in HSPC, we restricted expression in HSPC using the miR126 target elements[25, 26]. Transgene expression in HSPC in globoid leukodystrophy was found to be toxic to HSPC, which was overcome by placing miR126 target sites in the vector[22]. While this may cause some reduction in the HSPC miR126 pool, studies in normal HSC show that miR126 attenuation does not adversely affect HSPC function, and may even expand them[36].

Our studies on functional correction of FHLH2 in the $Prf1^{-/-}$ mouse model were performed with LSK transplantation and myeloablation to simulate a human clinical gene therapy trial. Unexpectedly, with this modified protocol, we noted blunted cytopenias and IFN-γ responses in TxPrf1-/- mice compared to previous methodologies [14]. Moreover, TxWT mice also experienced some illness and mortality after LCMV infection. We suspect these differences relate to relatively slower immune reconstitution with the revised protocol. While there are limitations to the murine model with a narrow margin for successful correction of HLH pathology, this approach is favored from a translational perspective. Patients will receive transduced CD34+ HSPC, following a similar myeloablation. It may be beneficial to consider transplant of genetically modified T cells along with genetically modified HSC, to prevent adverse consequences from peri-transplant infectious complications while a new immune system emerges from the gene-corrected HSC.

Despite these caveats, transfer of the perforin gene into HSC led to a significant correction of the IFN-γ levels and *ex-vivo* cytotoxicity with the MND4T and PRF0T LV vectors, and partial correction with the PGK4T vectors, as compared to $TxPrf1^{-/-}$ mice. Notably, the MND4T LV outperformed in all aspects, with nearly half the VCN and lower gene-modified cell chimerism. Also, despite similar VCN and perforin expression from the PGK4T and PRF0T mice, there was much better reduction in IFN-γ levels and longer survival in the PRF0T mice, suggesting a regulated promoter may be beneficial; although the eventual dismal outcome in both appears to be from subnormal perforin expression and inability to clear/suppress LCMV; TxWT and MND4T mice had LCMV mRNA levels consistent with low grade chronic LCMV infection and/or clearance. It is to be noted that the PRF promoter comprises of a small fragment (1.4Kb) of an otherwise 150 Kb upstream cis regulatory region that regulates

perforin expression[32]. Hence identification and inclusion of important cis-regulatory elements in the perforin promoter that can boost the level of perforin expression per cell may improve this cellular promoter.

The importance of perforin in the feedback control of immune activation has been studied in the context of HLH, and there exists a perforin-dependent reciprocal relationship between dendritic cells and CD8+ T cells[37, 38]. In the absence perforin and in face of a viral infection; Prf1$^{-/-}$ CD8+ T cells are conceivably incapable of providing feedback control of immune activation, besides being incapable of reducing the viral load, which rises and/or stabilizes in the liver and spleen of infected mice [31]. Our data suggests that moderate, but subnormal levels of perforin in CD8+ T and NK cells is sufficient to provide the feedback to decrease the exaggerated inflammation and immune response; however, complete correction and recovery from HLH, including viral clearance, requires a much higher level of perforin expression, that is near normal levels, and was achievable by the MNDU3 promoter/enhancer. It will be of interest to study the expression of this promoter in primary CD8 T cells and NK cells of perforin deficient HLH patients for robust validation.

In conclusion, our data suggests that for gene therapy for FHLH2, cellular promoters are capable of providing only partial phenotypic correction and complete correction of the phenotype requires robust perforin expression that can be facilitated by a strong MND enhancer in LV.

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S.T., A.H., C.T., M.J., K.A.R., and P.M. designed the experiments and wrote the manuscript. S.T. performed the cloning and production of the lentiviral vectors. S.T. and C.T. performed the murine experiments. A.H. was responsible for the NK cell differentiation and the chromium release assays. P.A. and M.C. provided input and preliminary data for the design of the experiments. All authors have contributed to their respective portions of the manuscript and reviewed the manuscript. A special thanks to Anastacia Loberg, Katie Burke, Sarah Figuera and Michael Wourms for their contribution in the murine experiments.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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