Molecular Therapy
Methods & Clinical Development
Original Article

Human Lentiviral Gene Therapy Restores the Cellular Phenotype of Autosomal Recessive Complete IFN-γR1 Deficiency

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Autosomal recessive (AR) complete interferon-γ receptor 1 (IFN-γR1) deficiency, also known as one genetic etiology of Mendelian susceptibility to mycobacterial disease (MSMD), is a life-threatening congenital disease leading to premature death. Affected patients present a pathognomonic predisposition to recurrent and severe infections with environmental mycobacteria or the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine. Current therapeutic options are limited to antibiotic treatment and hematopoietic stem cell transplantation, however with poor outcome. Given the clinical success of gene therapy, we introduce the first lentiviral-based gene therapy approach to restore expression and function of the human IFN-γR-downstream signaling cascade. In our study, we developed lentiviral vectors constitutively expressing the human IFN-γR1 and demonstrate stable transgene expression without interference with cell viability and proliferation in transduced human hematopoietic cells. Using an IFN-γR1-deficient HeLa cell model, we show stable receptor reconstitution and restored IFN-γR1 signaling without adverse effect on cell functionality. Transduction of both SV40-immortalized and primary fibroblasts derived from IFN-γR1-deficient MSMD patients was able to recover IFN-γR1 expression and restore type II IFN signaling upon stimulation with IFN-γ. In summary, we highlight lentiviral vectors to correct the IFN-γ mediated immunity and present the first gene therapy approach for patients suffering from AR complete IFN-γR1 deficiency.

INTRODUCTION

For almost two decades now, hematopoietic stem cell gene therapy (HSCGT) has been proven to be an effective treatment for a variety of different monogenetic diseases. Currently, lentiviral vectors in HSCGT are evaluated in a dozen clinical trials highlighting a promising therapeutic success for various primary immunodeficiencies (PIDs).[1] The so-far positive outcome of these clinical trials further underlines that HSCGT is comparably effective and in certain cases of rare diseases even superior to allogeneic HSC transplantation.[2] The obvious advantage of HSCGT over allogeneic transplantation is donor-compatibility and the absence of graft-versus-host disease. Furthermore, the inapparent immunogenicity of autologous cells leads to a decreased level of pre-transplant conditioning and immunosuppressive therapy.[1]

Considering the previously outlined advantages and the promising success of HSCGT in other PIDs, the use of lentiviral vectors might be broadened to a genetic disorder leading to a selective susceptibility to mycobacterial infection.[3] The rare hematopoietic condition referred to as Mendelian susceptibility to mycobacterial disease (MSMD) is defined as a predisposition to weakly virulent mycobacteria, such as non-tuberculous environmental mycobacteria (EM) or bacillus Calmette-Guérin (BCG) vaccine.[4,5] The severity of the clinical manifestation depends on the underlying mutation of genes involved in the interferon-γ (IFN-γ) mediated immunity, which either interferes in the production (*IL12B*, *IL12RB1*, *IL12RB2*, *IL23R*, *ISG15*, *TYK2*, *RORC*, *NEMO*) or the response to IFN-γ

Received 24 February 2020; accepted 2 April 2020;
https://doi.org/10.1016/j.omtm.2020.04.002.

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(*IFNGR1*, *IFNGR2*, *STAT1*, *JAK1*, *CYBB*) or both (*IRF8*, *SPPL2A*).[6,7] Autosomal recessive (AR) complete deficiency of IFN-γ receptor ligand-binding chain 1 (IFN-γR1) is the most severe form (OMIM: 209950) of MSMD with a disease onset before 3 years of age. Clinical hallmarks of MSMD are disseminated, persistent, or recurrent infections with BCG or EM, including subspecies such as *M. avium* complex, *M. kansasii*, and *M. smegmatis*.[8–11] The absent or dysfunctional IFN-γ-receptor on the cell surface of macrophages leads to an impaired type II IFN immune response and hampers the activation of the respective downstream signaling cascade, e.g., phosphorylation of signal transducer and activator of transcription 1 (STAT1), thereby enabling intraphagocytic survival of pathogens.[5,12–14] Given the important role of non-responding macrophages in IFN-γR1-deficiency, patients suffering from this disease show also an increased prevalence of other infections, e.g., with *M. tuberculosis*, further bacterial infections (e.g., *Salmonella spp.*), and fungal (e.g., Candida spp.). In rare cases, patients can have viral (e.g., human cytomegalovirus [CMV]; human gamma herpesvirus 8 [HHV-8]) infections. However, whether or not the predisposition to the aforementioned pathogens is directly linked to defects of the IFN-γR1 remains still elusive.

Current forms of treatment for MSMD are very limited and depend on long-term administration of antibiotics, which only provide symptomatic relief, but not lasting remission of the disease. Moreover, for AR complete IFN-γR1 deficiency, exogenous infusion of IFN-γ is ineffective due to the absent functional surface receptor.[8,15] The only curative treatment option available is HSCT. However, allogeneic HSCT is impeded by (re)current infections and high serum levels of IFN-γ, which interferes with the cell cycle of transplanted donor HSC leading to either delayed engraftment or complete graft rejection within a short period of time.[15–17] Due to the lack of effective treatment options, the prognosis for AR complete IFN-γR1 deficiency is fatal within the first two decades of life.[6,8,18] As a promising alternative, lentiviral vector mediated genetic correction of *Ifnγr1* in a murine mouse model of MSMD could prove the feasibility of HSCGT for AR complete IFN-γR1 deficiency. In this study, transplantation of genetically corrected HSC into lethally irradiated IFNγR1$^{-/-}$ mice could protect mice from disseminated BCG disease (BCG-osis) following intra-pulmonary infection of mice with BCG. Using lentiviral vectors expressing the *Ifngr1* transgene from a myeloid specific microRNA223 (miR223) promoter, we could even unveil corrected alveolar macrophages as one important cellular component that mediates prolonged survival of BCG challenged animals.[19]

Given this proof-of-concept study in the murine IFNγR1$^{-/-}$ system, we now aimed to translate these findings into the human system and to establish the first gene therapy approach for human AR complete IFN-γR1 deficiency. In our study, we developed lentiviral vectors, which constitutively express the human IFN-γR1 either from a spleen focus forming virus (SFFV) or human elongation factor 1α short (EFS) promoter element and demonstrate stable transgene expression without interference with cell viability and proliferation in transduced human hematopoietic cell lines. Moreover, transduction of both SV40-immortalized and primary fibroblasts derived from AR complete IFN-γR1-deficient MSMD patients was able to recover IFN-γR1 expression and restore the function of cells upon stimulation with IFN-γ. Thus, we highlight lentiviral vectors to correct the IFN-γ signaling and present the first gene therapy approach for patients suffering from AR complete IFNγ-R1-deficient MSMD.

RESULTS

Design and Evaluation of Lentiviral Vectors Expressing Human IFN-γR1

Given the promising results of HSCGT in a murine model for IFN-γR1$^{-/-}$,[19] we here aimed to generate third-generation self-inactivating (SIN) lentiviral vectors equipped with the human complementary DNA (cDNA) of *IFNGR1*. In order to easily follow transgene expression, the *IFNGR1*-cDNA was coupled to an enhanced green fluorescent protein (EGFP) via an internal ribosomal entry side (IRES). For our proof-of-concept study, we utilized a constitutive expression cassette that drives transgene expression from a SFFV promoter (Lv.SFFV.IFNγR1.iGFP). A vector expressing only the GFP reporter gene (Lv.SFFV.GFP) served as a control (Figures 1A and 1B, respectively). To evaluate vector functionality, we transduced the human myeloid cell line K562 with the Lv.SFFV.IFNγR1.iGFP and cell surface expression of GFP, and we detected IFN-γR1 (CD119) via flow cytometry. Analysis revealed profound GFP expression and an enhanced CD119 expression level on transduced cells in comparison to non-transduced K562 control cells. Of note, as K562 cells showed endogenous expression of CD119, only a mild overexpression was noted (Figure 1C).

In order to exclude potential side effects of IFN-γR1 overexpression on cellular functionality, we performed analysis for stability of transgene expression, as well as proliferation and apoptosis, in fluorescence-activated cell sorting (FACS)-purified SFFV.IFNγR1.iGFP transduced K562 cells. Here, stable transgene expression over the entire observation period of 5 weeks was demonstrated using flow cytometric analysis of GFP expression (Figure 2A). Moreover, cell viability analysis by propidium iodide (PI) staining showed no significant difference between transduced and non-transduced control cells (Figures 2B and 2C). In addition, labeling of IFN-γR1 overexpressing cells with a fluorescence dye showed equal dilutions of the fluorescent signal in control K562 and SFFV.IFNγR1.iGFP transduced K562 cells over time, thus indicating a normal cell proliferation (Figure 2D). Furthermore, quantification of differences in the mean fluorescent intensity of day (d)9 and d13 revealed no significant changes between both transduced and non-transduced cells, indicating equal proliferation capacity (Figure 2E). Thus, transduction with our Lv.SFFV.IFN-γR1.iGFP vector leads to stable transgene expression in hematopoietic K562 cells. Furthermore, constitutive overexpression of CD119 did not induce changes in cell viability, apoptosis, or proliferation of K562 cells.

Restored Functionality upon IFN-γR1 Overexpression in IFN-γR1$^{-/-}$ HeLa Cells

Following the transduction of K562 cells, initial vector expression and safety studies in this cell line, we next aimed to evaluate whether the designed lentiviral vector is able to restore a defective IFN-γ signaling

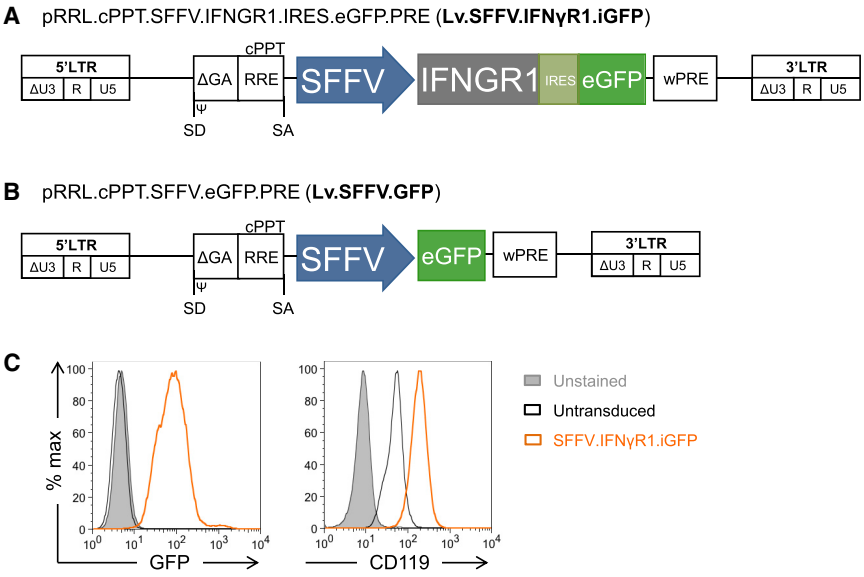

Figure 1. Lentiviral Vector Design and Evaluation of Transgene Expression

(A and B) Schematic overview of the third-generation SIN lentiviral vectors encoding (A) *IFNGR1* cDNA coupled to a GFP reporter via an internal ribosomal entry side (IRES) and (B) a control vector encoding only for the GFP reporter. Transgene expression is driven by a spleen focus forming virus (SFFV) promoter in both constructs. (C) Flow cytometric analysis of GFP and IFN-γR1 (CD119) expression in untransduced and Lv.SFFV.IFNγR1.iGFP-transduced K562 cells (human myeloid cell line; gray filled: unstained untransduced cells, black: stained untransduced cells, orange: Lv.SFFV.IFNγR1.iGFP-transduced K562 cells).

pathway. To test restoration of type II IFN response, we made use of a commercially available HeLa cell line, previously genetically modified by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology. This cell line (referred to as IFN-γR1$^{-/-}$) harbors a homozygous insertion of a selection cassette in exon 1 in the *IFNGR1* locus, resulting in a frameshift mutation. Absence of IFN-γR1 surface expression in the knockout line was confirmed by flow cytometry (Figure 3A). Transduction of IFN-γR1$^{-/-}$ HeLa cells with Lv.SFFV.IFNγR1.iGFP restored IFN-γR1 cell surface expression to wild-type (WT) levels ($p = 0.77$). While no expression of CD119 could be seen in (Lv.SFFV.GFP) control transduced IFN-γR1$^{-/-}$ cells, a 5.2 ± 1.7-fold increase in CD119 expression could be observed after transduction with Lv.SFFV.IFNγR1.iGFP (mean ± SD, n = 3, Figures 3A and 3B). Functionality of the IFN-γR1 expressed from the Lv.SFFV.IFNγR1.iGFP vector was demonstrated by the ability of transduced IFN-γR1$^{-/-}$ cells to phosphorylate the transcription factor STAT1 upon IFN-γ stimulation. In this assay, non-corrected IFN-γR1$^{-/-}$ cells showed only background levels of phosphorylated STAT1 (pSTAT1), whereas Lv.SFFV.IFNγR1.iGFP transduced IFN-γR1$^{-/-}$ cells showed restored pSTAT1 isoforms (Figures 3C and 3D). We also evaluated the expression of other IFN-γ downstream targets such as the IFN-induced 35 kDa-protein (IFP35), the immunoproteasome low molecular weight protein 7 (LMP7) or the major histocompatibility complex (MHC) class II after stimulation. Evaluation of corrected IFN-γR1$^{-/-}$ HeLa cells revealed the induction of IFP35 or LMP7 expression after stimulation with IFN-γ (Figure 3E). Moreover, Lv.SFFV.IFNγR1.iGFP transduced HeLa cells were able to upregulate MHC-II after stimulation, which was in clear contrast to non-transduced IFN-γR1$^{-/-}$ HeLa control cells (Figure 3F). Similar to the aforementioned studies, we also could detect γ-activated factor (GAF)-DNA binding activity in IFN-γ-stimulated WT and Lv.SFFV.IFNγR1.iGFP transduced IFN-γR1$^{-/-}$ cells, but not in IFN-γR1$^{-/-}$ HeLa cells using an electrophoretic mobility shift assay (EMSA) with a γ-activating sequence (GAS) probe (Figure 3G).

While the constitutive overexpression of IFN-γR1 could restore IFN-γ downstream signaling in IFN-γR1$^{-/-}$ HeLa cells, we also tested whether physiological type I IFN responses were maintained in IFN-γR1 overexpressing cells. GAS-binding after IFN-α stimulation could be detected in all samples, highlighting a normal type I IFN signaling cascade also in transduced cells (Figure S1A). A similar observation was made when analyzing expression of IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) and IFN-stimulated gene 15 (ISG15). Here, similar to the aforementioned IFN-α studies, a normal induction pattern could be detected in all samples, irrespectively of IFN-γR1 overexpression (Figure S1B). Thus, our designed lentiviral vector system is able to correct type II IFN downstream signaling pathway, while maintaining type I IFN response.

As a next step, we aimed to investigate whether the level of IFN-γR1 expression correlated with the cellular response to IFN-γ using the aforementioned HeLa IFN-γR1$^{-/-}$ cell line. Transduced IFN-γR1$^{-/-}$ HeLa cells were FACS-purified based on their IFN-γR1 cell surface expression level and further cultured as "low," "middle," and "high" expressing populations (Figures 4A). Following a cultivation period of more than 2 weeks, we could establish transduced IFN-γR1$^{-/-}$ HeLa cell lines harboring different expression levels of IFN-γR1, which range from median fluorescence intensity 8.05 ± 1.6 × 1,000 (low), 10.4 ± 2. × 1,000 (middle), and 11.1 ± 2.3 × 1,000 (high, all mean ± SD, n = 4; Figure 4B). Irrespective of the different IFN-γR1 expression levels, analysis of pSTAT1 in the presence of IFN-γ showed a similar, approximately 20-fold induction of pSTAT1 in all three conditions, which was comparable to WT levels (Figures 4C and 4D). Similar to the type I IFN studies mentioned before, we also tested GAS-binding after stimulation with IFN-α in the different cell lines. We could not observe any detrimental effects on the formation of the STAT1 complex in IFN-γR1 overexpressing cells after IFN-α stimulation, further highlighting the proper type I IFN downstream signaling (Figure 4E).

Lentiviral Vector Restores IFN-γR1 in Fibroblasts from an IFN-γR1$^{-/-}$ MSMD Patient

After demonstrating functionality and safety of our Lv.SFFV.IFN-γR1.iGFP lentiviral vector in two different cell lines, we next aimed

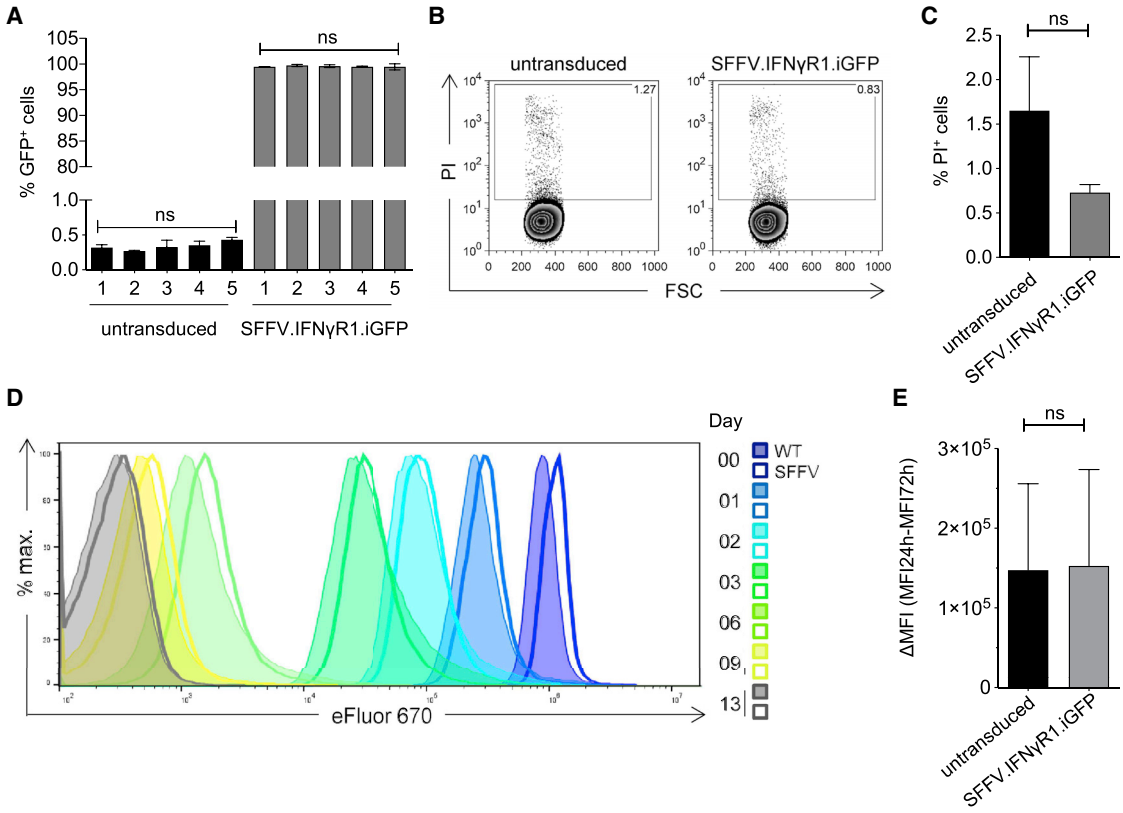

Figure 2. Analysis of Vector Safety in Fluorescent-Activated Cell Sorting (FACS) purified K562 Cells

(A) The GFP expression of untransduced and FACS-purified Lv.SFFV.IFNγR1.iGFP-transduced K562 cells was evaluated over a period of 5 weeks. Viable cells were pre-gated according to their size and granularity using FSC/SSC plots (mean ± SD; n = 3; ns = not significant, statistical analysis was performed using two-way ANOVA with Tukey's post hoc testing). (B) Representative dot plot of untransduced K562 cells (left plot) and sorted Lv.SFFV.IFNγR1.iGFP transduced K562 cells (right plot) stained with propidium iodide (PI). (C) Percentage of not-viable, PI^+ cells as analyzed in flow cytometry (mean ± SD; n = 3; ns = not significant, statistical analysis was performed using unpaired t test). (D) Representative histogram of untransduced (WT) and FACS-purified Lv.SFFV.IFNγR1.iGFP transduced (SFFV) K562 cells, stained with cell proliferation dye eFluor 670 and analyzed by flow cytometry over a period of 13 days post staining (day 0, 1, 2, 3, 6, 9, and 13). Untransduced cells (WT) are depicted with a tinted graph; Lv.SFFV.IFNγR1.iGFP-transduced cells (SFFV) are depicted with a solid line. (E) Difference of mean fluorescence intensities (ΔMFIs) 24 h and 72 h post-staining with eFluor 670 proliferation dye (MFI24h – MFI72h) in untransduced and FACS-purified Lv.SFFV.IFNγR1.iGFP transduced K562 cells (mean ± SD; n = 3; ns = not significant, statistical analysis was performed using unpaired t test).

to evaluate this vector in cells derived from an IFN-γR1$^{-/-}$ MSMD patient. In a first set of experiments, we used fibroblasts from an AR complete IFN-γR1-deficient MSMD patient (c.107ins4/c.200+1G > A, patient 10i in Lancet, 2004[8]), which have been immortalized by the introduction of the large T antigen (TAg) of Simian virus 40 (SV40). First, SV40 fibroblasts were transduced with SFFV.IFNγR1.iGFP vector and subsequently evaluated for transgene expression. Here, fluorescence microscopy revealed a vector-mediated GFP-expression and a typical morphology of transduced patient fibroblasts compared to control fibroblasts (Figure 5A). As a second step, we analyzed IFN-γR1 (CD119) cell surface expression. Flow cytometry revealed absence of the IFN-γR1 on non-transduced SV40 fibroblasts (median fluorescence intensity, MFI: 3960) in contrast to their healthy counterparts (MFI: 10610). Following transduction with our lentiviral vector, IFN-γR1$^{-/-}$ SV40 fibroblasts showed a marked expression of GFP and IFN-γR1 on their cell surface (MFI of CD119 in GFP^+ cells: 8985; Figure 5B). As a next step, we subjected the transduced cells to IFN-γ studies, investigating the corrected downstream signaling cascade. While non-transduced IFN-γR1$^{-/-}$ patient SV40 fibroblasts showed no phosphorylation of STAT1 in response to IFN-γ, patient fibroblasts corrected with our lentiviral vector and WT fibroblasts were able to phosphorylate STAT1 in the presence of IFN-γ (Figure 5C).

After showing vector functionality in the patient-derived cell line, we aimed to evaluate the vector in primary material derived from a second MSMD patient (homozygous mutation c.131delC, p.Pro44-Leufs*18 in exon 2 of *IFNGR1*). Thus, primary fibroblasts derived from the IFN-γR1-deficient patient were transduced with our SFFV.IFNγR1.iGFP vector and analyzed for expression of IFN-γR1 by flow cytometry. Transduced patient fibroblasts stained positive for CD119, while no expression could be observed in non-transduced

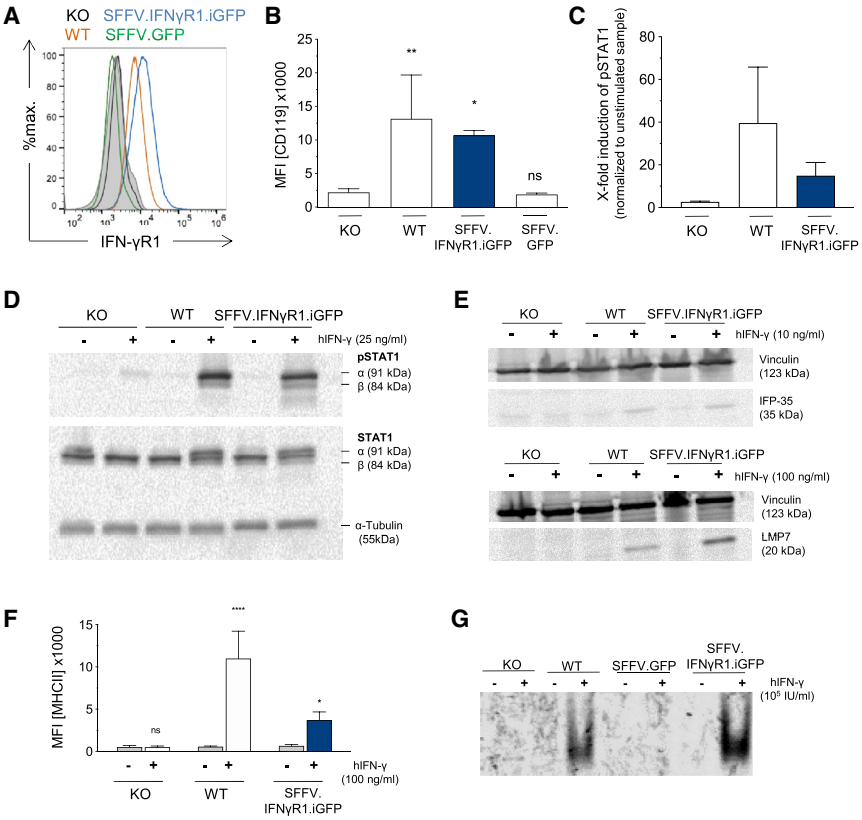

Figure 3. Evaluation of IFN-γR1 Expression and Functionality in Lv.SFFV.IFNγR1.iGFP Transduced IFN-γR1$^{-/-}$ HeLa Cells

(A) Representative histograms depicting IFN-γR1 (CD119) expression on WT (orange), IFN-γR1$^{-/-}$ (knockout [KO], black), and Lv.SFFV.GFP (green) or Lv.SFFV.IFNγR1.iGFP (blue) transduced KO HeLa cells (unstained control cells are depicted in gray). (B) Quantification of median fluorescence intensities (MFIs) of IFN-γR1 (CD119) expression in WT, KO, and transduced HeLa cells (n = 3–4; mean ± SD, statistical analysis was performed using one-way-ANOVA with Tukey's post hoc testing. All shown values are compared to KO cells, *p ≤ 0.05; **p ≤ 0.01; ns, not significant). (C and D) Quantification of phosphorylated STAT1 (pSTAT1)induction in different HeLa cell lines after stimulation with 25 ng/mL hIFN-γ (n = 3; mean ± SD, values are shown as fold-induction normalized to unstimulated controls) (C) and representative western blot (D). (E) Western blot showing expression of interferon-induced 35 kDa-protein (IFP-35, upper panel) and the immunoproteasome low molecular weight protein 7 (LMP-7, lower panel) in unstimulated (–) and hIFN-γ-stimulated (+) KO, WT, and Lv.SFFV.IFNγR1.iGFP transduced KO cells with vinculin as loading control. (F) MHCII expression in WT, KO, and transduced HeLa cells with or without stimulation with hIFN-γ for 72 h (n = 4; mean ± SD, statistical analysis was performed using two-way-ANOVA with Sidak's multiple comparison post hoc testing. *p ≤ 0.05, ****p ≤ 0.0001. ns, not significant, all values were compared to respective unstimulated controls). (G) Electrophoretic mobility shift assay (EMSA) using γ-activated sequence (GAS) probe in LI-COR 700 (4 μg of nuclear lysis) and stimulation with 10^5 IU/mL hIFN-γ for 20 min.

control cells (Figure 5D). Of note, detection of CD119 on the cell surface of Lv.SFFV.INFyR1.iGFP transduced patient cells could also be linked to functional correction of these cells. Investigation of the IFN-γR1-STAT1-signaling pathway revealed absence of pSTAT1 in non-transduced patient cells, whereas a clear signal of pSTAT1 was detected following genetic correction (Figure 5E). These results indicate that the Lv.SFFV.IFNγR1.iGFP vector is also able to restore expression and functionality of the IFN-γR1 in patient-derived cells.

EFS-driven IFN-γR1 Expression Restores Signaling in Fibroblasts from an IFN-γR1$^{-/-}$ MSMD Patient

Of note, all aforementioned studies have been performed in a proof-of-concept format using the strong viral SFFV promoter/enhancer element. In order to direct the clinical translation of our approach, we also designed a third generation SIN-lentiviral vector, in which the human cDNA of *IFNGR1* is expressed from a physiological truncated EFS promoter (Figure 6A). To assess vector functionality, we first evaluated this construct in the previously mentioned IFN-γR1$^{-/-}$ HeLa cell model. Transduced IFN-γR1$^{-/-}$ HeLa cells showed an increase in IFN-γR1 expression, which was again associated with restoration of the IFN-γR1 signaling pathway, as demonstrated by the ability of transduced cells to induce pSTAT1 upon IFN-γ stimulation (Figures 6B and 6C). We also transduced primary fibroblasts from an IFN-γR1$^{-/-}$ deficient patient with the designed Lv.EFS.IFNγR1.iGFP vector construct. Similar to the previous studies, we detected IFN-γR1 expression in primary fibroblasts of the patient following lentiviral transduction (Figure 6D). In addition, we could also prove functional correction of these cells by their ability to phosphorylate STAT1 in the presence of IFN-γ, highlighting the functionality of the EFS-based vector construct. In summary, our results demonstrate that both designed vectors are able to restore expression and functionality of the IFN-γ cytokine-receptor signaling pathway and thus highlight the feasibility of a human gene therapy approach for AR complete IFN-γR1 deficiency.

DISCUSSION

Patients suffering from MSMD due to AR complete IFN-γR1 deficiency show a severe progression of the infectious clinical phenotype, characterized by disseminated infections with EM or BCG. This PID results in fatality in the first two decades of life and the only therapeutic option, HSCT, remains challenging due to pre-existing infections and high serum levels of IFN-γ.[6,8] Previous work could demonstrate the feasibility of HSCGT using lentiviral gene transfer to reconstitute IFN-γ mediated immunity in HSC-derived macrophages. Furthermore, this study was able to unveil increased survival of IFN-γR1$^{-/-}$ mice, which received genetically corrected HSCs before infection with BCG.[19] In the present study, we demonstrate for the first time translation of the promising HSCGT approach into the human

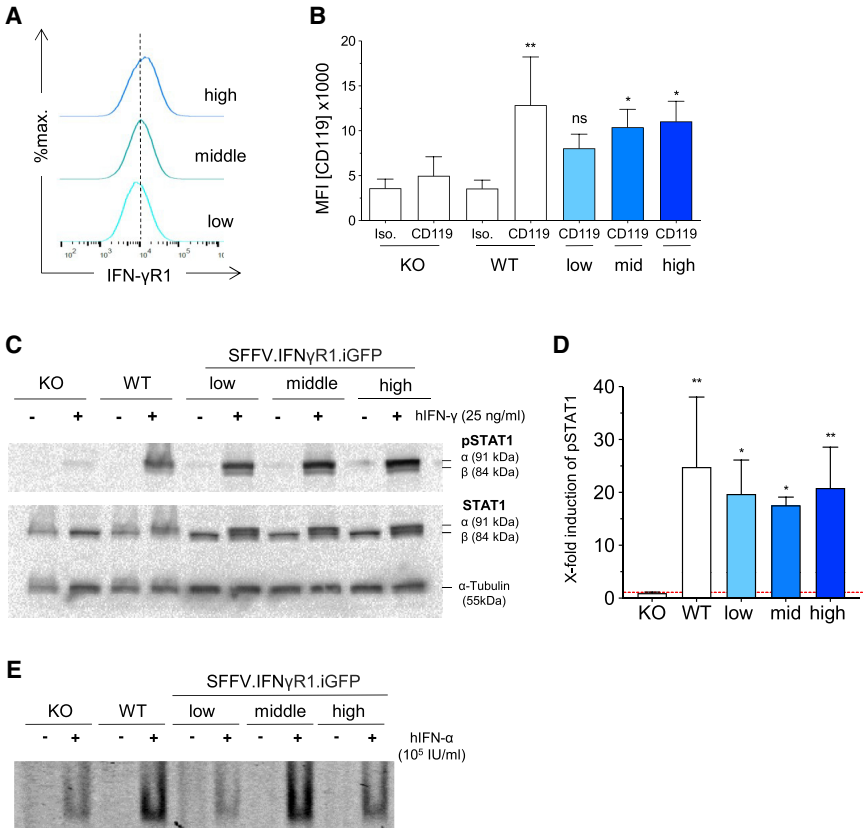

Figure 4. Different IFN-γR1 Expression Levels in HeLa Cells

(A) Representative flow cytometry data showing the results of FACS-purification in low, middle (mid), and high IFN-γR1 (CD119) expression in Lv.SFFV.IFNγR1.iGFP transduced KO HeLa. (B) IFN-γR1 (CD119) expression in different HeLa cell lines (n = 4; mean ± SD, statistical analysis was performed using one-way-ANOVA with Dunnett's multiple comparison post hoc testing. *p ≤ 0.05; **p ≤ 0.01; ns, not significant. Iso, isotype control. All statistical values are compared to CD119 expression of KO cells, MFI, median fluorescence intensity). (C) Representative western blot showing pSTAT1 and total STAT1 in unstimulated (–) and hIFNγ-stimulated (+) (25 ng/mL hIFN-γ) KO, WT, and Lv.SFFV.IFNγR1.iGFP transduced HeLa KO cells (low, middle, high) with α-tubulin as loading control. (D) Quantification of pSTAT1 induction in different HeLa cell lines (n = 4, except for middle n = 3; mean ± SD, values are shown as fold induction normalized to unstimulated controls, statistical analysis was performed using one-way-ANOVA using Dunnett's multiple comparison testing. *p ≤ 0.05, **p ≤ 0.01, red line is drawn at 1. (E) EMSA using GAS probe in LI-COR 700 (4 μg nuclear lysis of different HeLa cell lines). Stimulation was performed with 10^5 IU/mL hIFN-α for 20 min.

system and the generation and evaluation of lentiviral vectors expressing the human *IFNGR1* cDNA. Transduction of IFN-γR1$^{-/-}$ HeLa cells and cells derived from IFN-γR1 deficient MSMD patients was able to restore receptor expression and recover IFN-γ-/IFNγR1-signaling without adverse effects on cell functionality and survival.

With regard to clinical application, third generation SIN lentiviral vectors were chosen for gene transfer due to their preferred integration profile and reduced risk of insertional mutagenesis in comparison to gamma-retroviral vectors.[20] The suitable use of lentiviral vectors in HSCGT has already been proven in the treatment of several PIDs such as the Wiskott-Aldrich syndrome or X-linked severe combined immunodeficiency without evidence of severe side effects.[21–23] In contrast to the studies mentioned before, transcription of *IFNGR1* cDNA in our study was initiated by a SFFV promotor owing to its high activity in hematopoietic cells.[24] Transduction of cell lines and patient cells with Lv.SFFV.INFγR1.iGFP led to a stable reconstitution of IFN-γR1 expression and its respective signaling pathway without evidence of cell functionality impediment. Whereas previous studies demonstrated high ubiquitous transgene expression by the SFFV promoter in cell lines, in our studies expression of IFN-γR1 was only moderately increased.[25] The significantly stronger expression of the GFP reporter gene compared to transgene expression may be explained by the tight endogenous regulation of IFN-γR1 surface expression.[26,27]

Given the strong viral enhancer elements within the SFFV promotor, which can lead to insertional mutagenesis by activation of proto-oncogenes,[28] a physiological EFS promotor was introduced in a second vector construct. This weaker promotor is characterized by increased biosafety and already established for the gene therapy of further monogenetic hematological diseases.[23,29] In our studies, the application of lentiviral vectors driven by an EFS promotor also enabled receptor replenishment and activation of the downstream transcription factor STAT1 in presence of IFN-γ in an IFN-γR1$^{-/-}$ cell model and patient cells. However, further evaluation will be needed to strengthen suitability of this promotor. Based on the lentiviral gene therapy approach in an Ifnγr1$^{-/-}$ mouse model indicating a direct correlation between MSMD disease progression and non-functional macrophages,[19] prospective lentiviral vectors equipped with a myeloid-specific promotor may improve safety and efficacy in a gene therapy approach. Since the disease is primarily characterized by infections with intracellular pathogens and the formation of undifferentiated granulomas, lineage-restricted transgene expression in myeloid target cells may prevent adverse side effects on hematopoietic stem/progenitor cells and other hematopoietic cell types.[13,30,31]

In our studies, we could demonstrate sustained transgene overexpression in transduced human myeloid cells without evidence of alterations in cell proliferation and death. Introduction of our vector construct into an IFN-γR1$^{-/-}$ human cell line showed its ability to

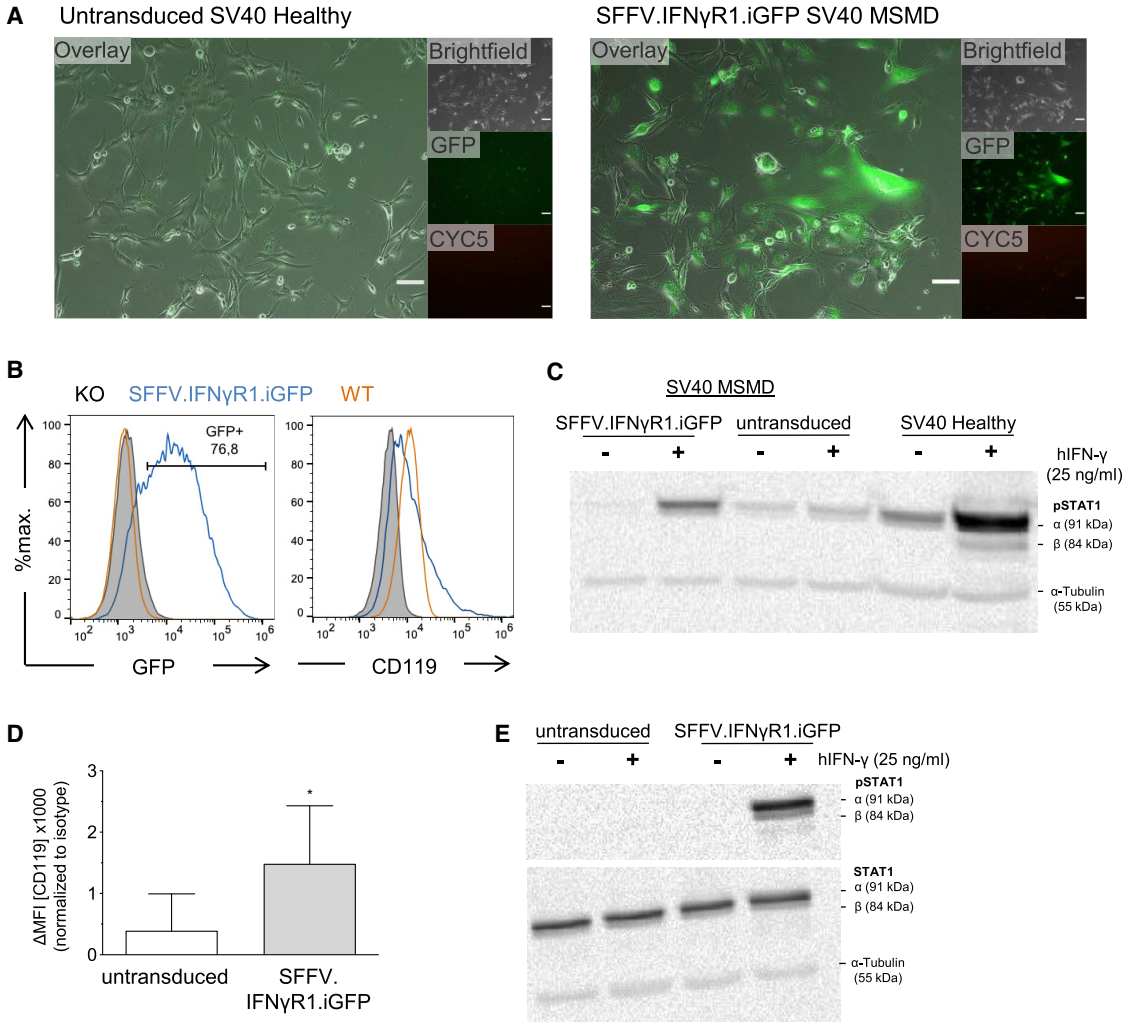

Figure 5. Vector Analysis in SV40 Fibroblasts derived from an IFN-γR1$^{-/-}$ MSMD Patient

(A) Detection of green fluorescent protein (GFP) expression and morphology of Lv.SFFV.IFNγR1.iGFP-transduced SV40-immortalized fibroblasts from an IFN-γR1$^{-/-}$-MSMD patient by fluorescence microscopy. Untransduced SV40-immortalized fibroblasts from a healthy donor shown as control (scale bar, 100 μM). (B) Flow cytometric analysis of IFN-γR1 (CD119) expression in untransduced IFN-γR1$^{-/-}$-MSMD SV40-immortalized fibroblasts (gray filled), healthy SV40 fibroblasts (orange), and Lv.SFFV.IFNγR1.iGFP-transduced IFN-γR1$^{-/-}$-MSMD SV40 fibroblasts (blue). (C) Western blot analysis of pSTAT1 upon stimulation with 25 ng/mL hIFN-γ in untransduced SV40-immortalized fibroblasts from a healthy control, untransduced, and Lv.SFFV.IFNγR1.iGFP-transduced SV40-immortalized fibroblasts from an IFN-γR1$^{-/-}$-MSMD patient with α-tubulin as loading control. (D) ΔMean fluorescence intensity (MFI; corrected to isotype) of IFN-γR1 (CD119) expression in corrected and untransduced patient fibroblasts (n = 8 for untransduced cells, n = 4 for transduced cells, mean ± SD; statistical analysis was performed using unpaired t test, *$p \leq 0.05$). (E) Western blot showing pSTAT1 and total STAT1 in unstimulated (–) and hIFN-γ-stimulated (+) (25 ng/mL hIFN-γ) untransduced patient fibroblasts and Lv.SFFV.IFNγR1.iGFP transduced patient fibroblasts with α-tubulin as loading control.

induce functional receptor expression under disease conditions. Evaluation of the downstream signaling pathway of IFN-γ revealed correction of the MSMD phenotype and ability of transduced cells to respond appropriately as analyzed in assays with IFN-γ or IFN-α stimulation and different receptor expression levels. Similar to the aforementioned data, the lentiviral gene therapy approach was able to correct the disease phenotype in immortalized fibroblasts and primary fibroblasts derived from IFN-γR1-deficient MSMD patients. With respect to the later target cell, these vector studies may have to be strained in patient-derived CD34$^+$ cells and thereof derived macrophages. While the genetic etiology of AR complete IFN-γR1-deficient MSMD is rare and the disease onset is below the age of 3 years, availability of a sufficient number of CD34$^+$ cell for *in vitro* studies is very limited.[6,8]

One alternative could be the use of patient-specific induced pluripotent stem cells (iPSCs). Because iPSCs provide an unlimited source of differentiated target cells, they have been used to model and study a variety of different PIDs and other diseases. We also previously applied iPSC technology not only to validate lentiviral vectors for

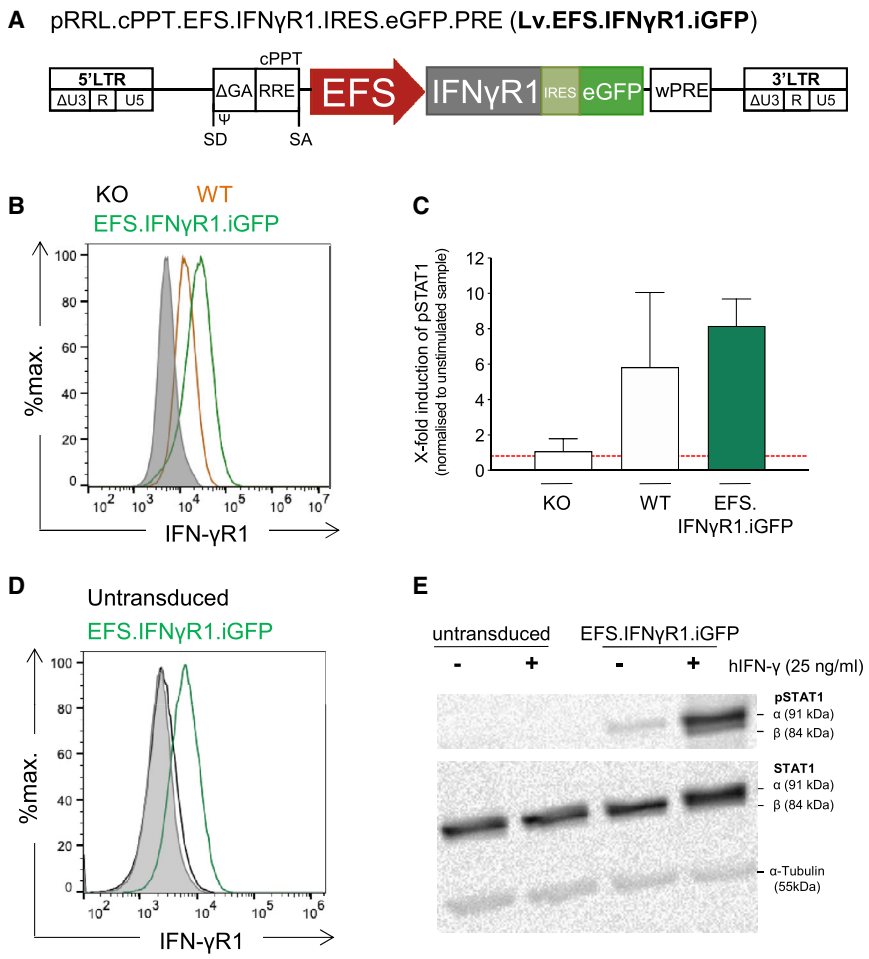

Figure 6. Vector Analysis in Fibroblasts Derived from an IFN-γR1$^{-/-}$ MSMD Patient

(A) Schematic overview of a third-generation SIN lentiviral vector encoding *IFNGR1* cDNA coupled to a GFP reporter via an IRES initiated by an elongation factor 1 α short (EFS) promoter. (B) Representative histograms depicting IFN-γR1 (CD119) expression on WT, IFN-γR1$^{-/-}$ (KO), and Lv.EFS.IFNγR1.iGFP transduced KO HeLa cells (gray filled: stained KO cells, orange: stained WT cells, green: Lv.EFS.IFNγR1.iGFP-transduced KO cells). (C) Quantification of pSTAT1 induction in different HeLa cell lines after stimulation with 25 ng/mL hIFN-γ (n = 3; mean ± SD, values are shown as fold-induction normalized to unstimulated controls). Red line is drawn at 1. (D) Representative histogram depicting IFN-γR1 (CD119) expression on untransduced patient fibroblasts and Lv.EFS.IFNγR1.iGFP transduced patient fibroblasts (gray filled: unstained KO cells, black: stained KO cells, green: Lv.EFS.IFNγR1.iGFP-transduced KO cells). (E) Western blot showing pSTAT1 and total STAT1 in nstimulated (–) and hIFN-γ-stimulated (+) (25 ng/mL hIFN-γ) untransduced patient fibroblasts and Lv.EFS.IFNγR1.iGFP transduced patient fibroblasts with α-tubulin as loading control.

gene therapy of pulmonary alveolar proteinosis, but also for disease modeling of MSMD etiologies.[32,33] As iPSCs can be differentiated into CD34^{+} hematopoietic stem/progenitor cells,[34] as well as macrophages,[35] this platform might serve as an interesting basis for further evaluation of the vector constructs.

In a next step, the presented benefits of a lentiviral gene therapy approach may also be transferred to further etiologies of MSMD with a comparable severe phenotype and where treatment with recombinant IFN-γ is not indicated. AR complete IFN-γR2 deficiency and AR complete STAT1 deficiency show a severe phenotype with a high mortality rate, and induction of STAT1 in mice lacking endogenous signaling has already been shown to improve immune defense against pathogens.[36–38]

To conclude, we introduced SIN lentiviral vectors (Lv.SFFV.IFN-γR1.iGFP, Lv.EFS.IFNγR1.iGFP) that were able to restore IFN-γR1 expression sufficiently in cells derived from patients with IFN-γR1 deficiency. Further development of this technology may lay the foundation for a cause-directed and long-lasting therapy for individuals suffering from MSMD.

MATERIALS AND METHODS

Cultivation of Cells

All cell lines and primary cells were incubated at 37°C, 5% CO_2 and 95% humidity in their respective medium supplemented with 10% fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and 1% penicillin/streptomycin (P/S; Life Technologies, Darmstadt, Germany). Adherent SC-1 and suspension K562 cell lines were kept in 6-well plates in Dulbecco's modified Eagle's medium (DMEM; GIBCO; Life Technologies, Paisley, UK) or RPMI 1640 media (GIBCO; Life Technologies), respectively. The commercially acquired HeLa IFN-γR1$^{-/-}$ cell line (#CL0011273003A; EdiGene, Beijing, China) was created by CRISPR/Cas9 genome editing of an original HeLa cell line derived from American Type Culture Collection and is harboring a homozygous insertion of a selection cassette in exon 1 in the *IFNGR1* gene locus resulting in a knockout mutation. HeLa IFN-γR1$^{-/-}$ and their complimentary WT cells were kept in DMEM high glucose medium. Primary patient-derived fibroblasts were harboring an exon 2 c.131delC p.Pro44Leufs*18 homozygous IFN-γR1 defect[4] and were kept in DMEM high glucose medium supplemented with 20%. SV40 fibroblasts have been immortalized by the introduction of the large TAg of Simian virus 40 and were derived from a MSMD patient harboring a c.107ins4/c.200+1G > A mutation (patient 10i in Lancet, 2004[8]) and were maintained under the same conditions.

Design and Cloning of Lentiviral Vectors

Human IFNGR1 cDNA was commercially acquired from BioCat (BC005333; BioCat, Heidelberg, Germany; PubMed: 3459) and

introduced into a third-generation SIN lentiviral vector as previously described.[39] For generation of the Lv.SFFV.IFNγR1.iGFP vector, the transgene was flanked with Age1 and Sal1 restriction sites, whereas cloning for the Lv.EFS.IFNγR1.iGFP vector was performed using BamH1 and Sal1_BamH1 restriction sites. Insertion of a GFP reporter gene coupled to an IRES was done using restriction digestion with Sal1. Plasmids for lentiviral production were isolated from XL-10 gold bacteria using the Nucleo Bond Xtra Maxi kit according to the manufacturer's instructions.

Virus Production

Viral particle preparations were manufactured by transient transfection as described previously.[40] Briefly, human embryonic kidney 293 (HEK293) cells were seeded at 7×10^6 per 10 cm dish the day before transfection to reach 90% confluence. Appropriate DMEM high glucose medium was supplemented with 10% FCS, 1% P/S, 20 mM of HEPES (Life Technologies, Paisley, UK), and 25 μM of chloroquine (Sigma-Aldrich, Steinheim, Germany). Transient transfection of 8 μg gag/pol, 5 μg pRSV-Rev, 2 μg vesicular stomatitis virus glycoprotein (VSVg), and 5 μg lentiviral vector plasmid was performed using calcium phosphate co-precipitation method. Viral supernatants were harvested 24 h and 48 h post transfection, sterile filtrated (0.22 μm) and concentrated via ultracentrifugation to increase viral titers. Lentiviral titers were calculated on the basis of GFP expression in murine fibroblasts (SC-1) using serial dilution transduction.

Transduction of Cells

Transduction of cell lines was done in the presence of 0.1% protamine sulfate (Sigma Aldrich). Cell lines were transduced at a density of 10^5 cells per well in an appropriate 12-well plate with multiplicities of infection (MOIs) of 0.3 to 3. Transduction of primary cells was accomplished at a cell count of 10^5 with a MOI of 0.3 to 1.3 in 24-well adherent plate. Subsequent determination of transduction efficiency was done by flow cytometric analysis of GFP expression or antibody staining.

Flow Cytometric Analysis

Analysis of cell surface protein expression was performed by staining of surface antigens with hCD119-PE (#12-1199-42) and isotype control murine IgG1κ-PE (#12-4714-81; both Life Technologies, Carlsbad, CA, USA). Cells of interest were incubated with 1.5 μL of fluorochrome-conjugated antibodies (0.75 μL of respective isotype control) for 45 min at 4°C in the dark. For analysis of MHC-II expression, HeLa cells were starved in X-VIVO15 (Lonza, Verviers, Belgium) O/N and stimulated with 100 ng/mL hIFN-γ for 72 h before antibody staining with 3.3 μL hMHC-II-APC (HLA-DR clone L243; Biolegend, San Diego, CA, USA) per 500,000 cells for 45 min at 4°C in the dark. Prior to flow cytometric analysis cells were washed, filtered, and resuspended in phosphate-buffered saline (PBS). Flow cytometric analysis was performed using a CytoFLEX cytometer (Beckman Coulter) and data was evaluated using FlowJo 10 software (Treestar).

Cell Viability and Proliferation Analysis

To analyze cell viability, we stained cells with 50 μg/mL PI (eBioscience, San Diego, CA, USA) for 5 min on ice and subsequently analyzed at the CytoFLEX cytometer. To analyze proliferation, we stained cells with 5 μM Cell Proliferation Dye eFluor 670 (#65-0840; eBioscience, Frankfurt am Main, Germany) according to the manufacturer's instructions.

Western Blotting

HeLa cells or patient-derived fibroblasts were starved in X-VIVO15 with 1% P/S, 1% L-Glutamine, and 0.1% 2-Mercaptoethanol (both Life Technologies Limited, Paisley, UK) O/N. For analysis of pSTAT1 (Phospho-Stat1 [Tyr701, 58D6], Cell Signaling Technology, Frankfurt am Main, Germany) and STAT1 (Stat1 [D1K9Y], Cell Signaling Technology) samples were stimulated with hIFN-γ (PeproTech, Hamburg, Germany) for 30 min or with 1,000 IU/mL hIFN-α (PeproTech) for 24 h at 37°C. In terms of IFP-35 (#MA5-25662) (or LMP7 [#PA1-972]) (both Invitrogen, Rockford, IL, USA) analysis, samples were stimulated with 10 ng/mL (100 ng/mL) hIFN-γ for 72 h at 37°C. Cell lysates were prepared using radioimmunoprecipitation assay buffer in accordance to the manufacturer's instructions. For protein concentration quantification Pierce BCA protein assay kit was used and evaluated with Bio-Rad Model 680 microplate reader at 560 nm. Protein probes were separated by 10% [12%] SDS-PAGE. Electroblotting was performed using Bio-Rad Wet/Tank Blotting System Mini Trans-Blot Cell at 300 mA for 90 min in a 4°C room. Antibodies used for detection were STAT1, phospho-STAT1, LMP7, IFP-35, IFIT1 (Cell Signaling Technology, Danvers, MA, USA), and ISG15 (Santa Cruz Bio Technology, Dallas, TX, USA) and their respective horseradish peroxidase (HRP) coupled secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA; for IFIT1: goat anti-rabbit IgG; for ISG15: goat anti-mouse IgG1; for IFP-35: donkey IgG anti-mouse IgG; for STAT1, pSTAT1 and LMP7: donkey IgG anti-rabbit IgG). Documentation was done with Bio-Rad ChemiDoc XRS+ System or Image Studio Lite (LI-COR Biosciences). To validate the amount of target proteins, we used α-tubulin (or vinculin; or β-actin; Sigma Aldrich, Munich, Germany; Abcam, Cambridge, UK) with a molecular weight of 55 kDa (123 kDa; 42 kDa) as a loading control.

EMSA

EMSA was performed as previously described.[41] In brief, WT or IFN-γR1$^{-/-}$ HeLa cells were stimulated with 10^5 IU/mL hIFN-γ or 10^5 IU/mL hIFN-α for 20 min. 4 μg nuclear extract of respective cells was incubated with IRDye 700 GAS probe. Analysis of DNA binding activity was performed by polyacrylamide gel electrophoresis of the mixture and subsequent detection using Li-Cor Odyssey CLx system (Li-Cor, Lincoln, NE, USA).

Ethics

The patient with c.107ins4/c.200+1G > A mutation in *IFNGR1*, was referred to the Laboratory of Human Genetics of Infectious Diseases (HGID) for cellular and genetic diagnosis. Written informed consent forms were signed by the parents of the patient. This study was conducted in accordance with the Helsinki Declaration and approval was obtained from the French Ethics Committee "Comité de Protection des Personnes" (CPP), the French National Agency for Medicine

and Health Product Safety (ANSM) and the *Institut National de la Santé et de la Recherche Médicale* (INSERM) in France. Primary fibroblasts from the patient with an exon 2 c.131delC p.Pro44Leufs*18 homozygous IFN-γR1 defect were obtained according to the Declaration of Helsinki with the approval of the local Medical Ethical Committees.

Statistics

GraphPad Prism 8 was applied to perform unpaired Student's t test or analysis of variance (ANOVA). For experiments, mean ± SD is given. All statistical tests used are described in the corresponding figure legend. Asterisks denote: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.04.002.

AUTHOR CONTRIBUTIONS

N.L., K.H., and L.P. designed the study; K.H., L.P., M.A., and N.L. wrote the paper; L.P., K.H., J.N., A.H.H.N., A.-L.N., and S.F.H.W. performed experiments and analyzed data; and L.P., K.H., A.-L.N., U.B., J.-L.C, J.B., F.P., M.A., M.H., and A.S. provided conceptual advice. All authors of this manuscript received and edited the manuscript and discussed results.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

The authors thank Theresa Buchegger and Doreen Lüttge (both Hannover Medical School) for excellent technical assistance. Moreover, the authors are grateful to M. Ballmaier (Cell Sorting Facility, Hannover Medical School). Lentiviral vectors kindly provided by Axel Schambach (Institute of Experimental Hematology, MHH). All authors thank families for their contribution to this study. The presented research was derived from a HeLa cell line. Henrietta Lacks, and the HeLa cell line that was established from her tumor cells without her knowledge or consent in 1951, have made significant contributions to scientific progress and advances in human health. We are grateful to Henrietta Lacks, now deceased, and to her surviving family members for their contributions to biomedical research. This work was supported by grants from the Else Krööner-Fresenius St Stiftung (2015_A92 to N.L.) and the German Center for Infection Research (DZIF) (DZIF stipends K.H. and L.P.). This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 852178). A.-L.N. was supported by state funding from the Agence Nationale de la Recherche under the "Investissements d'avenir" program (ANR-10-IAHU-01) and Fondation Bettencourt Schueller.

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