Title: Hematopoietic stem cell gene therapy for IFNγR1 deficiency protects mice from mycobacterial infections

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Key points:

1. Hematopoietic cell and gene therapy can prevent in vivo infections by *Mycobacteria spp.* using different SIN lentiviral vectors

2. Cellular repair of macrophages *in vivo* highlights phagocytes as key players in the disease progression of MSMD

Abstract

Mendelian Susceptibility to Mycobacterial Disease (MSMD) is a rare primary immunodeficiency, characterized by severe infections caused by weakly virulent mycobacteria. Bi-allelic null mutations in genes encoding interferon gamma (IFNγ) receptor 1 or -2 (*IFN*G*R1, IFN*G*R2*) result in a life-threatening disease phenotype in early childhood. Recombinant IFNγ therapy is inefficient and hematopoietic stem cell transplantation (HSCT) has a poor prognosis. Thus, we developed a HSC gene therapy approach using lentiviral vectors expressing *Ifnγr1* either constitutively or myeloid-specifically. Transduction of mouse *Ifnγr1*$^{-/-}$ HSCs led to stable IFNγR1 expression on macrophages, which rescued their cellular responses to IFNγ. As a consequence, genetically corrected HSC-derived macrophages were able to suppress T-cell activation and showed restored anti-mycobacterial activity against *Mycobacterium avium* and *Mycobacterium bovis* Bacillus-Calmette-Guérin (BCG) *in vitro*. Transplantation of genetically corrected HSC into *Ifnγr1*$^{-/-}$ mice prior BCG infection prevented manifestations of severe BCG disease and maintained lung and spleen organ integrity, which was accompanied by a reduced mycobacterial burden in lung and spleen and a prolonged overall survival of transplanted animals. In summary, we demonstrate an HSC-based gene therapy approach for IFNγR1 deficiency, which protects mice from severe mycobacterial infections, thereby laying the foundation for a new therapeutic intervention in the corresponding human patients.

Introduction

Hematopoietic stem cell gene therapy (HSCGT) is a promising approach for the treatment of a growing number of primary immunodeficiencies (PIDs). To date, numerous clinical trials are underway in order to improve the quality of life for patients suffering from severe combined immunodeficiency (SCID-X1 or ADA-SCID), Wiskott-Aldrich syndrome (WAS), or Chronic Granulomatous Disease (CGD) [1-4]. Improvements in vector design and the use of safety improved self-inactivating (SIN) lentiviral vectors, have laid the foundation for today's new generation of HSC-based gene therapy [5]. Taken the aforementioned success, HSGCT may be extended to hematopoietic diseases in which the impaired blood and tissue leukocytes are unable to fight mycobacterial infections.

Along this line, bi-allelic mutations in *IFN*G*R1* have been associated from 1996 onward with a selective predisposition to severe infections by weakly virulent mycobacteria, including BCG vaccines and environmental mycobacteria (EM). This condition is known as Mendelian Susceptibility to Mycobacterial Disease (MSMD) (OMIM #209950) [6,7]. Mutations in several genes involved in the cross-talk between the myeloid and lymphoid cells that orchestrate IFNγ-mediated immunity have been described in patients with MSMD. As a consequence, tissue macrophages cannot destroy mycobacteria normally. MSMD-causing genes control either the production of IFN-γ (*IL12B*, *IL12RB1*, *TYK2*, *IRF8*, *ISG15*, *NEMO*) or the response to IFNγ (*IFN*G*R1*, *IFN*G*R2*, *STAT1*, *IRF8*, *CYBB*) [8]. The clinical features depend on the genotype.

The onset of MSMD in patients with the two most severe forms, autosomal recessive (AR) complete IFNγR1 and IFNγR2 deficiency, is typically before 3 years of age and is characterized by disseminated and persistent or recurrent infections by one or more mycobacterial species (e.g. BCG, *M. chelonae*, *M. avium*), which are life threatening [8-13]. Conservative treatment options for all patients primarily rely on vigorous treatment of mycobacterial infections by antibiotics. IFNγ substitution therapy can only restore macrophage function in patients whose cellular responses to IFNγ are intact or impaired but not abolished. Indeed, patients suffering from AR and complete IFNγR1 (OMIM #209950) or IFNγR2 (OMIM #107470) deficiency do not respond to IFNγ therapy [8]. Together, these two defects have a prevalence of around 1/100,000 births worldwide and have been found in most ethnicities. As of yet, 36 patients from 30 families have been described in the literature, while probably many more have been diagnosed [8,14-19].

In patients suffering from complete IFNγR1 or IFNγR2 deficiency, antibiotics cannot be discontinued and allogeneic HSCT (alloHSCT) is the only curative treatment[20-24].

However, substantial side effects such as recurrent infections during the transplantation scenario or even graft rejection still considerably limit the success of alloHSCT to treat AR complete IFNγR1 or IFNγR2 deficiency. The unusually high graft rejection is associated with high IFNγ serum levels present in IFNγR-deficient MSMD patients, as their cells are not able to clear the IFNγ from the blood [25]. This negative effect can even be exacerbated if IFNγ is produced by alloreactive T cells in the donor graft [26]. As a consequence, IFNγ may interfere with the cell cycle of donor HSCs or induce cell death by up-regulating Fas ligand in combination with TNFα [26-30]. With 17 reported deaths out of 36 patients, the overall prognosis of HSCT for patients suffering from IFNγR1 and IFNγR2 deficiency is poor, highlighting the need for improved therapeutic approaches.

As current forms of treatment are limited, we here envision to establish a HSCGT approach to restore the anti-mycobacterial function in hematopoietic cells *in vivo*. This approach would prevent the risks of graft rejection and graft versus host disease that are inherent to HSCT. To achieve this aim, we used lentiviral vector technology to correct the cellular response in the hematopoietic system to IFNγ and investigated the ability of genetically corrected macrophages to clear mycobacteria *in vitro* and *in vivo*.

Our findings suggest that restored expression of IFNγR1 on macrophages is directly linked to the cellular repair of these cells and highlight a new cell-based treatment approach for the clinical condition of MSMD.

Methods

Design of lentiviral vectors

Ifnγr1 cDNA was introduced into third generation SIN lentiviral vectors downstream of either a spleen focus forming virus (SFFV) promoter (Lv.SFFV.Ifnγr1), a miR223 promoter (Lv.miR223.Ifnγr1), or a synthetic, myeloid-specific SP-146/gp91 promoter (Lv.MSP.Ifnγr1) and all constructs were also coupled with a green fluorescent protein (GFP) through an IRES.

Cultivation of cells

All cell lines and primary cells were kept under standard culture conditions at 37 °C, 5% CO_2 and 95% humidity. RAW264.7 cells were kept in 6-well adherent plates. Cells were kept in high glucose DMEM Medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (both PAA).

Mice

B6.SJL-Ptprca-Pep3b/BoyJZtm (Ly5.1; CD45.1) mice were used as wild-type (WT) controls and B6.129S7-Ifngr1^{tm1Agt}/J (*Ifnγr1*$^{-/-}$) harboring a knockout in the *Ifnγr1* gene served as a disease model of MSMD. Mice were housed in the central animal facility of Hannover Medical School under specific-pathogen-free conditions in individually ventilated cages (IVC). Mice had had access to food and water *ad libitum*. All animal experiments were approved by the lower Saxony State animal welfare committee and were conducted according to the animal welfare law.

Isolation, cultivation and transduction of lineage negative cells

Lineage negative cells were isolated from total bone marrow using lineage cell depletion kit (Miltenyi) following the manufacturer's instructions. Pre-stimulation of lineage negative cells was performed for 24 hours in STIF medium (StemSpan medium supplemented with 1mM Penicillin/Streptomycin, 2mM L-glutamine, 100 ng/ml mSCF, 20 ng/ml TPO, 20 ng/ml IGF-2 and 100 ng/ml FGF2). Thereafter, transduction of lineage negative cells was performed on RetroNectin® (Takara) coated plates using STIF medium and MOIs between 20 and 50 as previously described [31]

Bone marrow transplantation

Ifnγr1$^{-/-}$ recipient mice were irradiated with a single dose of 9 Gy. Lineage negative bone marrow cells of *Ifnγr1*$^{-/-}$ or WT donor mice were isolated as described above and injected

intravenously into the tail vain of recipient mice 24 h post irradiation. Alternatively, lineage negative cells were transduced with SIN lentiviral vectors and transplanted by the same protocol. For some experiments, non-transplanted *Ifnγr1*$^{-/-}$ served as negative controls (also indicated in the respective figure legend).

Pulmonary BCG infections

After reconstitution of the hematopoietic system (>6 weeks post transplantation), transplanted mice were infected by intratracheal application of 1×10^7 CFU DsRed-labeled BCG-Pasteur under general anesthesia using *i.p.* injection of Ketamin/Rompun.

Differentiation of lineage negative cells

Lineage negative cells were differentiated into macrophages for 7-15 days in RPMI medium supplemented with 10% FBS, 1mM Penicillin/Streptomycin, and 30% of M-CSF supernatant produced by L929 cells as previously described [31,32]

Mycobacterium avium

Intracellular killing of *M. avium* by macrophages was assessed as described in [33] with and without stimulation with IFNγ for 24 h. Macrophages were infected for 1 h with *M. avium* at an OD_{600} of 0.1. Non-phagocytosed bacteria were removed by intense washing. At 1 and 24 h post infection cells were lysed using 0.1% Triton X100 in PBS and plated on Middlebrock 7H9 agar plates and incubated at 37°C. After 2 weeks colonies were counted.

BCG

Transduced lineage negative cells were sorted and differentiated into macrophages. 200,000 cells per condition were seeded. 24 h prior to infection the cells were washed twice with PBS to remove remaining antibiotics. Cells were stimulated with 50 ng/ml IFNγ in RPMI 1640 (Gibco) supplemented with 10% FBS. Cells were infected with BCG at an MOI of 100 and incubated for 1 h to allow BCG uptake. Following that, 10 μg/ml Gentamycin was added to remove all extracellular BCG. Cells were harvested and lysed 1 h and 24 h post infection by using a 24g canula to resuspend the cells 10 times. Lysates were plated at different dilutions on 7H10 plates and incubated for 2 weeks until the colonies were counted. CFU/ml values were then calculated.

IFNγ serum levels

Blood of mice was drawn from the retrobulbar vein plexus and cell free serum was collected by centrifugation in serum separation tubes. Serum IFNγ levels were determined using the eBioscience mouse IFNγ Ready-SET-Go! ELISA kit.

Light sheet microscopy

Light sheet microscopy was performed as described before [34].

Histology

For histopathological investigation, formalin-fixed, paraffin-embedded lung tissue was sectioned at 2-3 µm thickness, mounted on glass slides and stained with hematoxylin and eosin (HE). For immunohistochemistry, a polyclonal rabbit anti-eGFP antibody (1:10,000, catalog no.: orb303312, Biorbyt) was used. A microwave pretreatment over 20 minutes with citrate buffer was applied for antigen retrieval. As negative control, the primary antibody was replaced by rabbit serum. A peroxidase-conjugated avidin-biotin complex (Vector Laboratories) and 3.3- diaminobenzidine-tetrachloride in 0.1 M imidazole (Sigma-Aldrich) was used for visualization. Finally, sections were counterstained with Mayer’s hematoxylin (Merck).

Results

Design of lentiviral vectors and safety studies in primary hematopoietic cells

In order to establish a HSCGT approach for IFNγR1 deficiency, we made use of 3rd generation SIN vectors to express a murine *Ifnγr1* cDNA from a spleen focus forming virus promoter/enhancer (SFFV) element. In order to assess transgene expression, we coupled the *Ifnγr1* cDNA via an internal ribosomal entry site (IRES) to an enhanced green fluorescent protein (GFP) (Lv.SFFV.Ifnγr1). The same lentiviral vector backbone was used to establish the control vector, expressing the GFP transgene directly under the control of the SFFV promoter (Lv.SFFV.GFP) (**Figure 1A**). As a first step, we evaluated vector-mediated transgene expression in the murine fibroblast cell line SC1, resulting in detectable levels of IFNγR1 (CD119) in cell lysates and on the cell surface (**Figure S1A-C**). Transgene expression was next evaluated in lineage negative hematopoietic stem/progenitor cells (HSPCs) of mice which are deficient in *Ifnγr1* (*Ifnγr1*$^{-/-}$) and which faithfully recapitulate the MSMD phenotype upon infection with BCG [25,35]. Transduction of lineage negative HSPCs revealed co-expression of GFP and IFNγR1 in clear contrast to non-transduced *Ifnγr1*$^{-/-}$ control cells (**Figure 1B**). Of note, mean fluorescent intensity (MFI) values of CD119 indicated higher expression levels of IFNγR1 in transduced *Ifnγr1*$^{-/-}$ cells compared to their wild type counterparts. As a next step, we followed a straight forward differentiation protocol using M-CSF to differentiate HSPCs towards macrophages. After seven days of differentiation, macrophages derived from Lv.SFFV.Ifnγr1-transduced *Ifnγr1*$^{-/-}$ HSPCs showed typical morphology on cytospins and surface marker expression of CD11b, CD200R, and F4/80, which was very much comparable to HSPC-derived macrophages from wild type or non-corrected *Ifnγr1*$^{-/-}$ cells (**Figure 1C and D and Figure S2**). Furthermore, stable transgene expression as well as normal rates of cell death and cell proliferation were observed in Lv.SFFV.Ifnγr1-transduced Raw 264.7 cells, further highlighting the functional expression of IFNγR1 upon transduction of our lentiviral vector (**Figure S3A-C**).

Phenotypic correction of IFNγ functionality upon constitutive Ifnγr1 overexpression

After we evaluated SFFV-mediated IFNγR1 expression on *Ifnγr1*$^{-/-}$ KO and WT cells, we studied the direct effect of IFNγ on corrected macrophages. As expression of MHC class II on macrophages can be induced upon stimulation with IFNγ [36], we studied the upregulation of HLA-DR and of the co-stimulatory molecule CD86 (B7.2) upon treatment with IFNγ. Overexpression of IFNγR1 by the Lv.SFFV.Ifnγr1 construct was able to restore upregulation of HLA-DR in GFP^{+} macrophages upon stimulation with IFNγ, whereas non-corrected

Ifnγr1$^{-/-}$ cells showed no response (**Figure 2A**). A similar observation was made for CD86. Here similar to HLA-DR, only corrected GFP^{+} macrophages showed restored upregulation of CD86 compared to WT cells (**Figure 2B**). Proper downstream signaling of IFNγ is induced upon initial binding to IFNγR1, and subsequent dimerization of the two IFNγR1 chains and the assembly of the functional IFNγR1/2 heterodimer receptor complex. While the IFNγ:IFNγR1/2 receptor complex forms a STAT1 binding site at the intracellular domain of IFNγR1, receptor-associated-activated JAK kinases are able to phosphorylate STAT1 and to induce proper downstream signaling [37]. Cultivation of Lv.SFFV.Ifnγr1-transduced macrophages led to IFNγ consumption over time, so that after 24 h of cultivation only 60-80% of the original IFNγ input was detected (**Figure 2C**). A similar picture was observed for the ability to phosphorylate STAT1 in the presence of IFNγ. While WT or genetically corrected macrophages were able to phosphorylate STAT1, non-corrected *Ifnγr1*$^{-/-}$ macrophages showed no pSTAT1 upregulation in the presence of IFNγ (**Figure 2D**). Proper downstream signaling was also evaluated for known downstream target genes of IFNγ such as *Interferon regulatory factor 1* (*Irf1*), *nitric oxide synthase 2* (*Nos2*), or *indoleamine 2,3-dioxygenase* (*Ido*). Stimulation of macrophages with IFNγ proved a restored expression pattern of *Irf1*, *Nos2*, and *Ido* in macrophages transduced with the Lv.SFFV.Ifnγr1 construct, which was very similar to WT cells (**Figure 2E**). In contrast, macrophages from *Ifnγr1*$^{-/-}$ showed only background expression of the aforementioned genes (**Figure 2E**). While surface expression of HLA-DR and the secretion of IDO from macrophages have a direct effect on T cell behavior, we also analyzed the IFNγ-dependent ability of macrophages to suppress ovalbumin (OVA)-specific T cell proliferation in the presence or absence of antigen [38]. Cultivation of CD4^{+} T cells from OTII mice with OVA-loaded and IFNγ-primed macrophages showed a clear reduction in the proliferation of T cells when either WT or genetically corrected macrophages were present (**Figure 2F**). In contrast, more than 80% of T cells still proliferated if co-cultured with *Ifnγr1*$^{-/-}$ macrophages (**Figure 2F**). As a final assessment of our lentiviral construct, we elucidated the IFNγ-dependent intracellular degradation of *Mycobacterium avium* (*M. avium*) or BCG. Irrespective of the mycobacterium used, genetically corrected macrophages showed restored intracellular degradation of either *M. avium* or BCG, which was at a level comparable to WT macrophages (**Figure 2G and H**).

Lineage restrictive expression of Ifnγr1 mediates phenotypic correction in HSPC-derived macrophages

Following the promising *in vitro* results using the constitutive Lv.SFFV.Ifnγr1 lentiviral construct, we additionally designed 3rd generation SIN lentiviral constructs, which are able to express the murine *Ifnγr1* cDNA in a cell type-specific manner in cells of the myeloid lineage. In order to achieve this aim, we used either a fragment of the human miRNA223 promoter [39] (Lv.miR223.Ifnγr1) or a promoter harboring a 1.5kb minimal promoter sequence from the gp91^{phox} locus fused to the SP146 synthetic promoter (Lv.MSP.Ifnγr1; MSP=myeloid specific promoter) (**Figure 3A**), previously shown to express transgenes cell type specific in the myeloid lineage [40,41]. In order to evaluate the aforementioned constructs, we again isolated HSPCs from *Ifnγr1*$^{-/-}$ mice, transduced them and differentiated the cells towards macrophages. Irrespective of the constructs used, MFI values of transduced macrophages revealed distinct expression of IFNγR1, which was significantly higher than that in non-transduced *Ifnγr1*$^{-/-}$ macrophages (**Figure 3B and C**). Of note, expression mediated by the Lv.MSP.Ifnγr1 construct revealed the highest expression of IFNγR1 compared to WT and Lv.miR223.Ifnγr1-transduced macrophages (**Figure 3B and C**). Following the detection of CD119 on the cell surface, we also evaluated key macrophage functions, which are dependent on IFNγ stimulation. Similar to studies performed with the Lv.SFFV.Ifnγr1 construct, also myeloid-specific expression of IFNγR1 was able to restore the upregulation of HLA-DR and CD86 in GFP positive macrophages (**Figure 3D and E**). In addition, both, Lv.miR223.Ifnγr1 and Lv.MSP.Ifnγr1 gene corrected cells were able to restore the expression of the IFNγR downstream targets *Irf1* and *Nos2* after stimulation of macrophages with IFNγ (**Figure 3F**). As a final assessment for our myeloid cell-specific gene therapy approach, we also tested the ability of genetically corrected cells to reduce IFNγ levels in cell culture supernatant and to clear intracellular BCG. HSPCs transduced with either Lv.miR223.Ifnγr1 or Lv.MSP.Ifnγr1 constructs and differentiated towards macrophages showed restored consumption of IFNγ from the medium, reaching levels of 40% reduction within 32h post treatment, whereas IFNγ levels in cultures with non-corrected *Ifnγr1*$^{-/-}$ macrophages remained unaffected (**Figure 3G**). Similarly, genetically corrected GFP positive macrophages showed restored intracellular degradation of BCG within 24 h post infection, whereas GFP negative cells from the same transduction were not able to clear BCG (**Figure 3H**). Similar to Lv.SFFV.Ifnγr1-transduced cells, also Lv.miR223.Ifnγr1- or Lv.MSP.Ifnγr1-transduced Raw264.7 cells showed stable transgene expression, which was in accordance with normal rates of apoptosis and proliferation (**Figure S3A-C**).

Hematopoietic gene and cell therapy using genetically corrected HSPCs mediates protection against BCG infection in vivo

As an *in vivo* assessment of our gene therapy approach, we used *Ifnγr1*$^{-/-}$ mice and established a HSCGT approach, with the overall aim to protect transplanted mice from BCG infection (**Figure 4A**). After 9 Gy lethal irradiation, *Ifnγr1*$^{-/-}$ recipient mice received lineage negative cells either from WT C57Bl/6, or non-corrected *Ifnγr1*$^{-/-}$ donor mice. Alternatively, *Ifnγr1*$^{-/-}$ recipient mice were transplanted with genetically corrected lineage negative cells, transduced either with Lv.SFFV.Ifnγr1 or Lv.MSP.Ifnγr1 constructs (**Figure 4A**). Following a minimum time of 6 weeks to allow for reconstitution of the hematopoietic system, we performed intra-pulmonary infection of transplanted mice with DsRed-BCG [42], followed by an observation time period of 5-9 weeks (**Figure 4A**). Analyzing the vector copy numbers (VCN) in the bone marrow of transplanted animals, mice receiving Lv.GFP-transduced cells showed a VCN of 8-11, whereas cells from Lv.SFFV.Ifnγr1- or Lv.MSP.Ifnγr1-transplanted animals had a VCN of 1-2 or 0.5-2, respectively (**Figure 4B**). Of note, VCN values in the lungs increased post BCG infection in animals which received genetically corrected cells whereas mice transplanted with Lv.GFP-transduced HSC showed stable VCN values (**Figure 4B**). Detection of genetically corrected cells in the bone marrow and lung was associated with stabilized body weight ratios and with an increase in overall survival of transplanted mice following BCG infection (**Figure S4A and Figure 4C**). Irrespective of the viral construct used, *Ifnγr1*$^{-/-}$ mice transplanted with genetically corrected cells survived more than two months post infection, while control mice showed severe BCG related symptoms already 21 days post infection (**Figure 4C**). Similarly, mice, which received genetically corrected cells, showed low levels of IFNγ throughout the infection period, whereas Lv.GFP-transplanted mice showed increased values of IFNγ (**Figure 4D**).

Hematopoietic gene and cell therapy sustains organ integrity of Ifnγr1$^{-/-}$ *mice following BCG infection*

We next performed detailed analysis of hematopoietic organs following immunization with BCG. Transplantation of Lv.SFFV.Ifnγr1- or Lv.MSP.Ifnγr1-transduced cells into *Ifnγr1*$^{-/-}$ mice prevented splenomegaly and led to a normalized spleen morphology as well as spleen to body weight ratio (**Figure 5A and B**). In addition, mice receiving genetically corrected cells had a splenic parenchyma with characteristic regions described as red and white pulp (**Figure 5C**). Moreover, detailed histology of spleen sections receiving genetically corrected cells

revealed only mild multifocal granulomatous inflammation irrespective of the lentiviral construct used. In contrast, negative control mice showed moderate to severe multifocal granulomatous inflammation with multi-nucleated giant cells. In line with the aforementioned histopathology, mice that received genetically corrected cells showed a 10-100 fold decrease in the mycobacterial burden in spleen homogenates compared to negative control mice (**Figure 5D**). A similar protective effect was observed in the lung. *Ifnγr1*$^{-/-}$ mice which received Lv.SFFV.Ifnγr1- or Lv.MSP.Ifnγr1-transduced cells had a normal lung morphology comparable to that of mice receiving healthy wild type cells (positive controls), whereas mice which received non-corrected cells (negative control) showed clear signs of BCG-mediated infection (**Figure 6A**). The severity of BCG infection was also seen in light sheet microscopy analysis as DsRed positive BCG infections in the post-caval lung lobe of negative control mice (**Figure 6A and B**). In contrast, mice receiving healthy or genetically corrected HSCs had a much lower DsRed signal, highlighting a decreased BCG burden in the lung (**Figure 6A and B**) (**Supplemental Videos 1-4**). Similar observations were made for the inferior, middle, left and superior lung lobes (**Figure S4B**). Furthermore, total CFU analysis of lung tissue confirmed the reduced mycobacterial burden in the lung of transplanted mice. While negative control mice had a mean of $3x10^5$ CFU per lung, mice that received genetically corrected cells showed bacterial burdens reduced by an order of magnitude (approx. $3x10^4$ CFU per lung), irrespective of the lentiviral construct used (**Figure 6D**). Lung histology further supported the protective effect of genetically corrected cells in the lung. While Lv.MSP.Ifnγr1-transplanted animals showed a normal lung structure (**Figure 6E, picture 1 and 2**) and the presence of GFP-labeled macrophages in the bronchoalveolar space (**Figure 6F, picture 1 and 2**), Lv.GFP-transplanted negative control mice showed formation of pulmonary granulomas (indicated by *) with multinucleated giant cells surrounded by collagen fibers (**Figure 6E and F, picture 3 and 4**). Although Ziehl-Neelsen staining of macrophages in both Lv.MSP.Ifnγr1- and Lv.GFP-transplanted mice revealed the presence of acid fast bacteria (**Figure 6G**), only macrophages from mice transplanted with Lv.MSP.Ifnγr1-transduced cells were able to degrade BCG while Lv.GFP macrophages started to form granulomas (**Figure 6D-G**).

Discussion

In the present study, we demonstrate that a HSCGT approach for IFNγR1 deficiency is feasible and that lentiviral gene transfer can correct the IFNγ signaling pathway in HSC-derived macrophages *in vitro*. As a consequence, we established an HSCGT approach using *Ifnγr1*$^{-/-}$ mice and evaluated the therapeutic benefit of our HSCGT approach in a clinically relevant infection model using BCG. Thus, we highlight a new treatment approach, which is able to restore anti-mycobacterial activity of macrophages in lung and spleen. This observation is of clinical relevance, as patients suffering from AR complete IFNγR1 deficiency have poor prognosis and often die in early childhood due to disseminated infections with BCG and/or other EM [8,10].

The feasibility and clinical transfer of HSCGT using retroviral vector technology has already been proven for other PIDs such as WAS, CGD, SCID-X1, or ADA-SCID [1-4], leading to the first marketing approval of an *ex vivo* gene therapy [43]. In our model, similar to the aforementioned approaches, we have transplanted genetically corrected HSCs into lethally irradiated *Ifnγr1*$^{-/-}$ mice prior to BCG infection. In this transplantation scenario, HSC are able to migrate to the bone marrow and engraft in the host. However, this approach might not be feasible in a transplantation scenario in which genetically corrected HSCs will be transplanted into patients who carry disseminated mycobacterial infections. Here, high levels of IFNγ in the plasma of patients may interfere with the engraftment of donor HSCs. Similar to attempts of HSCT using HLA-identical relatives, IFNγ may induce proliferation of genetically corrected HSCs, which hampers the engraftment potential of the HSCs and could ultimately lead to graft rejection [22,27]. To circumvent this problem, humanized anti-IFNγ antibody (Fontolizumab; HuZAF) could be used in combination with rigorous antibiotic treatment to open a therapeutic window for the transplantation of autologous and genetically corrected HSCs [44].

As an alternative to the antibiotic/HuZAF therapy, systemic transplantation of genetically corrected or healthy macrophages could be performed in AR complete IFNγR1 or IFNγR2 deficient patients. Here, transplanted macrophages may be able to eradicate disseminated infections and to clear IFNγ from the plasma at the very same time, thus opening a therapeutic window for subsequent HSCGT. To strengthen this innovative approach, the intra-pulmonary transplantation of macrophages was shown to be very effective for the treatment of hereditary pulmonary alveolar proteinosis (herPAP) [45,46]. Macrophages transferred directly into the lungs of diseased PAP mice could engraft and improve PAP-related disease symptoms in a very short time frame, favoring this concept to be also suited as a bridging therapy of AR complete

IFNγR1 or -2 deficiencies. In our study, we have performed HSCGT using genetically corrected HSCs. The therapeutic benefit observed *in vivo*, however, is presumably based on corrected macrophages, which are derived from the donor graft. In fact, a direct link between the pathophysiology of MSMD and macrophages remains as of yet elusive. To shed light into the contribution of myeloid cells to the disease progression of MSMD, we have used lentiviral vectors, which express the therapeutic *Ifnγr1* transgene in a myeloid cell type specific fashion. Using these vectors, we could prove a direct link of genetically corrected myeloid cells to the improved disease parameters observed in transplanted mice, suggesting an important role of macrophages in MSMD. Of note, macrophages and especially tissue resident macrophages (TRMs) play a pivotal role in the progression of different diseases [47-50]. The important role of macrophages is further underlined by the detection of GFP-positive macrophages in the lung and the sustained lung tissue integrity, post BCG infection of transplanted animals. In fact, analyzing the VCNs in transplanted animals revealed increased VCNs in lung compared to bone marrow of mice which had exclusively received genetically corrected HSC, suggesting that there might be *in vivo* selection of macrophages post BCG infection.

The rise of modern technologies such as generation of macrophage from induced pluripotent stem cells [51,52] and the recent insights in tissue resident macrophage self-renewal and plasticity [53-55] further encourages the use of modern cell types to treat the life-threatening condition of IFNγR1 deficiency in human.

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Author contribution

M.H., A.M., P.B. and N.L. designed the study, wrote the paper and performed experiments. A.H.H.N., J.S., O.H., M.P.K., S.B., R.M., D.B., V.H., W.B., F.C.B., R.G performed experiments and analyzed data. R.F., B.G., J.B., J.L.C., A.S., D.J., O.H., and U.K provided conceptual advice, discussed results and edited the manuscript.

Conflict of Interest

All authors declare no financial interest.

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Figure Legends

Figure 1. Constitutive lentiviral *Ifnγr1* overexpression in *Ifnγr1$^{-/-}$* macrophages. (A) Schematic representation of the lentiviral vector Lv.SFFV.Ifnγr1 expressing the *Ifnγr1* cDNA by a spleen focus forming virus (SFFV) promoter coupled to a GFP reporter via an internal ribosomal entry site (IRES). (B) GFP expression in *Ifnγr1$^{-/-}$* macrophages without (left plot) and with transduction (middle plot) using the Lv.SFFV.Ifnγr1 vector. Histogram depicting IFNγR1 (CD119) expression in *Ifnγr1$^{-/-}$* (black), WT (blue) and Lv.SFFV.Ifnγr1-transduced (blue) macrophages compared to an isotype control (grey). (C) WT (left), *Ifnγr1$^{-/-}$* (middle), and Lv.SFFV.Ifnγr1 (right) macrophages presenting characteristic morphology in representative cytospins. (D) Characteristic macrophage surface marker expression of CD11b (left), CD200R (middle), and F4/80 (right) at similar levels on WT (green), *Ifnγr1$^{-/-}$* (red), and Lv.SFFV.Ifnγr1-transduced (black) macrophages compared to unstained samples (grey).

Figure 2. Restored macrophage functionality upon Lv.SFFV.Ifnγr1 transduction of *Ifnγr1$^{-/-}$* cells. (A) Representative histograms depicting MHC-II upregulation on IFNγ-stimulated (black) vs. unstimulated (grey) WT, *Ifnγr1$^{-/-}$*, and Lv.SFFV.Ifnγr1-transduced macrophages. Bar graphs depicting summarized results of three independent experiments (2-way ANOVA using Sidak's multiple comparisons post hoc testing, ns = not significant, ***p≤0,001, ****p≤0,0001). (B) Representative histograms depicting CD86 upregulation on IFNγ-stimulated (black) vs. unstimulated (grey) WT, *Ifnγr1$^{-/-}$*, and Lv.SFFV.Ifnγr1-transduced macrophages. Bar graphs depicting summarized results of three independent experiments (2-way ANOVA using Sidak's multiple comparisons post hoc testing, ns = not significant, *p≤0,05, **p≤0,01). (C) IFNγ internalization of WT, *Ifnγr1$^{-/-}$*, and Lv.SFFV.Ifnγr1-transduced macrophages over 24 h (normalized to 0h; n=4; 2-way ANOVA using Tukey's multiple comparisons post hoc testing, ****p≤0,0001). (D) Phosphorylation of STAT1 (pSTAT1) in unstimulated (-) and IFNγ-stimulated (+) WT, *Ifnγr1$^{-/-}$*, and Lv.SFFV.Ifnγr1-transduced macrophages compared to GAPDH loading control. (E) Relative mRNA expression of *Irf1*, *Nos2*, and *Ido* in IFNγ-stimulated WT, *Ifnγr1$^{-/-}$*, and Lv.SFFV.Ifnγr1-transduced macrophages normalized to unstimulated samples (*Irf1*: n=4, *Nos2*, *Ido*: n=3). (F) Induction of ovalbumin-specific T cell proliferation by WT (black), *Ifnγr1$^{-/-}$* (light grey), and Lv.SFFV.Ifnγr1-transduced (dark grey) macrophages upon stimulation with IFNγ (left bars) or IFNγ and ovalbumin (OVA, right bars). Bars depicting the percentage of eFluor670^{low} CD4^{+} T cells (n=3; 2-way ANOVA using Bonferroni's multiple comparisons post hoc testing, **p≤0,01, ***p≤0,001). (G) Bacterial burden of *Mycobacterium avium* in macrophages 0 h

(black) and 24 h (grey) after infection (n=3; 2-way ANOVA using Sidak's multiple comparisons post hoc testing, ***p≤0,001, ****p≤0,0001). (H) Bacterial burden of *Bacillus Calmette Guérin* in macrophages 8 days after infection (normalized to uptake value 4 h after infection; n=3; one-way ANOVA using Dunnett's multiple comparisons post hoc testing, **p≤0,01).

Figure 3. Expression and function of myeloid- specific lentiviral *Ifnγr1* overexpression in *Ifnγr1*$^{-/-}$ macrophages. (A) Schematic representation of the lentiviral vectors expressing the *Ifnγr1* cDNA by a synthetic myeloid specific promoter (Lv.MSP.Ifnγr1) or a miRNA223 promoter (Lv.miR223.Ifnγr1) both coupled to a GFP reporter via an internal ribosomal entry site (IRES). (B) Histogram depicting IFNγR1 expression in *Ifnγr1*$^{-/-}$ (grey), WT (green), Lv.MSP.Ifnγr1-transduced (blue) and Lv.miR223.Ifnγr1-transduced (orange) macrophages. Bar graphs depicting the mean fluorescence intensity (MFI) of IFNγR1 (CD119; n=3; one-way ANOVA using Dunnett's multiple comparisons post hoc testing, **p≤0,01, ****p≤0,0001). (C) Representative brightfield, GFP- and autofluorescence pictures of mock-transduced (left), Lv.MSP.Ifnγr1-transduced (middle), and Lv.miR223.Ifnγr1-transduced (right) macrophages. (D) Representative histograms depicting MHC-II upregulation on IFNγ-stimulated (black) vs. unstimulated (grey) WT, *Ifnγr1*$^{-/-}$, Lv.MSP.Ifnγr1-transduced, and Lv.miR223.Ifnγr1-transduced macrophages. Bar graphs depicting summarized results of four independent experiments (one-way ANOVA using Dunnett's multiple comparisons post hoc testing, *p≤0,05). (E) Representative histograms depicting CD86 upregulation on IFNγ-stimulated (black) vs. unstimulated (grey) WT, *Ifnγr1*$^{-/-}$, Lv.MSP.Ifnγr1-transduced, and Lv.miR223.Ifnγr1-transduced macrophages. Bar graphs depicting summarized results of four independent experiments (one-way ANOVA using Dunnett's multiple comparisons post hoc testing, ns = not significant). (F) Relative mRNA expression of *Irf1* and *Nos2* in IFNγ-stimulated WT, *Ifnγr1*$^{-/-}$, Lv.MSP.Ifnγr1-transduced, and Lv.miR223.Ifnγr1-transduced macrophages (grey) normalized to unstimulated samples (black; n=4). (G) IFNγ internalization of WT (black), *Ifnγr1*$^{-/-}$ (grey), Lv.MSP.Ifnγr1-transduced (red), and Lv.miR223.Ifnγr1-transduced (green) macrophages over 32 h (normalized to 0 h; n=3; 2-way ANOVA using Tukey's multiple comparisons post hoc testing; ****p≤0,0001). (H) Bacterial burden of *Bacillus Calmette Guérin* in Lv.MSP.Ifnγr1-transduced and Lv.miR223.Ifnγr1-transduced macrophages 24 h after infection sorted positive and negative for GFP expression (normalized to uptake value 0 h after infection; n=3).

Figure 4. Engraftment of lentiviral *Ifnγr1* overexpressing cells rescues *Ifnγr1$^{-/-}$* mice from lethal pulmonary BCG infection. (A) Schematic representation of the experimental outline. *Ifnγr1$^{-/-}$* mice were lethally irradiated and transplanted with lineage negative *Ifnγr1$^{-/-}$* cells transduced with Lv.SFFV.Ifnγr1 or Lv.MSP.Ifnγr1. After hematopoietic reconstitution, DsRed labelled BCG was instilled to the lungs of the transplanted mice. Final analysis was performed latest 9 weeks after infection. (B) Number of vector copies (VCN) integrated into cells in the bone marrow (BM) and lungs (L) of transplanted and infected mice. Equal symbols represent VCN of the same mouse in BM and L (n≥3; p-values are shown). (C) Kaplan-Meier-curve depicting the survival of mice after BCG infection in two independent experiments (positive ctrl n=6 (mice receiving WT cells), negative ctrl n=13 (mice receiving Lv.GFP-transduced *Ifnγr1$^{-/-}$* cells), Lv.SFFV.Ifnγr1 n=7, Lv.MSP.Ifnγr1 n=6). (D) IFNγ serum levels of mice 2, 4, 6 and 8 weeks after infection. Mean and SEM are shown for three independent experiments (positive ctrl n=9 (mice receiving WT cells), negative ctrl n=9 (mice receiving Lv.GFP-transduced *Ifnγr1$^{-/-}$* cells and non-transplanted *Ifnγr1$^{-/-}$* mice), Lv.SFFV.Ifnγr1 and Lv.MSP.Ifnγr1 n=7).

Figure 5. Prevention of severe splenomegaly upon constitutive or myeloid-specific *Ifnγr1* overexpression. (A) Representative macroscopic pictures of spleens isolated from mice 9 weeks after infection. Scale bar 2 cm (positive ctrl = mouse receiving WT cells, negative ctrl = non-transplanted *Ifnγr1$^{-/-}$* mouse). (B) Spleen-to-body weight ratio depicting the normalized spleen weight of individual mice 9 weeks after infection. Mean and SEM are shown for three independent experiments (positive ctrl n=10 (mice receiving WT cells), negative ctrl n=13 (mice receiving Lv.GFP-transduced *Ifnγr1$^{-/-}$* cells and non-transplanted *Ifnγr1$^{-/-}$* mice), Lv.SFFV.Ifnγr1 n=10, Lv.MSP.Ifnγr1 n=9, one-way ANOVA using Tukey's multiple comparisons post hoc testing). (C) H&E stained histologic sections of spleens isolated 9 weeks after infection. Positive ctrls (mice receiving WT cells), Lv.SFFV.Ifnγr1 and Lv.MSP.Ifnγr1 mice show typical spleen histology with clear separation between white pulp (areas within dotted lines, #) and red pulp (*), while this separation is absent in negative ctrl mice (mice receiving Lv.GFP-transduced *Ifnγr1$^{-/-}$* cells), 100x magnification. (D) BCG CFU counts of spleen homogenates 9 weeks after infection. Mean and SEM are shown for two independent experiments (positive ctrl n=7 (mice receiving WT cells), negative ctrl n=6 (mice receiving Lv.GFP-transduced *Ifnγr1$^{-/-}$* cells and non-transplanted *Ifnγr1$^{-/-}$* mice), Lv.SFFV.Ifnγr1 n=4, Lv.MSP.Ifnγr1 n=5).

Figure 6. Preservation of lung structure after pulmonary BCG infection upon constitutive or myeloid-specific *Ifnγr1* overexpression. (A) Representative macroscopic pictures of lungs isolated from mice 9 weeks after infection (positive ctrl = mouse receiving WT cells, negative ctrl = mouse receiving Lv.GFP-transduced *Ifnγr1*$^{-/-}$ cells). (B) Representative light sheet microscopy pictures of post caval lung lobes depicting the lung structure in green and infiltration of DsRed-labelled BCG in red (positive ctrl = mouse receiving WT cells, negative ctrl = non-transplanted *Ifnγr1*$^{-/-}$ mouse). (C) Quantification of light sheet microscopy depicting the percentage of infected volume of the total lung volume. Mean and SEM are shown for one representative experiment (positive ctrl n=9 (mice receiving WT cells), negative ctrl n=13 (non-transplanted *Ifnγr1*$^{-/-}$ mice), Lv.SFFV.Ifnγr1 n=10, Lv.MSP.Ifnγr1 n=9; one-way ANOVA using Tukey's multiple comparisons post hoc testing; ****p≤0,0001). (D) BCG CFU counts of lung homogenates 9 weeks after infection. Mean and SEM are shown for two independent experiments (positive ctrl n=6 (mice receiving WT cells), negative ctrl n=5 (mice receiving Lv.GFP-transduced *Ifnγr1*$^{-/-}$ cells and non-transplanted *Ifnγr1*$^{-/-}$ mice), Lv.SFFV.Ifnγr1 n=4, Lv.MSP.Ifnγr1 n=5; one-way ANOVA using Dunnett's multiple comparisons post hoc testing; *p≤0,05, **p≤0,01). (E) Representative H&E stained histologic sections of lungs isolated 9 weeks after infection are shown for Lv.MSP.Ifnγr1 mouse (1 and 2) and negative ctrl (mouse receiving Lv.GFP-transduced *Ifnγr1*$^{-/-}$ cells; 3 and 4). 1: Macrophages and multinucleated cells within the alveoli indicated by black arrows (200x). 2: Magnification of black box indicated in 1. 3: Granuloma indicated by * and dotted line with macrophages and neutrophils surrounded by collagen fibers (arrow; 200x). 4: Higher magnification picture of giant cells (indicated by black arrows). (F) Representative GFP-stained histologic lung sections isolated 9 weeks after infection are shown for Lv.MSP.Ifnγr1 mouse (1 and 2) and negative ctrl (mouse receiving Lv.GFP-transduced *Ifnγr1*$^{-/-}$ cells; 3 and 4). 1: GFP-positive cells (brown signals) are distributed equally throughout the lung tissue of the Lv.MSP.Ifnγr1 mouse lung and give rise to macrophages indicated by black arrows (200x). 2: Higher magnification of GFP-positive intraalveolar macrophages (600x). 3: GFP-positive cells are numerous in granulomas of negative ctrl mice indicated by * and dotted line with macrophages and neutrophils (black arrows) surrounded by collagen fibers (dotted arrow; 200x). 4: Higher magnification picture of clustered GFP-positive cells (indicated by black arrows). (G) Ziehl-Neelson staining of histologic lung sections indicating intracellular acid fast bacteria (black arrows, 1 and 2; 600x).