Development of novel efficient SIN vectors with improved safety features for Wiskott Aldrich Syndrome stem cell based gene therapy

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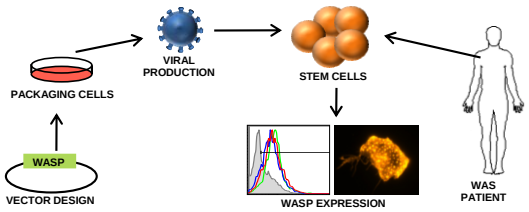

ABSTRACT

Gene therapy has proven as a promising therapeutic approach to treat primary immunodeficiencies. Indeed, the clinical trial for the Wiskott-Aldrich Syndrome (WAS) that is currently ongoing at the Hannover Medical School (Germany) has recently reported the correction of all affected cell lineages of the hematopoietic system in the first treated patients. However, an extensive study of the clonal inventory of those patients reveals that *LMO2*, *CCND2* and *MDS1/EVI1* were targeted preferentially. Moreover, a first leukemia case was observed, thus reinforcing the need of developing safer vectors for gene transfer into HSC.

Here we present a novel self-inactivating (SIN) vector for the gene therapy of WAS that combines improved safety features. We used the elongation factor 1 alpha (EFS) promoter, which has been extensively evaluated in terms of safety profile, to drive a codon-optimized human *WASP* cDNA. To test vector performance in a clinically relevant setting, we transduced murine HSPC as well as human CD34+ cells and also tested vector performance in their differentiated myeloid progeny. Our results show that our novel vector is as effective as the clinically used LTR-driven vector. Therefore, the described SIN vectors appear to be good candidates for potential use in a safer new gene therapy protocol for WAS, with decreased risk of insertional mutagenesis.

INTRODUCTION

Wiskott-Aldrich Syndrome (WAS) is an X-linked primary immunodeficiency disorder characterized by thrombocytopenia, recurrent infections, eczema and an increased incidence of autoimmune manifestations[1]. WAS is caused by mutations in the *WAS protein* (*WAS*) gene which encodes for a key regulator of actin polymerization expressed exclusively in cells of hematopoietic origin[2]. Defective WASP expression causes complex immunologic abnormalities affecting T cells, B cells, natural killer cells, dendritic cells, macrophages and granulocytes, respectively[3]. Patients with classical WAS usually die within the first decades of their life[4], unless treated by allogenic hematopoietic stem cell transplantation (HSCT)[5]. However, HSCT can be associated with severe morbidity and mortality secondary to infections, Graft-versus-Host-Disesase (GvHD), non-engraftment and post-transplant lymphoproliferative syndrome[6], especially in patients transplanted with HSC from HLA-mismatched donors and in patients transplanted at an age older than five years[7].

In disorders caused by mutations of single genes, gene therapy is an attractive concept to specifically revert the molecular defect underlying the disease. This holds true in particular for hematopoietic diseases where target stem cells are accessible with relative ease for *in vitro* manipulation and subsequent reinfusion[8]. The first human disease treated successfully by gene therapy was X-linked severe combined immunodeficiency (X-SCID)[5], soon followed by reports of successful treatment of ADA-SCID[9] and chronic granulomatous disease (CGD)[10], and more recently, the Wiskott-Aldrich syndrome[11]. In spite of the documented clinical benefit, the current technology is far from being perfect. Five out of 20 patients treated in two X-SCID clinical trials with transduced HSC developed malignant clonal disorders presenting clinically as leukemia/lymphoma[12, 13], and two adult patients with CGD developed a pre-myelodysplastic syndrome characterized by monosomy 7[14]. Also a first case of leukemia, possibly triggered by insertional upregulation of a neighboring *LMO2* allele, was observed in one of series of WAS patients we have treated so far with a gammaretroviral

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LTR-driven vector[15, 16]. This has underlined the need to advance the development of improved vector systems.

U3-deleted, so-called "self-inactivating" (SIN) vectors, confer a reduced risk for insertional mutagenesis and secondary cancer[17-19] because the promoter/enhancer regions have been deleted from the LTR. Both lentivirus- and retrovirus-based vectors have been tested in preclinical studies[20, 21]. Lentiviruses (LV), with their tendency to insert the genetic cargo into transcribed genes, have been shown to be less genotoxic than retroviruses (RV), which show a preference to integrate close to transcriptional start sites and regulatory gene regions[22, 23]. However, the latest advances in the field indicate that altering the enhancer-promoter elements of the vector have a greater effect on safety than the retroviral insertion pattern[24] and that the insertional gene activation is determined by the characteristics of the transcriptional regulatory elements carried by the vector, thus it is in part independent of the vector type[25]. The selection of the internal promoter is another important point to consider, given that cellular promoters show a reduced risk of activating neighboring proto-oncogenes in comparison to internal promoters of viral origin[18]. The potency and increased safety of the described gammaretroviral and lentiviral SIN vector backbones have already been described in previous studies[18]. We speculate that, in addition to the vector backbone and the choice of the internal promoter, the transgene itself may be of critical importance when it comes to questions of risk of leukemogenesis or myelodysplastic syndrome.

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Comentario [s3]: UM: Da gehst Du nachher gar nicht mehr so drauf ein.

We here present a gammaretroviral (and lentiviral) SIN-vector with re-designed backbone architecture. We included an improved version of a woodchuck posttranscriptional regulatory element (wPRE)[26]. Moreover, the sequence of the *WAS* transgene has been optimized, resulting in more stable RNA, which allows protein expression levels above the therapeutic threshold under the control of the human elongation factor 1-α (EFS) short promoter, a cellular promoter with a more physiologic activity in hematopoietic stem/progenitor cells (HSPC)[18]. Importantly, this study also provides evidence for the efficacy of this novel vector in WASP deficient CD34+ cells, the target cell population for gene therapy. Therefore, this

vector appears to be a good candidate vector to pursue the development of a safer second generation gene therapy protocol for WAS, with greatly decreased risk of insertional mutagenesis.

MATERIAL AND METHODS

Viral vectors and vector production

An advanced generation, self-inactivating (SIN) γ-retroviral vector[26,27], SRS11.EFS.WASP.iresGFP.pre (R.EFS.W.iG), was constructed to express a full-length human *WASP* cDNA under the control of the human elongation factor 1-a (EFS) short promoter (Fig. 1). WASP was cloned into XbaI and SalI unique restriction sites and iresGFP (iG) was cloned downstream into a unique BamHI restriction site (Fig. 1). To generate the lentiviral counterpart, the same expression cassette was cloned through a NotI (blunted by Klenow polymerase) / SpeI restriction into the XhoI (blunted by Klenow polymerase) / SpeI sites of pRRL.PPT.SF.GFP.pre*, a standard 3rd generation lentiviral vector[28,29]. The resulting vector was named pRRL.PPT.EFS.WASP.iresGFP.pre (L.EFS.W.iG) (Fig.1). The EFS internal promoter was substituted by the spleen focus-forming virus (SFFV) U3 promoter/enhancer to generate SRS11.SFFV.WASP.iresGFP.pre (R.SF.W.iG) and pRRL.PPT.SFFV.WASP.iresGFP.pre (L.SF.W.iG) (Fig. 1). The substitution of WASP cDNA by a codon-optimized WASP (coWASP) sequence (Epoch Biolabs Inc., Texas, USA) was carried out in all vectors through AgeI/XbaI and SalI unique restriction sites, thus generating SRS11.EFS.coWASP.iresGFP.pre (R.EFS.Wco.iG), SRS11.SFFV.coWASP.iresGFP.pre (R.SF.Wco.iG), pRRL.PPT.EFS.coWASP.iresGFP.pre (L.EFS.Wco.iG) and pRRL.PPT.SFFV.coWASP.iresGFP.pre (L.SF.Wco.iG), respectively (Fig. 1). All SIN vectors contain the safety improved woodchuck hepatitis virus post-transcriptional regulatory element (PRE*)[30] downstream of iG. The cloning of the CMMP.WASP.iresGFP (CMMP.W.iG) vector containing an intact myeloproliferative sarcoma virus (MPSV) LTR which been described previously[24, 31].

Retroviral cell-free vector supernatants were generated by transient co-transfection of 293T packaging cells with each transfer vector, together with packaging constructs coding for the gag-pol proteins (for lentiviral: pcDNA3.GP.4xCTE; for gammaretroviral: pcDNA3.MLVgp) and the ecotropic, GALV or VSV-G envelope, as previously described[25]. To produce lentiviral vectors, a RSV-Rev containing plasmid was aditionally co-transfected. Virus titrations were determined by flow cytometry on HT1080 and SC1 fibroblasts, respectively, and stored at -80ºC until use.

Patients

All experiments were performed upon informed consent/assent of patients or legal representatives. The study protocol was approved by the institutional review board (IRB) at Hannover Medical School.

Cell lines and primary cultures

Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (B-LCLs) were derived from peripheral blood mononuclear cells of WAS patients after infection with B95-8 cell supernatant in the presence of cyclosporine A. Cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin/streptomycin and 2mM glutamine.

Bone marrow (BM) lineage-negative (Lin-) cells of untreated 129 Wasp-/- mice were isolated from complete BM by magnetic sorting using the Lineage Cell Depletion Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) following the instructions given by the manufacturer. Briefly, total BM cells were magnetically labelled with a cocktail of biotinylated antibodies against a panel of lineage antigens (CD3, B220, CD11b, Gr-1 and Ter-119) and anti-biotin MicroBeads. Lin- cells were automatically separated using an AutoMACS device (Miltenyi Biotech), aliquoted and cryopreserved until used. Cells were grown in StemSpan HS2000

medium (CellSystems, St Katharinen, Germany) containing 100 U/mL penicillin/streptomycin, 2 mM glutamine, 50 ng/mL murine stem cell factor (SCF), 100 ng/mL hFlt-3 ligand, 100 ng/mL hIL-11 and 10 ng/mL mIL-3 (PeproTech, London, United Kingdom) at a density of 1-5x10^5 cells/mL, unless otherwise specified.

CD34+ cells were purified from human BM mononuclear cells of healthy individual donors and patients using immunomagnetic beads as described by the manufacturer (Miltenyi Biotec). Separation was performed using an AutoMACS device (Miltenyi Biotec) following the manufacturer´s instructions. CD34+ cells were cultured in StemSpan serum-free medium (StemCell Technologies, Inc.) supplemented with 100 U/mL penicillin/streptomycin, 2 mM glutamine, 100 ng/mL human SCF, 100 ng/mL human Flt-3 ligand, and 20 ng/mL human thrombopoietin (TPO) (PreproTech, London, UK).

Viral transduction

Transduction of B-LCL and Lin- cells was performed on RetroNectin-coated (10g/cm^2; TaKaRa, Otsu, Japan) suspension culture dishes preloaded with viral supernatant. Viral preloading was carried out by centrifugation of supernatant at 800g during 30 minutes at 4ºC. B-LCL were transduced with GALV-pseudotyped viruses at MOI=1.

Lin- and CD34+ cells were expanded for two days prior to transduction. On day three, cells were transduced with ecotropic or GALV-pseudotyped viruses, respectively, by incubation on viral preloaded suspension culture dishes. Virus preloading was carried out on RetroNectin-coated (10 g/cm^2; TaKaRa) dishes, as indicated above. Transduced cells were incubated for expansion 72h before proceeding to further experiments.

Detection of WASp by intracellular immunofluorescence staining

Cells were treated with the Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA), as described by the manufacturer. As primary antibodies, anti-human WASP monoclonal IgG2a

(Clone B-9, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse IgG2a isotype (Becton Dickinson GmbH, Heidelberg, Germany) were used. Goat F(ab0)2 fragment rat IgG-phycoerythrin (Immunotech) was used as a secondary antibody. Subsequently samples were analyzed by fluorescent activated cell sorting (FACS).

Western blot

Western blot analysis of WASP expression was performed as previously described[24]. The membrane was exposed to anti-WASP monoclonal (clone D-1) primary antibody (Santa Cruz, Biotechnology) diluted 1/400, and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Becton Dickinson GmbH) diluted 1/10000 was used as secondary antibody. Membranes were re-probed with GAPDH monoclonal antibody 6C5 (Santa Cruz Biotechnology) diluted 1/10000 or Erk polyclonal rabbit anti-Erk 2 (Santa Cruz Biotechnology) diluted 1/2000, followed by HRP-conjugated goat anti-mouse antibody.

Northern blot

Northern blots were performed according to standard procedures previously described[25]. As specific probes, ~ 700bp fragments of WASP, GFP and PRE cDNAs were used to detect RNA transcripts and 18S served as a housekeeping control.

Flow cytometry and cell sorting

Expression of GFP was detected by fluorescence-activated cell sorting (FACS) analysis. Data were collected with a FACSCalibur (BD-Pharmingen, SD, CA) equipped with the standard fluorescein filter set and the fluorescence distribution was analyzed using CellQuest™ software (BD Biosciences, San Jose, CA) and FlowJo® (Treestar, Ashland, OR, USA). For cell sorting, cells were harvested, washed with PBS and resuspended in PBS

supplemented with 10% FCS before being sorted in a FACSAria cell sorter (Cytomation Inc., Fort Collins, CO, USA).

In vitro differentiation of CD34+ cells and magnetic separation of CD14+ cells

Differentiation of CD34+ cells into myeloid progeny was performed as previously described[24]. In brief, eight thousand transduced CD34+ cells were seeded on a monolayer of mitomycin C-treated (10mg/mL) MS5 feeder cells [32] and incubated for two weeks in IMDM supplemented with 10% human AB serum (Sigma), 5% FCS, 100 U/mL penicillin/streptomycin, 2mM glutamine, 50 ng/mL hSCF, 20 ng/mL hTPO, 50 ng/mL hFlt3-ligand, 10 ng/mL granulocyte colony-stimulating factor (G-CSF) and 10 ng/mL granulocyte/monocyte colony-stimulating factor (GM-CSF) (CellSystems Biotechnology). To purify CD14+ cells, we used anti-CD14 magnetic beads and separated in an AutoMACS device (Miltenyi Biotech) following the instructions provided by the manufacturer.

Podosome staining and detection

Podosomes were detected as described [24]. In brief, $5x10^4$ cells were plated on $25mm^2$ coverslips in six-well plates previously coated with 10 mg/mL fibronectin (Roche Diagnostics GmbH) and incubated for 2 hours at 37ºC. Cells were fixed for 20 minutes in 4% para-formaldehyde, permeabilized for 5 minutes in 0.2% Triton-X-100 in PBS and blocked with 1% BSA for 30 minutes. Podosomes were stained with anti-vinculin antibody (Sigma, Pool, Dorset, UK; dilution 1/50), Cy5-conjugated rabbit anti-mouse IgG F(ab)2 fragment (Jackson ImmunoResearch Europe Ltd., Soham, UK; dilution 1/100) and phalloidin-TRITC (Sigma) at 0.1 mg/mL to detect F-actin. Nuclei were labelled with DAPI contained in the mounting solution. Preparations were transferred to slides and examined with a fluorescence microscope Axiovert 200 equipped with an Axioplan 2 imaging system (Carl Zeiss, Göttingen, Germany). Images were captured with a PLAN-APOCHROMAT 63X/1,4 oil

objective lens at room temperature and acquired with the Openlab 3.1.7 imaging software (Improvision Ltd., Coventry, UK). Percentage of cells displaying podosomes was counted by enumerating at least 200 cells per sample.

RESULTS

Development of self-inactivating vectors to substitute for the clinically used LTR-driven gammaretroviral vector

We designed a series of novel gammaretro- and lentiviral vectors with self-inactivating (SIN) configuration for the expression of WASP with a decreased risk of genotoxicity (Fig. 1). In those vectors, the expression of the therapeutic transgene (either wild-type or codon optimized for *Homo sapiens*) and the marker gene (GFP) is driven by the EFS (EF1a short) and the SFFV (spleen focus forming virus U3) internal promoters, respectively. All vectors were developed with a split packaging design to minimize the risk of RCR formation and to gain maximum space for the insertion of transgene cassettes. Titers of both SIN-gammaretro- and SIN-lentiviral vector supernatants did not significantly differ from those obtained with the gammaretrovirus with intact LTR that is currently being used in the Hannover clinical trial[11]. Importantly, the replacement of the wild-type *WASP* cDNA with the novel codon optimized sequence led to equally high titers as the vectors carrying the wild-type cDNA (in the range of 5x10e7 – 1.5x10e8 t.u.) (Fig. 2).

A SIN-gammaretroviral vector with a weak cellular promoter and a codon-optimized WASP cDNA with improved WASP expression

Next, we evaluated the feasibility of the novel codon-optimized WASP (Wco) sequence to improve the RNA processing and transgene expression. A comparative Northern blot analysis of HT1080 cells transduced with SIN-retroviral vectors (Fig. 3a) revealed that Wco drastically improved RNA processing, thus allowing to use a weaker cellular promoter (EFS) to reach the same protein levels. The combination of the more physiological cellular promoter, EFS, and the new transgene, Wco, yielded nearly the same RNA levels as the strong promoter of viral origin, SFFV, and the wild-type cDNA (wt) (Fig. 3c). The same effect was observed in the context of SIN-lentiviral vectors (Fig. 3b and 3d), even in the absence of

marker gene, in samples with very low number of viral integrations (≤0.9 copies per genome), as it is desired in a clinical scenario. Noteworthy, the SIN design of our retroviral vectors prevented the transcription of full-length retroviral RNA harboring the packaging signal ψ (see Fig. 3a, full length RNA would be expected between 5000-6000 bp), which improves the safety of these vectors.

To further characterize transgene expression, an immunoblot was performed to specifically detect the expression of WASP upon transduction with our new vectors, where we compared samples with similarly low number of viral integrations (Fig. 3e). The substitution of the wt by the Wco cDNA resulted in higher protein expression, a finding consistent with the phenomenon observed at the RNA level. As before, it was the combination of EFS and Wco, the one that yielded the same levels of protein expression than the LTR-driven retroviral vector used in the Hannover clinical trial.

Efficient transgene expression of murine Wasp-/- hematopoietic precursor cells.

To comparatively assess the efficiency of our vector, R.EFS.Wco.iG, directly in the hematopoietic target cells for gene therapy in the murine model, *Wasp*-/- Lin- cells were transduced with SIN-gammaretro- and SIN-lentiviral vectors containing Wco with multiplicities of infection (MOI) of 1, 3, and 5, respectively. Four days after transduction, samples were analyzed by flow cytometry. As expected, we were able to effectively transduce WASP-/- Lin- cells even at low MOIs with all tested vectors. Increasing the vector dose resulted in higher transduction rates in all cases (Fig. 4a). Transgene expression driven by cellular promoter was lower than the expression from by the promoter of viral origin (Fig. 4b; Y-axis: GFP mean fluorescence intensity) and transduction rates achieved with both promoters were very similar. SIN-gammaretroviral vectors were slightly more efficient than their lentiviral counterparts (Fig. 4a and c). This might be attributable to the differences in integration site preferences. These results show that our novel vectors, R.EFS.Wco.iG and L.EFS.Wco.iG, can efficiently transduce murine hematopoietic precursor cells. The EFS-

driven vectors were only 2(-3) fold weaker in terms of fluorescent intensities compared to their counterpart with the strong internal promoter SFFV.

An EFS-driven SIN vector corrects the WAS phenotype in patient-derived human hematopoietic cells

After demonstrating that our newly generated vector, R.EFS.Wco.iG, allows for higher RNA levels and improved transgene expression, we wanted to further evaluate whether it also provides, in a clinically more relevant scenario, the same efficiency and functionality as the earlier vector used in the clinical trial in Hannover. To this aim, we tested whether this vector could reconstitute the WAS phenotype in CD34+ derived $WASP^{-/-}$ monocytes. WASP-deficient macrophages and dendritic cells are characterized by the abscense of adhesion structures called podosomes[26], a hallmark of the disease. To assess the functionality of the protein expressed by Wco, we investigated whether retroviral gene transfer into WASP-deficient CD34+ cells could reconstitute the formation of podosomes in myeloid cells differentiated *in vitro*. CD34+ cells from two independent patients, WAS1 and WAS2, were transduced either with CMMP.W.iG or R.EFS.Wco.iG at an MOI=5 and three days later, they were differentiated into myeloid progeny. Subsequently, CD14+ cells were magnetically separated and stained for vinculin and F-actin intracellularly. The specific co-localization of vinculin and F-actin corresponds to the organized structure of podosomes.

Comentario [s4]: UM: How? How much after transduction?

The transduction of CD34+ cells from WAS1 with R.EFS.coWiG and CMMP resulted in 16.5% and 12.3% GFP+ cells, respectively. After subsequent myeloid differentiation, the percentage of CD14+ GFP+ cells was 19.5% with R.EFS.coWiG and 27.2% with CMMP (Fig. 5a). Podosomes were detected in 23% and 28% of CD14+ cells transduced with our novel vector and with CMMP, respectively (Fig. 5b and c).

The transduction of CD34+ cells from WAS2 with R.EFS.coWiG and CMMP resulted in 15.2% and 7.5% GFP+ cells, respectively. After subsequent myeloid differentiation, the

percentage of CD14+ GFP+ cells was 13.3% with R.EFS.coWiG and 15.6% with CMMP (Fig. 5a). Podosomes were detected in 30% and 37% of CD14+ cells transduced with our novel vector and with CMMP, respectively (Fig. 5b and c).

Comentario [KB5]: Do we know the percentage of WASP-positive in vitro diff monocytes here as well? This may be one obvious question from the reviewers...

As expected, *in vitro* differentiated WASP-deficient CD14+ cells completely failed to assemble vinculin and F-actin to form podosomes in both patients, whereas two independent healthy individual donors displayed 63% and 77% CD14+ cells with podosomes, respectively (Fig. 5b and c).

These findings are consistent with previous results obtained in immortalized B cells from two other independent WAS patients, named WAS3 and WAS4 (Supplementary Fig. 1), where the reconstitution of WASP expression upon transduction with R.EFS.W.iG was very similar to that obtained with the CMMP vector.

Taken together, these data show that the SIN-gammaretroviral vector, containing a novel codon optimized version of *WASP* cDNA driven by an internal promoter of cellular origin, can transduce the target cell population for gene therapy as efficiently as the clinically used vector. Importantly, the protein expressed by Wco is functional and allows for efficient reconstitution of the aberrant cytoskeleton of the myeloid progeny of WASP deficient CD34+ cells.

DISCUSSION

The clinical trial for WAS that is currently ongoing at the Hannover Medical School (Germany) has recently reported the successful correction of all blood cell lineages in the first two treated patients, including for the first time correction of autoimmunity and platelet numbers after transplantation of retroviral vector-transduced HSC in patients[11]. Noteworthy, the fractions of corrected lymphocytes increased over time, and both patients have markedly improved their clinical condition with respect to the susceptibility to infection, autoimmunity, and bleeding. These findings could be confirmed in additional patients treated with the conventional LTR-driven gammaretroviral vector CMMP in Hannover (priviledged communication, C. Klein). This clinical trial provides first proof-of-principle that gene therapy for WAS is feasible with a follow up of up to 4.5 years after gene therapy, indicating that early hematopoietic progenitor/stem cells contributing quantitatively to all cell lineages of blood were successfully corrected[11]. However, an extensive study of the clonal inventory of the first treated patients revealed that *LMO2*, *CCND2*, and *MDS1/EVI1*, were – among others – preferentially represented[11]. The activation of these proto-oncogenes can potentially cause severe side effects as seen in previous similar clinical trials[12,14], therefore. Although no persistent clonal imbalance has been observed so far in the first treated two WAS patients, the risk of insertional mutagenesis remains a drawback as evidenced by a recent leukemia case observed in one patient treated in this series [33, 34] (C. Klein, unpublished). Taken together, the WAS and SCID trials clearly show efficacy but also demonstrate the well-recognized insertional gene activation potential of first generation LTR-driven gammaretroviral vectors[33].

In this study, we present a novel vector for the gene therapy of WAS that combines improved safety features and similar efficacy as the clinically used vector. The risk of activating cellular proto-oncogenes has been significantly reduced by using a SIN-design[15,17] and the EFS promoter, which is much less likely to activate neighboring genes compared to strong viral promoters[16,35]. Moreover, among all cellular promoters that we comparatively tested (EFS,

PGK, and WASP; data not shown), EFS yielded the highest transduction rates and MFI values and was hence selected as the most efficient cellular promoter.

In an international consortial initiative, laboratories in Milano, London and Paris successfully used a lentiviral SIN vector harboring the natural 1.6kb WASP promoter driving the wild-type WASP cDNA and could correct the phenotype in a *Wasp* KO mouse model[36-38]. This is a promising concept since the WASP promoter, albeit weaker than the EF1a promoter, is primarily active in hematopoetic cells. Recently, a clinical trial with this WASP-promotor driven vector construct was initiated.

Comentario [CK6]: Würde ich keinesfalls so schreiben, denn bei WASp ist die gewebespezifische Expression ja völlig unbedeutend (im Ggs zu SCID etc....). Können wir das nicht komplett streichen?

Here, we chose a gammaretroviral vector as the current clinical trial in Hannover has established gammretroviral transduction conditions that are of sufficient potency for long-term disease correction. Furthermore, when the vector cassette does not contain strong enhancer elements, the gammaretroviral integration pattern could be advantageous as it may be less likely than the lentiviral to cause disruptions of cellular genes. Since *ex vivo* gene therapy targets hematopoietic cells, we considered that a hematopoiesis-specific promoter is not needed. We thus used the extensively safety-evaluated EF1a promoter [16, 39] driving a codon-optimized *WASP* cDNA. The wild-type *WASP* cDNA sequence was altered to optimal human codon usage for translation into protein, destabilizing *cis*-acting sequences, RNA secondary structures and possible splice sites were removed (see also Supplementary Fig. 2 providing a sequence alignment), and two STOP codons were added to ensure efficient termination. These modifications resulted in higher gene expression with all vectors in all cell types tested, probably reflecting the improved processivity of *coWASP*. Importantly, in the *WASP* coding sequence (encoding the GRSGPLPPXPP motifs and Poly-Proline motifs; compare Supplementary Figs. 2 and 3), a number of repetitive sequences were observed, which we tried to wobble to prevent hot spots for jumps of reverse transcriptase. Using a codon-optimization strategy, we can achieve good expression levels on RNA and protein level (see Fig. 3) despite using a moderately expressing internal promoter (such as the cellular promoter EF1a short, EFS).. To assess vector performance in a more clinically

relevant setting, we transduced murine HSPC, the target cell population for bone marrow transplantation in preclinical models, and also human CD34+ hematopoietic cells. When testing the gammaretroviral SIN vector R.EFS.Wco.iG in CD34+ cells from WAS patients, transduction rates obtained did not differ significantly from those with the clinically used gammaretroviral LTR-driven CMMP vector. The myeloid progeny of CD34+ cells treated with R.EFS.Wco.iG displayed a similar morphology and phenotypical correction compared to those treated with the CMMP vector and were indistinguishable from the healthy donor. These results demonstrate, in primary patient cells, that our novel vector can correct the aberrant cytoskeleton (Fig. 5), providing sufficient *WASP* expression to rescue the formation of podosomes as efficiently as the vector currently used in the clinical trial, despite the low MOI. The present data validate the ability to restore WASP expression in patients´ CD34+ cells while preserving their *in vitro* differentiation potential, two important requisites for clinical use.

This study also shows that this new safety-modified vector can be produced at high titers in human 293T cells, within the range necessary for clinical applications, as determined for several pseudotypes (VSVg, GALV, and ecotropic). Of note, our gammaretroviral vector backbone is devoid of viral gene remnants and devoid of any overlap between vector and helper plasmids[40], reducing the likelihood of formation of replication competent retroviruses and potentially immunogenic expression of viral proteins from transduced cells.

For these reasons, the new SIN vectors (e.g., R.EFS.Wco or its lentiviral counterpart) with the cellular EFS promoter and codon-optimized WAS appear to be reasonable candidates for potential use in a safer second generation gene therapy protocol for WAS, with decreased risk of insertional mutagenesis.

Comentario [KB7]: You may wish to refer to A Galy's paper here, showing that you require at least 70% expression levels of WASP for efficient reconstitution of podosome formation – thus implying that the levels of expression are fairly high and thus „physiological"

Comentario [s8]: UM: erwähnen, dass WAS Expression an sich nicht toxisch ist, also eine hohe Proteindosis wünschenswert ist.

ACKNOWLEDGEMENT AND DISCLOSURE STATEMENT

This work was supported by grants from German Ministry for Research and Education (iGene, PidNet, IFB-Tx (01EO0802), the German Academic Exchange Service (DAAD (0315187)), the Deutsche Forschungsgemeinschaft (SPP1230 and Cluster of Excellence REBIRTH (EXC 62/1) and the European Union (Integrated Project PERSIST). We would like to thank Jana Diestelhorst, Girmay Asgedom, Sabine Knoess and Diana Szepe for technical assistance. The authors disclose any financial interest in association with the submitted material.

SUPPLEMENTARY INFORMATION (SI)

Supplementary Fig.1. Reconstitution of WASP expression in WASP-deficient B cells.

Supplementary Fig. 2. Alignment of wildtype WAS vs. codon-optimized WAS cDNA.

Supplementary Fig. 3. Alignment of protein coding sequence of WAS vs. codon-optimized WAS proteins.

This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE LEGENDS

Fig. 1. Design of viral vectors used in this study.

(a) The self-inactivating (SIN) gammaretroviral vectors contain long terminal repeat sequences (LTRs) on both ends, whereas the promoter is deleted after reverse transcription. The elongation factor 1-a (E) or the spleen focus forming virus (SF) are used as internal promoters to drive the WAS cDNA (WASP) either wild-type (W) or codon optimized (Wco). Optionally, this is followed by an internal ribosome entry site (i), and the green fluorescent protein (G) as a marker. Furthermore, the vectors include the safety-improved woodchuck hepatitis virus post-transcriptional regulatory element (PRE*), a splice donor (SD), the primer binding site (Θ), and a packaging signal (Ψ). **(b)** The SIN-lentiviral counterparts include the rev responsive element (RRE), and the central polypurine track (PPT) in addition to the above described elements. **(c)** The vector used in the Hannover clinical trial exhibits an intact myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR), an extended packaging signal (Ψ+) and a splice acceptor (SA), other elements see description above.

Fig. 2. Titer analysis of viral supernatants used in this study.

Titers are expressed in transduction units per milliliter (t.u./mL) on the Y-axis. Bars indicate the average values obtained in three independent batches (n=3). Error bars correspond to the standard deviation. The gammaretroviral vector is abbreviated with R, the lentiviral counterpart with L.

Fig. 3. Characterization of our newly generated vectors in terms of mRNA processing and protein expression.

(a) Northern-blot analysis of cell lysates from HT1080ecat cells transduced with ecotropic bicistronic retroviral vectors. Blots were probed for detection of GFP (upper) and 18S RNA (lower), respectively. **(b)** Northern-blot analysis of HT1080ecat cells transduced with monocistronic ecotropic SIN-gammaretroviral and SIN-lentiviral vectors containing Wco as transgene. The blots were probed for PRE and 18S. Samples with similar number of viral integrations (meassured by Real-Time PCR) were selected for comparison purposes. **(c)** Quantification of relative GFP RNA levels of samples shown in Fig. 3a. **(d)** Quantification of relative viral RNA levels of samples shown in Fig. 3b. **(e)** Western-blot for detection of WASP expression in HT1080ecat cells transduced with ecotropic gammaretroviral vectors, either with or without marker gene. CMMP is the LTR-driven vector backbone used in the Hannover

WAS trial. Detection of Erk protein was used as loading control. The number of viral integrations per genome was calculated by Real-Time PCR (see copy number indicated above) and samples with similar copy number were selected for comparison purposes.

Fig. 4. Transgene expression in murine Wasp-/- hematopoietic precursor cells.

(a) Percentage of GFP+ cells (Y-axis) determined three days after transduction. **(b)** Analysis of the Mean Fluorescence Intensity (MFI) of the GFP expressing cells (Y-axis). The following MFI values were obtained: 296.30 (left plot), 109.57 (mid-left plot), 250.88 (mid-right plot), and 96.50 (right plot). **(c)** Contour plots corresponding to transductions with vectors containing Wco, where similar transduction rates were obtained (between 18 and 25%). Noteworthy, the MOI used to obtain these results was five times higher with lentiviruses than with retroviruses.

Fig. 5. Functional restoration of podosome formation after gene transfer.

CD34+ cells from patients WAS1 and WAS2 were transduced either with CMMP.W.iG or R.EFS.Wco.iG at an MOI=5 and differentiated into myeloid cells. Podosomes correspond to a specific co-localization of vinculin (red) and actin (green). Cell nuclei are recognized by DAPI stain (blue). Mock transduced cells express vinculin and actin but fail to co-assemble them into organized structures. **(a)** FACS analysis for detection of GFP in transduced CD34+ cells (upper panel) and differentiated CD14+ cells (bottom panel). Numbers in the upper right quadrant indicate the percentage of GFP positive cells. **(b)** Percentage of CD14+ cells with podosomes in an untransduced healthy donor (HD) and in transduced and untransduced WAS1 and WAS2 patients. **(c)** Representative staininings of: untransduced healthy donor cells (top panel), reconstituted cells from WAS1 (middle panel) and transduced cells from WAS2 (bottom panel), both compared to untreated patient cells.

Supplementary Fig. 1. Reconstitution of WASP expression in WASP-deficient B cells.

(a) Immunoblot (Western blot) analysis of immortalized B cells from a healthy donor and two independent WAS patients (WAS3 and WAS4) prior to transduction. Total protein extracts were analyzed for WASP and glyceraldehyde 3- phophate dehydrogenase (GAPDH) content. **(b)** FACS analysis of immortalized B cells 72h after transduction with CMMP.W.iG and R.EFS.W.iG gammaretroviral vectors. Grey and black bars indicate the percentage of GFP positive cells achieved with each vector in patients WAS3 and WAS4, respectively. **(c)**

Restoration of expression of WASP expression in sorted GFP positive cells. Healthy donor and WAS patients sorted GFP positive cells from transduced healthy donor or WAS patient cells were intracellularly stained and analyzed by FACS to specifically detect the expression of WASP. X-axes indicate the events and numbers corresponding to the mean fluorescence intensity (MFI) of WASP.

Supplementary Fig. 2. Alignment of wildtype WAS vs. codon-optimized WAS cDNA.

The original human WAS sequence (WASP) is compared to the codon-optimized WAS cDNA sequence (WASPco), which show 78% similarity (1186/1509 bp matched, depicted in red). The codon-optimized sequence was adapted to favored human codons, thereby increasing mRNA stability and translatability. In parallel, also potential splice sites were removed in the codon optimized form (e.g. potential splice acceptors ccctcctccAGgaccacgag and cctgcatccAGgtggagacc). In addition, sequences in the protein coding GRSGPLPPXP motifs (corresponding to amino acids 337-346 and 376-385) and poly-proline motifs (corresponding to amino acids 160-165, 312-319, 351-356, 359-362, 367-373,, 391-404, 410-417, 485-502) have been wobbled on the DNA level (see protein coding sequence in Supplementary Fig. 3).

Supplementary Fig. 3. Alignment of protein coding sequence of WAS vs. codon-optimized WAS proteins.

WASP and WASPco (codon-optimized) are shown, which show 100% identity. The GRSGPLPPXP and poly-proline (Poly-Pro) repetitive motifs are indicated.