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Safety Modality for X-linked Severe Combined Immunodeficiency Gene Therapy

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Abstract

X-linked severe combined immunodeficiency (SCID-X1), caused by a defect of the cytokine receptor common gamma chain (γc), has been successfully treated by gene therapy in the clinic. However, the occurrence of leukemia in several patients preceded by loss of oligoclonality revealed that treatment is associated with a risk inherent to the genetic modification of hematopoietic stem cells. In this study, we developed a safety approach that allows the specific elimination of gene-modified cells. For this, a small peptide sequence (myc-tag) was introduced into the murine γc protein. Cells expressing the modified chain can be detected with a myc-specific antibody by flow cytometry and are effectively depleted *in vitro* in the presence of complement factors. Further, thymic-derived T cells from mice reconstituted with myc-tagged γc-transduced bone marrow stem cells can be depleted by antibody administration *in vivo*. Similarly, specific complement-mediated lysis was observed for human T cells expressing the human myc-tagged γc. In a cell proliferation assay, the modified cytokine receptor chain showed no functional impairment compared to the wild-type chain. In sum, we show proof-of-principle of a safety mechanism for SCID-X1 gene therapy that would allow elimination of gene-corrected cells in a patient upon observation of monoclonal outgrowth.

Keywords: Common gamma chain; IL2RG; myc-tag; SCID-X1; Safety; Suicide gene

Abbreviations: SCID-X1: X-linked Severe Combined Immunodeficiency; γc: Cytokine Receptor Common Gamma Chain; HSC: Hematopoietic Stem Cells; ab: Antibody; wt: Wild-type; NK cell: Natural Killer Cell; HLA: Human Leukocyte Antigen; SIN: Self-inactivating; HSV-TK: Thymidine kinase of Herpes Simplex Virus; ER: Endoplasmatic Reticulum; MFI: Mean Fluorescence Intensity; CDC: Complement-dependent Cytotoxicity; PI: Propidium Iodide; TCR: T cell Receptor

Introduction

SCID-X1 is a profound immune disorder accounting for a high susceptibility to severe, recurrent infections and is caused by mutations in the γc gene [1]. Absence of γc-mediated cell signaling leads to defects in lymphocyte development and function. As a result, SCID-X1 patients have dysfunctional B cells and lack T cells and Natural Killer (NK) cells.

Transplantation of hematopoietic stem cells (HSC) from human leukocyte antigen- (HLA-) identical donors is an established therapy with high success rates; however, matched donors are only available for about a third of patients. In contrast, HLA-haploidentical transplantation bears a much higher risk of graft-versus-host disease, graft rejection and other complications [2-4].

As an alternative, gene therapy has been suggested for patients for whom HLA-identical donors cannot be found. In this approach, autologous bone marrow-derived HSC are genetically modified by a retroviral vector encoding for the intact γc gene and transplanted into the patient. The feasibility and efficacy of SCID-X1 gene therapy has been demonstrated in pioneer clinical trials [5,6]. However, therapy-related leukemia due to insertional mutagenesis occurred in 5 out of 20 treated patients [7,8] revealing the need to improve the safety of this otherwise successful strategy. The observed oncogenic transformation is thought to be caused by the skewed integration profile of retroviral vectors, which preferentially insert into transcriptionally active sites and enabling vector enhancer elements to act on nearby oncogenes [8-10]. Still, the processes leading to lymphomagenesis are not yet fully understood; and occurrence of lymphoma in one murine model of SCID-X1 has been shown to be independent from insertional mutagenesis [11].

Since clinical trials have been put on hold much effort has been undertaken to design safer vectors for SCID-X1 gene therapy. In self-inactivating (SIN) γ-retroviral vectors the 5' and 3' long terminal repeats are deleted upon integration while transgene expression is driven by an internal promoter. This reduces the number of *cis*- and *trans*-acting regulatory elements that could potentially influence transcription of surrounding genes. Such SIN vectors have been successfully tested in a murine model [12] and a multi-institutional phase I/II trial using a SIN vector has been initiated [13]. Similarly, the use of SIN lentiviral vectors has been studied [14,15]. Lentiviruses display a genomic integration pattern distinct from γ-retroviruses [16,17], which might have an impact on lymphomagenesis. Also, the application of enhancer-less promoters has been described [18,19] and insulator elements that prevent de-regulation of neighboring genes have been incorporated into γc-encoding vectors [14]. A different option to increase safety of

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gene therapy is the use of suicide genes such as thymidine kinase of herpes simplex virus (HSV-TK) which facilitates elimination of gene-modified cells upon administration of the prodrug ganciclovir [20,21]. HSV-TK has also been suggested as a safety strategy for SCID-X1 therapy [22] but faces the limitation of immunogenicity, slow response and exclusion of ganciclovir as antiviral treatment. More importantly, insertion of suicide genes requires the introduction of an additional open reading frame and promoter thus complicating the vector design.

In this study, we propose a novel safety strategy for SCID-X1 gene therapy based on a transgene-intrinsic mechanism. This safety modality allowed the specific elimination of gene-modified cells *in vitro* and *in vivo* by administration of a specific antibody (ab).

Material and Methods

Mice

B6 mice were purchased from Charles River (Sulzfeld, Germany). Rag-1$^{-/-}$ mice (B6.129S7-Rag1^{tm1Mom}) were obtained from The Jackson Laboratory (Bar Harbor, USA). Animal experiments were approved by the responsible institution and performed according to national and regional regulations.

Molecular cloning of wt and myc-tagged γc and retroviral vectors

The murine or human γc genes were amplified by PCR from cDNA clone IRAVp968C0452D (RZPD, Berlin, Germany) or from the plasmid IL2RGPRE (kindly provided by A. Thrasher, London, UK), respectively. The resulting products were cloned into the MP71 retroviral vector [23] (kindly provided by C. Baum, Hannover, Germany) via *Not*I/*Eco*RI or *Not*I/*Bsr*GI restriction sites, respectively. Insertion of one or two myc-tags and the linker peptide was facilitated using site-directed mutagenesis PCR. Primer sequences can be provided on request. Oligonucleotides were obtained from TIB MOLBIOL (Berlin, Germany). All generated vectors were checked for accuracy by sequencing.

Cell culture and transduction

58 cells [24] and primary human PBLs were grown in RPMI1640 medium (Invitrogen, Karlsruhe, Germany) with 10% FCS (PAN Biotech, Aidenbach, Germany), 10 mM HEPES (Sigma, Taufkirchen, Germany) and 100 IU/ml penicillin/streptomycin (Invitrogen). PBLs were additionally provided with 100 IU/ml recombinant human interleukin-2 (hIL-2, Chiron, Marburg, Germany). The same medium but with 30% FCS was used to culture the SCID-X1 B cell line (kindly provided by M. Cavazzana-Calvo, Paris, France). 293T cells (ATCC CRL-11268, American Type Culture Collection, Manassas, USA) and Plat-E cells [25] were cultured in DMEM (Invitrogen) with 10% FCS (Biochrom, Berlin, Germany) and 100 IU/ml penicillin/streptomycin. Murine HSC were expanded in StemPro-34 SFM (Invitrogen) supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin/streptomycin, 5% FCS (PAN Biotech), 50 ng/ml mSCF, 10 ng/ml mIL-3 and 50 ng/ml mIL-6 (all Peprotech, Hamburg, Germany).

Isolation and transduction of human PBLs as well as transduction of 58 cells was performed as described [26]. The B cell line was transduced similarly, but due to low proliferation rate transduction was repeated three times with one-week intervals to increase efficiency. HSC were isolated from the bone marrow of murine tibia and femur using murine Sca1 selection kit (StemCell Technologies, Köln, Germany) according to the manufacturers instructions. Transduction of HSC was performed on day three and four after isolation by seeding 2×10^5 cells suspended in 500 µl medium into a Retronectin- (Takara, St Germain en Laye, France) and virus-coated 24-well of a non-tissue culture plate (BD Bioscience, Heidelberg, Germany). The production of virus supernatant has been described before [26].

Antibodies and flow cytometry

Fluorochrome-labeled ab directed against murine CD3 (clone 145-2C11) and γc (clone 4G3) as well as human γc (clone TUGh4) were purchased from BD Biosciences. The myc-specific monoclonal abs 3A7 (IgG2a) and 9E10 (IgG1) used for depletion were obtained from US Biologicals (Swampscott, USA) or purified from hybridoma supernatant (ATCC CRL-1729), respectively. For FACS staining of myc-positive cells, a rabbit anti-myc ab with a secondary goat anti-rabbit ab (both Santa Cruz Biotechnology, Santa Cruz, USA) was used. Fluorescence intensity was measured using a FACS Calibur flow cytometer and CellQuestPro software (BD). Data analysis was performed with FlowJo software (Tree Star, Ashland, USA).

CDC assay

Exponentially growing 58 cells or Ficoll-Hypaque-purified PBLs were seeded in a 96-well plate (Corning Costar, Amsterdam, Netherlands) with 1×10^5 cells/well in RPMI1640 medium plus 25 mM HEPES and 0.3% BSA. Cells were labeled with 1 µg myc-specific ab/well (clone 3A7) for one hour at 4°C, washed and incubated with rabbit complement (for 58 cells: LOW-TOX-M; for PBLs: Rabbit Complement MA, both Cedarlane, Hornby, Canada) diluted 1:6 or 1:9 for two to three hours at 37°C. For live and dead cell discrimination cells were stained with 1 µg PI (Sigma) for ten minutes and analyzed by flow cytometry. Cells incubated with medium or complement alone served as controls. For calculation of cytotoxicity all numbers were first corrected for the percentage of dead cells in culture without treatment: (% cytotoxicity – % dead cells in culture) / (100% – % dead cells in culture) × 100. Specific depletion was then calculated with the corrected cytotoxicity values: [% cytotoxicity (ab+complement) – % cytotoxicity (complement alone)] / [100% – % cytotoxicity (complement alone)] × 100.

MACS enrichment

Myc-positive cells were labeled with anti-myc-beads (µMACS c-myc tagged protein isolation kit, human; Miltenyi Biotec, Bergisch-Gladbach, Germany) and positively selected via MACS columns. Positively selected PBLs were subsequently restimulated with irradiated human peripheral blood mononuclear cells (63 Gy), 60 ng/ml anti-human CD3 ab (clone OKT3, Cilag, Sulzbach, Germany) and 300 IU/ml hIL-2.

Results and Discussion

The strategy of the safeguard was to insert a small peptide tag into the γc molecule which can be recognized by a specific ab leading to cell depletion. The myc-tag (a 10-amino acid long peptide derived from the human c-myc protein) is a linear epitope that is bound by several myc-specific abs and due to its human origin is unlikely to be immunogenic in patients. We chose to insert the myc-tag at the extracellular N-terminus of the γc chain as we assumed that this part of the protein would be best accessible for an ab.

Retroviral vectors encoding wild-type (wt) or several variants of myc-tag-modified γc gene were constructed (Figure 1A). The tag was either cloned directly between the endoplasmatic reticulum (ER)

localization signal and the mature N-terminus of the protein or was spaced from the N-terminus by a short flexible peptide linker encoding the amino acids (GGSGGGGSGG) in order to possibly increase tag-specific ab accessibility. Further, either one or two copies of the tag were used to facilitate higher avidity of ab binding. To analyze expression of the modified γc gene the murine T cell line 58 was transduced and stained with anti-murine γc and anti-myc ab and analyzed by flow cytometry (Figure 1B). As 58 cells express endogenous γc, transduction efficiency cannot be concluded from the γc-specific staining although in some samples γc levels are elevated after transduction. However, binding of a myc-specific ab could be detected in all variants harboring the myc-tag, but not in wt γc-expressing or untransduced cells. Mean fluorescence intensity (MFI) of myc-specific staining was increased when two tags were present. As expression of myc-tag and γc are linked, we conclude from these data that all variants of the modified chain are processed and transported to the cell surface.

Next, the possibility to deplete cells via the introduced tag was analyzed. For this, 58 cells transduced with the myc-tagged γc variants were subjected to a complement-dependent cytotoxicity (CDC) assay by incubation with anti-myc ab and complement factors. Percentage of dead cells was determined by flow cytometry analysis of propidium iodide- (PI) stained cells (Figure 2A) and specific lysis was calculated (Figure 2B). CDC was observed for all myc-tagged γc variants, but not for wt γc. Also, in agreement with the myc-specific staining, double-tag variants led to more efficient depletion than single-tag variants. As Li-doubleMYC led to highest cell lysis, this construct was used for further experiments.

In a proof-of-principle experiment we wanted to test whether *in vivo* myc-ab treatment can deplete myc-tagged γc-expressing cells that

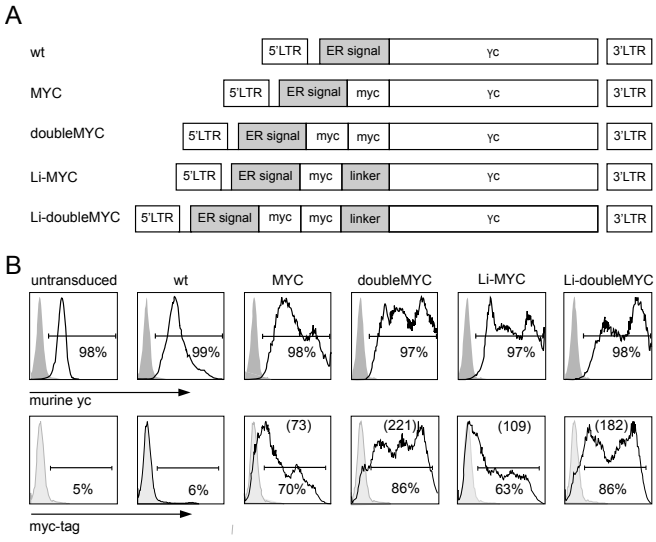

Figure 1: Variants of the myc-tagged murine γc are expressed on 58 cells and are differentially detected by anti-myc ab. (A) Composition of retroviral vectors encoding wt murine γc and differently tagged variants. The DNA sequences for the murine γc chain or its variants with single or double myc-tag and with or without linker were inserted into the retroviral vector MP71. (B) 58 cells were transduced with wt or myc-tagged murine γc variants shown in (A). γc surface expression was quantified by flow cytometry using anti-murine γc (upper panel) and anti-myc ab (lower panel). Tinted curves show isotype control staining (for anti-γc ab) or staining of untransduced cells (for myc-specific ab), respectively. Numbers in brackets indicate the MFI of myc-specific staining in the myc-positive population. (LTR: long terminal repeat, ER: endoplasmatic reticulum).

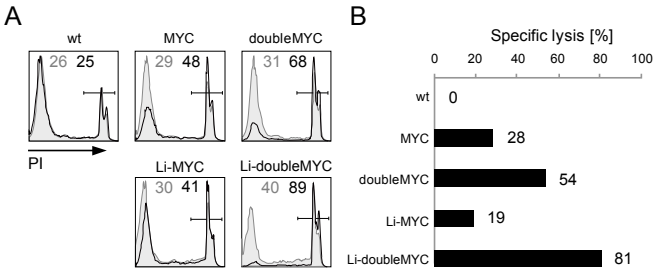

Figure 2: 58 cells expressing murine γc with two myc-tags can be depleted by anti-myc ab and complement *in vitro*. (A) CDC assay of wt and myc-tagged murine γc-transduced 58 cells was performed by subsequent incubation with anti-myc ab and complement factors (solid line, black numbers), or complement alone as a control (tinted, grey numbers). Dead cells were detected with PI and quantified by flow cytometry. (B) Calculated specific lysis of the differently tagged cells.

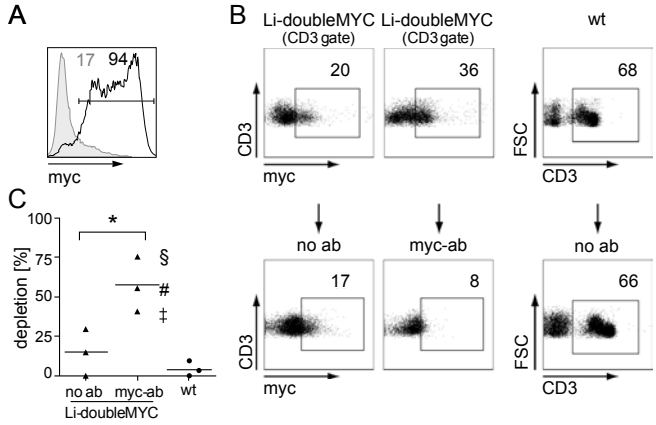

Figure 3: T cells derived from γc-transduced HSC can be depleted by anti-myc ab *in vivo*. HSC of B6 mice were isolated and transduced with either wt or Li-doubleMYC γc. (A) Before transfer, HSC were analyzed by staining with anti-myc ab and flow cytometry (wt: tinted, grey number; Li-doubleMYC: solid line, black number). (B) HSC were injected into sublethally irradiated Rag1$^{-/-}$ mice (wt: n=3, Li-doubleMYC: n=6). Six weeks after transfer, blood of reconstituted mice was stained with anti-CD3 and anti-myc abs. The samples from mice with Li-doubleMYC-modified HSC are gated on CD3-positive cells. For depletion, mice were treated with myc-ab (data shown for clone 3A7) or no ab and blood was analyzed again one day later. (C) Specific depletion was calculated (wt: based on CD3-positive cells, Li-doubleMYC based on myc-positive cells before and after ab treatment). Symbols represent single mice; bars indicate group averages. For no ab vs. myc-ab, $P < 0.03$ using unpaired Student's t-test. (§: IgG2a, #: IgG1 and IgG2a, ‡: IgG1).

have been derived from γc-engineered HSC. C57BL/6 (B6) HSC were transduced with either wt or Li-doubleMYC-modified γc retroviral vectors and 1×10^6 total HSC were transferred intravenously into Rag1$^{-/-}$ mice sublethally irradiated with 5.5 Gy. To assess transduction of HSC, transgene expression was confirmed by flow cytometry using anti-myc ab prior to injection (Figure 3A). Successful reconstitution of the lymphoid system was verified by flow cytometric analysis of peripheral blood cells six weeks after HSC injection. In all mice receiving Li-doubleMYC HSC (n=6), a proportion of myc-positive cells among CD3-positive cells (4-36%, mean: 20%) was detectable as compared to the group receiving wt HSC (n=3) where only CD3- but not myc-positive cells were detected. Figure 3B (upper panel) shows the staining of representative mice of each group. To test *in vivo* depletion, mice were injected intravenously with 2 mg anti-myc ab (clone 9E10), 0.15 mg anti-myc ab (clone 3A7), both abs, or no ab, respectively. The

number of myc-positive cells among CD3-positive cells was again determined by flow cytometry one day after ab treatment as shown in Figure 3B, lower panel (representative mice). The calculated depletion in Figure 3C shows that a large fraction of the myc-positive cells can be depleted by ab administration. These preliminary data also suggest that treatment with clone 3A7 (when used alone or in combination) was more efficient than clone 9E10. This might either be due to the fact that murine IgG2a abs – in contrast to murine IgG1 abs – can elicit a complement- as well as cell-mediated response [27], or might result from differences in ab affinity. However, this requires further confirmation. It is unlikely that the reduction in the number of myc-positive cells after anti-myc ab treatment is caused by mere masking of the epitope as a polyclonal ab was used for staining of myc-positive T cells.

Finally, we wanted to analyze whether the developed safety approach could also be applied to human γc. A human Li-doubleMYC γc variant was cloned and introduced into human PBLs by retroviral transduction. Figure 4A shows that also human myc-tagged γc can be detected by a myc-tag ab as opposed to wt γc. Further, specific depletion of myc-tagged γc-expressing PBLs was observed in a CDC assay (Figure 4B and 4C). In comparison, complement-mediated depletion of 58 cells was more efficient than that of human PBLs. This, however, might represent an inherent resistance of human T cells to rabbit complement factors.

In contrast to mice, there exist human γc-deficient B cell lines, which we used to compare the function of wt and myc-tagged human γc. Probably because SCID-X1 B cells are lacking autocrine and paracrine γc cytokine-mediated signaling, the cell line we used was inherently slowly proliferating and only grew when supplemented with 30% FCS. In a proliferation assay with low concentration of FCS, we observed that untransduced B cells turned into a resting state while wt and myc-tagged γc-modified cells proliferated equally suggesting that myc-tag insertion does not interfere with the signaling capacity of the γc receptor complex (Figure 5).

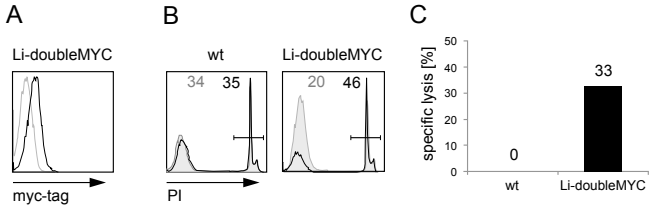

Figure 4: Myc-tagged human γc expressed on PBLs can be detected by anti-myc ab and mediates complement lysis. (A) PBLs were transduced with human Li-doubleMYC γc, enriched for myc expression by MACS and analyzed by flow cytometry using anti-myc ab. Wt γc-transduced PBLs were used as a control (wt: tinted, Li-doubleMYC: solid black line). (B) CDC assay of wt and myc-tagged human γc-transduced PBLs was performed by subsequent incubation with anti-myc ab and complement factors (solid line, black numbers), or complement alone as a control (tinted, grey numbers). Dead cells were detected with PI and quantified by flow cytometry. (C) Calculated specific lysis.

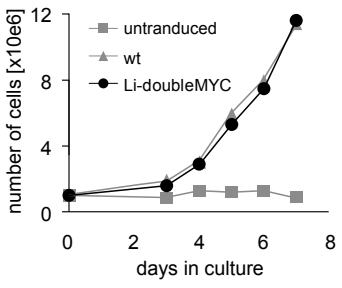

Figure 5: Myc-tagged human γc delivers proliferation signals comparable to wt γc in a SCID-X1 B cell line. A γc-deficient human SCID-X1 B cell line was transduced with Li-doubleMYC human γc and enriched for myc expression by MACS. Wt γc-transduced cells were used as a control. For analysis of proliferation, 1 x 10^6 SCID-X1 B cells were seeded on day 0 and were grown in culture medium containing 15% FCS. After an adaption phase, cells were counted daily.

In sum, we have introduced a technique that increases safety of SCID-X1 gene therapy by allowing *in vitro* and *in vivo* depletion of γc-modified cells. This technique is based on administration of a specific ab binding to a small modification within in the transgene. This intrinsic mechanism ensures that all transgene-modified cells are in parallel also equipped with the safeguard. Despite improved vector design, adverse events in the ongoing clinical study cannot be excluded. Therefore, close monitoring of the reconstituted immune cells in combination with a possibility to rapidly eliminate gene-modified cells upon loss of polyclonality is desirable. As ab therapy to treat lymphoma is also applied in the clinic [28] we believe that a transgene-directed ab may be suitable for prevention or treatment of lymphoma as a side effect of gene therapy. In a previous study, we have shown that a similar safety strategy allows the *in vivo* depletion of autoreactive T cell receptor- (TCR-) modified T cells *in vivo* [26]. So far, every TCR molecule analyzed in our laboratory allowed efficient myc-ab-mediated lysis (data not shown). Together with the present study, these data suggest that a myc-tag/myc-specific ab-based safety approach can be applied to a number of cell surface-expressed transgenes. Hence, it would be highly desirable to have a clinically approved ab available.

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The authors declare no conflict of interest.

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