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REVIEW article

Title:

Humanized mice are precious tools for evaluation of hematopoietic gene therapies and preclinical modeling to move towards a clinical trial

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Abstract

Over the last decade, incrementally improved xenograft mouse models, which support the engraftment and development of a human hemato-lymphoid system, have been developed and represent an important fundamental and preclinical research tool. Immunodeficient mice can be transplanted with human hematopoietic stem cells (HSCs) and this process is accompanied by HSC homing to the murine bone marrow. This is followed by stem cell expansion, multilineage hematopoiesis, long-term engraftment, and functional human antibody and cellular immune responses. The most significant contributions made by these humanized mice are the identification of normal and leukemic hematopoietic stem cells, the characterization of the human hematopoietic hierarchy, screening of anti-cancer therapies and their use as preclinical models for gene therapy applications. This review article focuses on several gene therapy applications that have benefited from evaluation in humanized mice such as chimeric antigen receptor (CAR) T cell therapies for cancer, anti-viral therapies and gene therapies for multiple monogenetic diseases.

Humanized mouse models have been and still are of great value for the gene therapy field since they provide a more reliable understanding of sometimes complicated therapeutic approaches such as recently developed therapeutic gene editing strategies, which seek to correct a gene at its endogenous genomic locus. Additionally, humanized mouse models, which are of great importance with regard to testing new vector technologies *in vivo* for assessing safety and efficacy prior to clinical trials, help to expedite the critical translation from basic findings to clinical applications. In this review, innovative gene therapies and preclinical studies to evaluate T- and B-cell and HSC-based therapies in humanized mice are discussed and illustrated by multiple examples.

1. Introduction

Great advances have been achieved in the field of gene therapy in the last two decades, which have evolved from gamma-retroviral (gRVs) to lentiviral vectors (LVs) to gene editing tools. Indeed, several gene therapy trials are currently completed or ongoing using LVs or gene editing strategies [1-5]. The two approaches differ from each other significantly. gRV- and LV-mediated gene therapies are mostly employed to overexpress a functional transgene permanently due to stable integration into the host cell genome. They can also include regulatory elements, which are needed for cell-specific controlled transgene expression. Therefore, they are used mainly to repair a loss of function mutation. LVs are considered safer than γ -retroviral vectors due to a different genomic insertion pattern away from gene regulatory sequences [6]. Gene editing approaches allow gene disruption, gene correction or specific transgene integration into a precise site such as the endogenous locus in the genome. The latter has the advantage of allowing a spacio-temporal control of the transgene expression. Gene editing is based on the induction of DNA double-strand breaks (DSBs) at a specific site in the genome by endonucleases such as TALENS, zinc finger or Cas9, followed by the activation of cell repair mechanisms [7]. The most frequent event that takes places after DSB is non-homologous end-joining (NHEJ) repair, which leads to insertion or deletion of nucleotides, often introducing frameshift mutations, totally or partially blocking gene transcription and translation [8]. In contrast, homology-directed repair (HDR) results in complete gene correction by homologous recombination with the sister chromatid or a delivered repair template [7, 8]. HDR is mainly utilized for gene correction or gene-targeted insertion in a desired locus. This can be performed using endonucleases that will induce double strand breaks to a preselected genome site. This break can be sealed by homology directed repair when an exogenous DNA template is provided flanked by sequences that are

homologous to the site of interest. The frequency of HDR increases when a healthy gene copy or a cDNA with homologous regions to the endogenous gene is provided either by electroporation of ssDNA/ssODN [9] or viral vectors such as integrase-defective lentiviruses (IDLVs) or adeno-associated viruses (AAVs) [10, 11]. However, since HDR is restricted to the S/G2 phase of the cell cycle, gene modification in primary cells remains a challenge for the scientific community.

Humanized mouse models have been essential for preclinical testing of gene therapies using retroviral vectors or gene editing tools for many indications as shown in Fig. 1. These allowed to retrieve preclinical data on therapeutic efficacy, toxicity, safety and stability of the therapy, which often constitutes the information required by regulatory agencies to proceed with a clinical trial. The most commonly-used humanized preclinical mouse models consist of transplantation of gene-modified human T cells and CD34⁺ hematopoietic stem and progenitor cells (called CD34⁺ cells hereafter) into immunodeficient mouse models. The most frequently used mouse model for CD34⁺ cell engraftment in the context of HCS-based gene therapy evaluation is the non-obese diabetic (NOD)/severe combined immunodeficiency (SCID), γ c^{-/-} mouse model (called NSG; [12, 13]). However, CD34⁺ cell humanized NSG mice poorly support human myeloid and erythroid lineage development and is thus not adapted for functional testing of genetic diseases affecting myeloid function or differentiation. Importantly, c-Kit receptor-mutant mice (c-Kit mutant mice) on the NSG background support unprecedented levels of human engraftment including myelo-erythroid differentiation [14-19]. Only very recently did c-Kit-mutant mice become commercially available (NBSGW and NSGW41 mouse model), which next to the MISTRG mouse model support an improved development of an innate immune system compared to NSG mice [20].

Additionally, humanized mice became indispensable for testing anti-HIV gene therapies since they are engrafted with the spectrum of human cells susceptible to HIV, which leads to

chronic viremia accompanied by HIV-induced hCD4 T cell loss. Moreover, they recapitulate HIV latency and generate human immune responses as in HIV patients [21]. The humanized mouse models mostly used to evaluate HIV gene therapies are Rag 2^{-/-}, Rag1^{-/-}, γ c^{-/-} (RG), NOD/shi-SCID, γ c^{-/-} (NOG) and immunodeficient NOD/SCID, γ c^{-/-} (NSG) mice [21-23]. HSC transplantation gives rise to all human immune cell subtypes (T, B, NK cells, macrophages and dendritic cells) and recapitulates both humoral and cell-mediated immune responses [24]. These mice are engrafted with either only HSCs (eg CD34⁺ engrafted NSG mice) and will not be fully immune-competent or are engrafted with HSCs and a functional autologous thymus, the latter allowing education of T cells on human HLA (BLT-mice) [23]. For cancer gene therapy multiple human xenograft mouse models have been developed. The most widely used mouse models for human tissue/tumor xenotransplantation is the NSG mouse, which supports development of a human hematopoietic and immune system to some extent [25]. This mouse model has become the platform to study the interaction between the human immune system and cancer, though there is room for improvement as detailed in point 3.

2. Preclinical evaluation of HSC gene therapy in humanized mice (Table 1)

2.1 ADA-SCID

Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) is a severe monogenetic disease characterized by impaired immune responses and other symptoms such as systemic toxicity[26]. Bone marrow transplant from a matched donor is the treatment of choice but is out of reach for 80% of patients and enzyme replacement is a non-curative treatment [27]. Therefore, *ex vivo* gene therapy of patient BM CD34⁺ stem and progenitor cells is considered a good option [28-30]. Recently, CD34⁺ cells transduced with a retroviral vector coding for human ADA cDNA was accepted as one of the first commercial

gene therapy products called Strimvelis. Evaluation of this product in humanized mice has been key to acceptance by the European Medicine Agency (EMA). In view of the difficulties in obtaining patient CD34⁺ progenitor cells from ADA-SCID patients due to the low prevalence of this disease [26], healthy hCD34⁺ cells from cord blood were transduced with the therapeutic ADA-vector and transplanted into NSG mice after conditioning [31]. Efficient human hematopoiesis was confirmed without aberrant development and vector insertion analysis and bio-distribution studies showed in all hematopoietic tissues a low vector copy number/cell indicating a low risk of insertional mutagenesis [31]. These safety data convinced the EMA to accept this new medicine for treatment of ADA patients.

2.2 SCID-X1

X-linked severe combined immunodeficiency (SCID-X1) is caused by a deficient interleukin-2 receptor gamma (IL2RG). An SCID-X1 patient has a severely compromised immune system marked by absence of T and natural killer (NK) cells and impairment of B-cell function [32, 33]. In previous gene therapy trials, 20 children received autologous BM stem cells that were transduced with a murine gRV encoding for the correcting IL2RG cDNA [34, 35]. The therapy was successful and resulted in reconstitution of T cells and disease-free survival in 18 patients. However, 5 of them developed leukemias that were the result of transactivation of proto-oncogenes by enhancer sequences in the viral promoters-enhancers (long terminal repeats) of the neighboring integrated vector [36]. This gRV has a preference for integration into promoter and enhancer sequences near the transcriptional start site of genes and therefore represents an increased risk for activation of genes [37]. This was overcome by designing a gRV lacking these enhancer sequences, called (self-inactivating) SIN vector. A clinical-grade preparation of this vector encoding for IL2RG transduced 60-70% of hCD34⁺ as confirmed by long term reconstitution of NSG mice [38]. These data

together with *in vitro* T cell differentiation from corrected SCID-X1 patient CD34⁺ cells paved the way to a clinical trial with this ‘safer’ vector [39].

Another solution was utilizing LVs, which are characterized by a safer genomic insertion pattern [6]. However, to increase safety even more, SIN LV vectors were engineered as was done for gRVs. Greene et al. [40] showed that SIN LVs encoding for SCID-X1 cDNA allowed efficient transduction of hCD34⁺ cells that engrafted long term into NSG mice without adverse effects. Moreover, they performed vector integration site analysis on the CD34⁺ cell progeny [40]. This revealed that engrafted CD34⁺ cells and their derived progeny harbored identical vector integration sites, which proved that initially true HSCs were gene modified. This preclinical research in the humanized mouse model is fundamental for progression of lentiviral SCID-X1 gene therapy into the clinic in the near future.

Another way to ensure safe gene therapy for SCID-X1 relies on targeted genome editing, which allows site-specific gene correction of the mutated SCID-X1 gene leaving it under the control of its own regulatory sequences. This therapeutic strategy was successfully performed in the IL2RG gene locus and transfer of gene-edited HSCs in NSG resulted in normal long term hematopoiesis [41, 42]. This approach has the benefit of maintaining the cell intrinsic expression pattern, thereby reducing the likelihood of side effects and may represent therefore a new therapeutic opportunity for SCID-X1 patients.

2.3 IPEX syndrome

Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome is characterized by severe autoimmunity[43]. This disease is due to mutations in FOXP3 (Forehead Box P3), a transcription factor assuring regulatory T cell (Treg) function and therefore the maintenance of immune tolerance [43, 44]. FOXP3 loss of function has been also associated with hyper-proliferation of lymphocytes and development of cancers [45, 46].

Santoni and coworkers engineered an LV encoding for constitutive expression of FOXP3. Human (h) CD34⁺ cells transduced with this vector were transplanted into NSG mice and unexpectedly revealed that hHSCs, which normally do not express FOXP3, preserved their quiescence and improved the maintenance of the most primitive HSCs in vivo [47]. However, as a consequence, constitutive expression of FOXP3 in HSCs impaired T cell differentiation and TCR-mediated T cell responses in NSG mice, which was exactly the opposite to the objective pursued by this gene therapy approach [47]. This problem was solved by Makiul et al. utilizing a vector placing FOXP3 under the control of regulatory elements derived from the endogenous FOXP3 locus. Thanks to humanization of NSG mice with gene modified HSC cells, correct differentiation of HSCs into Tregs was confirmed due to lineage specific and functional expression of FOXP3 in Treg [48]. These preclinical humanized mouse data might be the foundation for a translation of HSC-based gene therapy to IPEX patients.

2.4 Granulomatous Disease (X-CGD)

Chronic Granulomatous disease is a primary immunodeficiency which is characterized by the inability of phagocytes to produce bactericidal superoxide anions ($O_2^{\cdot-}$) due to mutations in any of the five subunits of the NADPH-oxidase complex [49]. Frequent and life-threatening infections caused by certain opportunistic microorganisms lead to high morbidity in affected individuals. Two-thirds of all patients have mutations in the *CYBB* gene encoding for the catalytic gp91phox subunit on the X-chromosome (hence X-CGD) and most therapeutic developments focused on this subgroup of patients. A variety of different gene therapy approaches has been developed, and the most important measure of therapeutic efficacy is the reconstitution of NADPH-oxidase activity in mature myeloid cells, i.e. neutrophil granulocytes and monocytes/macrophages. As previously mentioned, generation of human myeloid cells after transplantation of hHSC into NSG mice is generally poor. Nonetheless,

high titer LVs expressing a codon optimized gp91phox cDNA, mediated reconstitution of NADPH-oxidase activity after transplantation of X-CGD HSC transduced with these LVs in this mouse model [50]. Consistent with previous reports, only a fraction of myeloid cells fully matured and became capable of Phorbol 12-myristate 13-acetate (PMA) induced $O_2^{\cdot-}$ production as indicated by the variable fraction of 13-83% healthy donor cells showing oxidative burst activity in a functional assay *in vitro*. The same vector has been evaluated in the NSG mouse model in the context of preclinical studies, which showed that almost all CD33⁺ myeloid cells derived from healthy hHSC produced high levels of $O_2^{\cdot-}$, thus highlighting improved maturation in addition to higher myeloid cell engraftment levels in NSG mice [51]. This model was also chosen to show myeloid lineage specificity of LVs containing an engineered synthetic promoter element in combination with a miRNA-126 target element to repress unwanted expression of the transgene in HSCs [52]. More recently, NSG mice were used in a landmark paper showing CRISPR-Cas9 mediated genetic correction and engraftment of X-CGD patient derived HSCs [53]. The C676T point mutation in the *CYBB* gene, which is frequently found in X-CGD patients, was reverted in 14% of long-term engrafted cells after ex-vivo delivery of CRISPR-Cas9 and a co-delivered corrective single stranded oligonucleotide (ssODN) into patient HSCs, a level which is far higher than what had previously been possible. The recent generation of a novel humanized mouse strain based on NSG-background [54], carrying a disruption of the murine *CYBB* gene abrogates $O_2^{\cdot-}$ production from mouse granulocytes. These mice display a higher propensity to spontaneous infection with bacterial and fungal pathogens, essentially phenocopying the human disease. This model will prove very useful to assess the ability of human gene therapy treated cells to prevent or fight infections with certain pathogens [55, 56], and are essential to better predict therapeutic outcomes in clinical applications. Additionally, novel Kit-mutant

immunodeficient mouse strains [17] support improved generation of diverse mature myeloid cells, which will facilitate functional assessment of gene therapy approaches for CGD.

2.5 Hemoglobinopathies

Hemoglobinopathies encompass a group of diseases affecting red blood cells, including the particularly frequent Sickle Cell Disease (SCD) and Thalassemia. These are among the most common inherited genetic diseases in the world, affecting millions of people [57]. Thalassemia is characterized by deficiency of alpha or beta globin chains, which leads to poor hemoglobinization of RBCs and severe anemia in patients [58]. Sickle cell disease is caused by a single amino acid exchange in the beta-globin chain [59]. Sickle hemoglobin has an increased propensity to polymerize under low oxygen conditions, which leads to the characteristic sickle shape of red blood cells and to a plethora of different physiological manifestations, some of them life threatening. Although gene therapy for SCD has been under development for more than 20 years, the bar for clinical translation is particularly high due to the requirement of high levels of tightly regulated transgene expression and the fact that this disease is in most cases clinically manageable with Hydroxyurea, antibiotics and/or blood transfusion regimen [60]. Until recently, evaluation of gene therapy approaches has been mostly performed in immune competent mouse models which express human sickle hemoglobin instead of its murine counterparts [61, 62]. However, they do not allow for the evaluation of gene therapy approaches in human cells, which is essential for human sequence specific gene editing methodology. Humanized mouse models were not frequently used because human erythropoiesis in available models was extremely poor. Mature red blood cells (RBC) were virtually absent in the peripheral blood of humanized mice, even in the NSG mice [17]. As a result, humanized mice were used for engraftment and biodistribution studies

[63], but for functional analyses engrafted cells had to be isolated from the bone marrow and subjected to erythroid *in vitro* differentiation for efficacy assessments in red blood cells [64]. The c-Kit mutant NSG mouse model, which sustains human erythrocyte development, has been used to evaluate the efficacy of gene editing for SCD and for specific forms of Thalassemia [65, 66]. Genome editing based therapies rely on targeted correction of the sickling mutation for SCD [11, 67] or on the destruction of an erythroid specific enhancer in the BCL11A gene, which is a repressor of fetal globin gene transcription for as well SCD as beta-Thalassemia. Reactivation of fetal globin expression in SCD patient cells was achieved by subjecting HSCs to CRISPR-Cas9 mediated disruption of the BCL11A enhancer [65]. After engraftment into c-Kit mutant mice, therapeutic efficacy could be shown, for the first time, directly in mature human red blood cells from the bone marrow of transplanted animals. High induction of protective fetal hemoglobin and concomitant downregulation of adult sickling globin led to protection from sickling in SCD-HSC derived erythroid cells, while in Thalassemia patient-derived-grafts, induction of fetal hemoglobin led to improvements in RBC hemoglobinization and normalization of red blood cell parameters. The use of humanized mice in these studies enabled the simultaneous assessment of toxicity and efficacy in human cells, using similar reagents and materials as envisioned for clinical translation and thus to generate the data required to initiate a clinical trial.

2.6 Bone marrow failures

Inherited bone marrow failure syndromes (IBMFS) are a complex group of disorders characterized by deficiency in blood cell production that finally leads to bone marrow failure. In IBMFS, the most frequent disorders are the Fanconi anemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), Shwachman-Diamond (SD), severe congenital neutropenia (SCN) and congenital amegakaryocytic thrombocytopenia (CAT) [68].

Transplantation with HSCs from a matched allogenic donor is the only curative treatment for these disorders [69]. However, this can lead to severe complications, is not readily available and graft versus host rejection can occur [70-72]. Therefore, gene therapy has emerged as a potential alternative therapeutic approach for these disorders. However, in contrast to other disorders previously treated by HSC-based gene therapy, in the case of IBMF the harvest of enough CD34⁺ cells from the patients to be able to correct the disease can also constitute a limitation for the treatment by gene therapy [73, 74]. In this context, where the quality and number of HSCs required to correct a disease is severely compromised, the demonstration that corrected cells from the patients can engraft in immunodeficient mouse models is a key factor before moving to the clinic.

FA is a very heterogeneous genetic disease [75, 76], with the *FANCA* gene being the most frequently mutated in patients (>60% of FA patients worldwide) [77, 78]. Several studies in FA mouse models demonstrated the possibility of conducting gene therapy by using gRVs carrying a healthy copy of the mutated gene [79-83]. However, initial clinical trials conducted in Fanconi anemia patients did not give the expected results [84, 85], likely due to the low number of cells infused and the long *ex vivo* culture in the presence of cytokines during several days, thus leading to exhaustion of HSCs and thus loss of true HSCs [69].

Recent studies in *FANCA* disease have been focused on answering the questions arising from previous trials. For the first time two mobilization agents, G-CSF and plerixafor, were employed to increase the number of mobilized HSCs in Fanconi anemia patients. Importantly, to demonstrate the feasibility of this new protocol to correct HSCs from FA patients, mobilized peripheral blood CD34⁺ cells from patients were transduced with the clinical grade LV carrying the human *FANCA* gene and transplanted into NSG mice [86]. This confirmed for the first time the engraftment capacity of corrected cells *in vivo*, suggesting that the protocol used for the mobilization of HSCs from FA patients and the short *ex vivo* culture

required for transduction maintained the engraftment capacity of corrected FA cells. The analysis of myeloid and lymphoid subpopulations confirmed that differentiation capacity of corrected FA cells was similar as compared to healthy donor cells [86].

Importantly, the proliferative advantage of gene corrected human FA HSC cells was confirmed for the first time in NSG mice. This phenomenon had been observed in mosaic patients, in which the spontaneous reversion of an FA mutation, led to a marked proliferative advantage of the reverted cells and allowed correction of the hematological problems in some patients [82, 87, 88]. These preclinical studies in NSG mice paved the way for the development of a gene therapy clinical trial in Fanconi anemia patient subtype A, in which the authors confirmed the selective advantage of corrected HSCs in FA patients [89, 90].

However, due to the scarcity of FA patient HSCs and because the murine *Fanc^{-/-}* mouse model does not fully recapitulate human FA BMF, it was difficult to uncover the mechanism leading to BMF in FA. Therefore, healthy donor CD34⁺ cells were transduced with shRNA LVs targeting FA genes to develop FA-like cells [91]. This methodology allowed the generation of a higher number of FA-like HSCs. Transplantation of these FA-like HSCs into NSG mice demonstrated that these FA HSCs were impaired due to p53/p21 activation and G0/G1 cell cycle arrest in response to DNA damage and replication stress.

These FA-like HSCs are of course not perfectly mimicking FA patient cells since residual FANC protein levels were still present. In contrast, CRISPR/Cas9 gene editing technology can permit the generation of FA knock-out CD34⁺ cells that are comparable to FA CD34⁺ cells from patients and can be used to study the disease and potential therapeutic alternatives both *in vitro* and *in vivo*. In the context of gene editing in FA, immunodeficient mouse models have been an essential tool to demonstrate the engraftment capacity of edited HSCs. In a study by Diez et al. healthy donor CD34⁺ cells were gene-edited to insert hFANCA into the AAV1 genomic locus using an integrase defective LV (IDLV) carrying the intact gene [92]

combined with electroporation of mRNA encoding for zinc finger nucleases (ZFNs) targeting the *AAVS1* locus. This gene editing approach allowed the correction of FA-CD34⁺ cells by homologous directed repair (HDR). It also confirmed that the engraftment capacity of the gene-edited CD34⁺ cells in NSG mice was maintained after *in vitro* manipulation with IDLVs and ZFNs targeting the *AAVS1* locus. More recently the possibility to correct HSCs from FA patients by NHEJ-mediated repair and the *in vitro* and *in vivo* proliferative advantage of corrected cells was demonstrated in the NSG mouse model [93]. Furthermore, in NSG mice the authors confirmed the high editing efficiency mediated by NHEJ in long term repopulating stem cells [93].

2.7 Human Immunodeficiency virus (HIV) gene therapy employing HSCs or T cells

1.8.2 Anti-HIV gene therapy strategies

HIV is indeed a highly evolved retrovirus with a great tendency to mutations and the current combination antiretroviral therapies (HAART) are still not curative due to drug resistant HIV mutant viruses and the persistence of latent HIV reservoirs [94, 95]. Therefore, a permanent gene therapy is an attractive option. Ironically, the tool used most frequently to introduce anti-HIV therapeutics stably into hCD4⁺ T cells or hHSC are LVs, which are derived from HIV but devoid of all pathogenic HIV features and have built-in multiple safety features.

Although mature CD4⁺ T cells are attractive targets for anti-HIV gene therapy, they might only provide a transient anti-viral effect since they may not persist long term *in vivo*. In contrast, using HSC-based gene therapy might allow a permanent cure for HIV since all target cells should be protected. Anti-HIV gene therapy strategies are very diverse and so different in the target genes that only some representative examples are show here to emphasize the

enormous value of humanized preclinical mouse models for the evaluation of new anti-HIV gene therapies (see Fig. 2).

Broadly neutralizing antibodies are among the most promising strategies to control HIV in the long term [96, 97]. Kuhlmann et al. transduced hCD34⁺ cells with an LV encoding an HIV neutralizing antibody and demonstrated upon transplantation into NSG mice secretion of anti-HIV antibodies by hT and hB cells [98]. Moreover, they showed a delay in HIV-1 viremia accompanied by hCD4⁺ maintenance *in vivo*, indicating a protection against HIV-1.

Another strategy consists of the use of HIV entry inhibitors. Falkenhagen and co-workers developed an LV that secreted a soluble CD4 receptor (sCD4), one of the HIV entry receptors, which by binding to the HIV envelope inactivates the virus [99]. hCD34⁺ cells were transduced with these sCD4 encoding vectors and injected into NSG mice. Upon engraftment, HIV challenge of these humanized mice revealed a reduction of the viral load over time. Moreover, the authors demonstrated in these humanized mice protection of unmodified hCD4⁺ T cells underlining a bystander effect of the secreted CD4 [99]. Two other HIV entry inhibitors, which inhibit fusion of HIV with the cell membrane were introduced by an LV into hCD4 T cells as a membrane anchored protein and mice infused with these hT cells were protected against HIV [100, 101].

Another intriguing strategy is the knock-down of one of the co-receptors of HIV, CCR5. This strategy was inspired by a report on an HIV patient that developed leukemia and underwent transplantation with allogenic HSCs missing the CCR5 HIV co-receptor. This patient cleared its HIV infection [102]. Therefore, many anti-HIV therapeutic strategies are based on silencing of the CCR5 receptor. LVs were engineered encoding siRNA, shRNAs or microRNAs against CCR5 and transduction of hCD34⁺ cells with these vectors resulted in the desired CCR5 downregulation in HIV target cells and the production of HIV-resistant progeny cells upon transplantation into NSG or BLT mice [103-105]. The CCR5 receptor can

also be disrupted by gene editing. An LV encoding for endonucleases, CRISPR/Cas9, and gRNAs directed against CCR5 mediated efficient deletion of the receptor in hCD34⁺ cells with no influence on immune cell differentiation *in vivo* in humanized mice [106]. Other studies achieved disruption of CCR5 by Zinc Finger nucleases introduced into HSCs by electroporation of the CCR5-specific zinc finger nuclease mRNA. These authors showed normal hematopoietic potential of edited HSCs upon injection into NSG mice, which was a requirement for a first HIV-1 clinical trial [107-109]. Additionally, RNA guided gene editing systems such as CRISPR/Cas9 have been designed to mutate or excise the integrated HIV genome from latently infected cells [110, 111]. In addition, a CRISPR/Cas9 activator system was designed in which the endonuclease is not active and fused to a transcription activator. The gRNAs can target this Cas9 to the integrated HIV-1 provirus and reactivate dormant HIV-1 and thus revert latency [112]. Both systems might be curative for HIV since they may allow to eliminate the latent HIV reservoir in the patients.

Finally, a last example of an anti-HIV strategy is based on engineered T-cells called chimeric antigen receptor T cell (CAR) therapy. CAR T cell therapy involves changing a patient's own immune cells to recognize, fight and kill its own HIV infected cells (see also point 2).

Zhen et al. [113] gene-modified CD34⁺ cells with a CAR T directed against HIV, which developed into functional T and natural killer cells *in vivo* in humanized mice and were thus rendered resistant to HIV infection and suppressed HIV replication *in vivo*. This suggest that CAR T cell therapy might be an option in the future for HIV treatment. More details on this anti-HIV CAR T cell therapy are summarized in Kitchen et al. [114] and in a recent study using a two-molecule CAR architecture, termed duoCAR, which targets multiple sites on the HIV envelope [115].

Unfortunately, a single anti-HIV therapy led to escape mutants and therefore combinatorial approaches were evaluated in humanized mice. Two examples consist of a combination of

three highly potent anti-HIV genes into a single LV. These anti-HIV LV-transduced CD34⁺ cells were able to ensure multilineage hematopoiesis upon engraftment into immunodeficient mice and most importantly hCD4 T cells showed resistance to HIV-1 *in vivo* [116, 117].

2.8 Evaluation of novel lentiviral vector pseudotypes for human HSC transduction

A major breakthrough was the invention of novel envelopes to target LVs to HSCs or enhance HSC transduction. This was achieved by incorporating measles virus (MV) glycoproteins (MV-LVs) or baboon retroviral envelopes (BaEV-LVs) on the LV surface [118, 119]. Remarkably, using these new LVs, resting hCD34⁺ cells were transduced at levels not reported before (30-70%) [119, 120]. BaEV-LV and MV-LV transduced hCD34⁺ cells were transplanted into NSG mice to evaluate their long-term reconstitution capacity. In primary NSG recipients equivalent high transduction levels were sustained in hCD34⁺ cells, myeloid lineages and lymphoid lineages. hCD34⁺ cells isolated from the BM of primary recipients were injected into secondary NSG mice. Similarly, high transduction levels were detected in all hematopoietic lineages in the BM of these secondary recipients and in some cases transduction was increased as compared to the primary recipient, pointing towards transduction of bonafide HSCs [119, 120]. To further confirm these results, resting CD34⁺ cells were transduced with MV-LVs encoding for a barcode library. This marked each CD34⁺ cell with a unique barcode sequence which is propagated to the daughter cell upon differentiation. Upon reconstitution of NSG mice for 4 months, human progenitors, B cells, T cells and myeloid lineage cells were isolated and the barcodes present in each subpopulation were evaluated. For MV-LV CD34⁺ cell transduction, several barcodes present in the CD34⁺ cells were also found in the different lineages derived from them, which confirmed that initially true HSCs were transduced [120].

Selective high-level transduction of HSCs was also achieved by pseudotyping LVs with engineered MV glycoproteins, which contain scFv targeting sequences for CD133 or CD105, two markers present on the hHSC cell surface. NSG mice were used to show the superiority of CD133-targeted LVs using X-CGD as model disease [121]. LVs pseudotyped with an MV envelope targeting the limiting dilution competitive transplantation assays highlighting the value of humanized mice for quantitative assessments of genetically modified HSC engraftment.

3. Human T cell-based gene therapies evaluated in humanized mice

3.1 Anti-cancer CAR T cell therapy

Despite progress made in the treatment of many leukemias, lymphomas and solid cancers, therapeutic outcomes remain refractory and better treatment options are required. A promising anti-cancer strategy is based on engineered T-cells called chimeric antigen receptor T-cell (CAR-T) therapy. Anti-cancer CAR-T cell therapy directs a patient's own immune cells to kill cancer cells inside the body [122]. CARs are synthetic proteins consisting of antigen-binding moiety, usually a single-chain variable CD133 surface marker on HSCs showed preferential transduction of long-term repopulating stem cells as compared to VSV-G-LVs in NSG mice. This was shown by barcoding experiments and fragment (scFv) derived from non-human mAbs recognizing a cancer antigen, linked by a spacer and transmembrane sequences to an intracellular signaling molecule. Ongoing clinical trials have described durable rejection of previously refractory B-cell malignancies in patients after CD19-directed CAR therapy [123-127] with a complete response rate as high as 86% in leukemia patients. In 2017 this has led to the acceptance of two CAR T cell therapies by the regulatory agencies in the USA (Food and Drug agency; FDA) and Europe (European Medicine Agency; EMA) for B-cell

leukemia. The successful application of CARs directed against leukemia has now more recently encouraged the application to other cancers including solid cancers [128].

To fully understand CAR-therapy in terms of its limitations and capacities, preclinical testing and *in vivo* evaluation in a humanized mouse model has become the gold standard to validate these T cell therapies and get regulatory approval. Several humanized mouse models are employed for evaluation of CAR T cells. The first one is the xenograft mouse model, in which a human tumor cell line is engrafted in immunocompromised mice (mostly the NSG model), followed by infusion of human CAR T cells (Fig. 3A). Barret and colleagues demonstrated in this model that antiCD19-directed CAR T cells were able to kill the CD19⁺ cancerous B cells resulting in prolonged survival of this NSG leukemia xenograft model [129] and this set the basis for the first clinical trial. One study developed a bi-specific CAR targeting two B-cell specific molecules, CD19 and CD20, to minimize the risk of antigen escape by leukemic cells which lose CD19 expression. In contrast to CD19-CAR T cells, the bispecific CD19/CD20-CAR T cells also eradicated leukemic cells that lost CD19 expression at the surface in a xenograft NSG model [130].

Importantly, the early CARs harbored a murine antigen binding domain, which could potentially induce an immune response in patients leading to premature elimination of CAR T cells and might result in tumor relapse. To avoid this problem, one team developed a fully human CD19-specific CAR, malignancies which proved functional for elimination of a human lymphoma xenograft in NSG mice [131]. Blum et al. developed a humanized CAR against the B cell maturation Antigen (BCMA) [132]. BCMA-CAR T cells eradicated the tumor cells both in a multiple myeloma and a B cell lymphoma xenograft model [132]. BCMA CAR T cells were also able to target other B cells in humanized NSG mice in another study [133]. It became clear that a fully humanized CAR may reduce the risk of immune rejection when compared to a murine based CAR.

It is important to emphasize though that the human cancer cell line xenograft models lack a functional human immune system and other human tissues, which may modulate the anti-cancer activity *in vivo*. Nevertheless, they were instrumental to establish a first proof of concept. In addition to the examples here, many more CAR T studies rely on this xenograft mouse models for primary evaluation of efficacy.

Another more relevant humanized mouse model has been established for evaluation of CAR T cell efficacy. The patient derived xenograft (PDX) model consists of injection of a primary tumor biopsy from a patient instead of human cell lines (Fig. 3B). In this setting, the tumor cells as well as the tumor microenvironment (e.g. immune cells) are present *in vivo* to some extent. Therefore, PDX models are increasingly used for personalized anti-cancer T cell therapies and are more relevant than xenografted cancer cell line models for translation into the clinic.

As early as in 2010, a patient's tumor and autologous T cells were engrafted in NSG mice to show that the patient's T cells can function as CAR T cells [134]. Another study showed the efficacy of CAR T cells in PDX mice for independent hepatocarcinoma grafts from 3 different patient tumors. Two PDX mice eradicated the tumors upon CAR T cell therapy, while one was resistant and showed upregulation of immune check-point inhibitor molecules [135]. This might suggest that a combinatorial CAR T and immune check-point inhibitor treatment could be recommended for this particular patient. PDX models allowed the assessment, to some extent, of human immune responses to primary cancer cells, which is relevant for clinical translation. These PDX models still have their limitations since a normal functional human immune system is not present in these humanized mice and they cannot fully predict what might happen in cancer patients. That is why very recently a more complex humanized mouse model was developed (Fig. 3C). Jin et al. developed a mouse model for acute B lymphoblastic leukemia (hB-ALL), in which cancer and immune cells are autologous

[136]. They produced two humanized mice using hCD34⁺ progenitor cells from the same donor (Fig. 3C). Firstly, they engrafted one NSG recipient mouse with hCD34⁺ cells and fetal thymus to generate a humanized mouse with human immune-competence since T-cells are educated on human thymic tissue. In parallel, they generated a second mouse engrafted with fetal CD34⁺ cells from the same human donor transduced with a B-ALL relevant oncogene. These mice developed hB-ALL autologous to the blood system of the first mice generated. Then they engrafted these autologous B-ALL cells into the firstly developed immune-competent humanized mice to have a valid B-ALL model similar to the patient (Fig. 3C). In the next step, they isolated T cells from the first humanized mice, which they modified with an anti-CD19-CAR and reinfused into the B-ALL human like mouse model (Fig. 3C).

This model has important, unique characteristics: 1) the humanized mice have human hematopoiesis and a human functional immune system; 2) the B-ALL tumor cells are autologous and induced by a relevant human oncogene; 3) the CAR T cells are modified autologous T cells and they are educated on a human thymus. Altogether this makes this kind of model, though complex in its generation, much more relevant for the evaluation of human CAR T cell efficacy, resistance and toxicity.

Another huge step forward in CAR T cell therapy is the invention of LVs that permit the generation of CAR-T cells directly *in vivo* in humanized mice. Details can be found under point 4. and in [137].

3.2 Progenitor T cell correction to speed up T cell development in vivo

Upon HSC-mediated gene therapy (see point 1), patients can be immunocompromised for over a year because the complete restoration of a polyclonal T-cell repertoire after conditioning is slow [138, 139]. Mature T cells are mainly short-lived *in vivo*. T-cell gene therapy would therefore strongly benefit from gene transfer into long-lived T-cells. Immature

targets for gene modification are the T-cell progenitors, which can be generated from CD34⁺ hematopoietic stem and progenitor cells (HSPCs) in a feeder-cell-free culture system based on Delta-like ligand-4 (DL-4) [140, 141]. These T-cell progenitors derived from CD34⁺-cells cannot be efficiently gene modified by classical LVs pseudotyped with VSV-G [142]. In contrast, LVs pseudotyped with the modified glycoproteins (gps) of measles virus (MV-LVs) or baboon endogenous retrovirus (BaEV-LVs) [143], achieved high-level gene transfer in T cell progenitors (up to 80%; [142]). Transduction of hCD34⁺-cells cultured on DL-4, resulted in the expansion of gene-marked human early thymic progenitors - the most primitive T-cells *in vitro*. Upon engraftment of these progenitor T cells in NSG recipient mouse, high transduction levels (80%) were maintained in all T-cell subpopulations. These T cell progenitors retained their *in vivo* intra-thymic T-cell differentiation capacity and displayed a conventional CD4, CD8 and TCR $\alpha\beta$ /TCR $\gamma\delta$ repertoire [142]. Moreover, T-cell lineage reconstitution was accelerated in NSG recipients after T-cell progenitor injection as compared to hematopoietic stem cell (HSC) transplantation. BaEV-LV mediated correction of CD34⁺ and progenitor T cells from patients with X-linked Severe combined immunodeficiency (X-SCID) was shown *in vitro* through de novo expression of the γ C receptor [142]. This suggests that in the future injection of BaEV-LV corrected autologous HSCs and T-progenitors might produce both short-term recovery of T-cell immune response and long-term correction in X-SCID patients. This strategy is supported by co-engraftment of autologous hHSCs and progenitor T cells in NSG mice [140].

4. Human B-cell based gene therapy studies in humanized mice

4.1 B cells as gene therapy targets

B cells are attractive targets for gene therapy of genetic diseases or immunotherapy strategies. They could provide an innovative and promising approach for active immunotherapy against

pathogens like viruses or cancer cells through their secretion of very robust specialized antibodies [144]. Additionally, B-cell gene therapy is an important option for induction of potent tolerogenic antigen presenting B cells in autoimmune diseases such as diabetes or in the case of de novo secreted therapeutic proteins such as coagulation factors [145]. Besides these features, primary plasma B cells can persist for an entire life span which means that one single administration of modified B cells might suffice for life-long cure.

However, genetic modification of B cells to make them secrete a therapeutic antibody has proven to be very inefficient since classical LVs pseudotyped with VSV-G transduce hB cells very poorly [146]. In contrast, LV pseudotypes with the modified glycoproteins of measles virus (MV-LVs; [147-149], the baboon endogenous retrovirus (BaEV-LVs) [150] or Gibbon ape leukemia virus [143], achieved high-level gene transfer in B cell receptor-stimulated B cells. These novel LV pseudotypes paved the way for B-cell gene therapy as a new option for treatment.

Importantly, a new method was developed to generate human plasma B cells (PCs) *in vivo* to evaluate these therapies. hB cells can be differentiated into PCs by adoptive transfer of mature CD19⁺ B cells and autologous hCD4⁺ T cells into immuno-deficient NSG mice (Fig. 4). This hB/hCD4 T cell humanized mouse model was previously shown to be suitable for the study of the production of human Ig [151]. Moreover, it was demonstrated that B cell engraftment is strongly ameliorated if autologous CD4⁺ cells are co-injected in the context of immunocompetent mice [152]. In the context of an immunodeficient mouse, hB cells are activated and differentiated into PCs under the polyclonal activation signal triggered by the human xenograft due to massive activation of the hCD4⁺ T cells by the mouse xeno-antigens [151]. This method allowed robust generation of human plasmablasts and PCs in NSG mice in a 4-5 week time-span (Fig. 4). The humanization of NSG mice with B cells and CD4⁺ T cells allowed the evaluation of immunotherapies and homing of these therapeutic PC targets

in vivo.

4.2 B-cell gene therapy for Hemophilia B

Hemophilia B is an X-linked disorder caused by a deficiency in coagulation factor IX. It can result in frequent bleeding and early death if left untreated [153]. Currently, treatment is based on repetitive intravenous administration of recombinant FIX. However, some patients develop neutralizing antibodies (inhibitors) against FIX. Since management of FIX inhibitor development is limited, repetitive administration can become life-threatening for the patient [154]. Therefore, alternative therapeutic approaches such as gene therapy are needed [155]. FIX tolerance induction to prevent FIX inhibitors could be a preferable solution and therefore modification of primary B cells to secrete FIX might be ideal since B cells have been shown to function as tolerogenic APCs against IgG fusion proteins they secrete [156, 157]. One study developed BaEV-LVs encoding for FIX, which transduced hB cells up to 100%. *In vivo* human plasma B cell development upon CD4⁺ hT cells and hB cell engraftment in NSG mice demonstrated hFIX secretion in the serum of these mice (Fig. 4). This hFIX proved to be functional and was secreted at therapeutic levels for hemophilia treatment in this humanized mouse model supporting the potential of B cell gene therapy for this monogenetic disease [150].

4.3 B-cell gene therapy for the treatment of viral infections

As mentioned above, B cells could represent powerful tools for secretion of high-affinity, pathogen-specific antibodies against viruses such as HIV [98, 158], influenza, respiratory syncytial virus (RSV) [159] and hepatitis C virus [160].

Indeed, Fusil *et al.* designed an LV encoding for an antibody against the E2 surface protein of hepatitis C virus (HCV). Since protective vaccines should elicit both long-lived memory B

cells and antibody secreting plasma cells, these authors made sure that mature B cells expressed this antibody as a membrane anchored IgG form, which allows these cells to rapidly respond and differentiate into antibody secreting cells upon hepatitis C virus infection. BaEV-LV encoding for HCV-IgG transduced hB cells efficiently and were engrafted together with autologous hCD4⁺ T cells into the NSG mice, in which they underwent *in vivo* maturation into PC B cells that secreted the functional anti-HCV neutralizing antibody in the serum of the humanized NSG recipient (Fig. 4) [160]. This means that they obtained a physiologically regulated membrane anchored and secreted antibody expression depending on the hB-cell maturation status *in vivo*. Therefore, this humanized mouse model allowed to validate this promising immunotherapy approach.

Though LVs are considered safer than gRVs, they still randomly integrate into transcriptionally active regions of the B cell genome, which carries the risk of introducing mutations, a phenomenon called insertional mutagenesis. This represents a safety risk for future clinical applications. Therefore, gene-editing technologies are considered very attractive for precision engineering of B cells [161, 162]. Targeted gene editing of the endogenous IgH locus in primary B cells by CRISPR/CAS9 for the expression of a *de novo* antibody allows its expression under the control of the endogenous promoters and enhancers ensuring physiological expression levels. This strategy was shown to be successful for inducing protective antibody responses against HIV and respiratory syncytial virus (RSV) [159, 163]. Humanized mouse models could be of great value for *in vivo* evaluation of these gene edited B cells.

5. The humanized mouse: a model for testing *in vivo* gene delivery tools targeted to specific human blood cells

5.1 Advantages of in vivo gene therapy

The delivery of a relevant therapeutic gene directly into the organism is called *in vivo* gene delivery or *in vivo* gene transfer. *In vivo* targeted gene delivery to HSCs, T cells or B cells (see point 2) would mean a big step forward in the field of gene therapy for multiple reasons. This would reduce the risk of cell differentiation and loss of homing/engraftment capacity of the cells, as there is no need for purification and culture of the target cells. *Ex vivo* expansion of CAR expressing T cells before reinfusion clearly influences their phenotype and long term *in vivo* persisting T cells might disappear during the amplification process. Stimulation and culture of HSCs *ex vivo* also might induce the loss of ‘stemness’ of these cells. Additionally, *in vivo* gene transfer could target all HSCs in their stem cell niche, including those cells that are “missed” by purification based on surface antigens. Moreover, for the majority of diseases there is a requirement of a minimal number of gene-corrected cells to be re-infused to allow for efficient long-term correction. This requisite might become a limiting factor when treating children with inherited disorders, due to the low number of bone marrow CD34⁺ HSCs that can actually be isolated. All these obstacles could be overcome by using efficient *in vivo* HSC-specific LVs.

Vectors for *in vivo* HSC and T cell transduction ideally should be specific for the target cell, in order to avoid vector spreading and transduction of antigen presenting cells (APCs), which carries the risk of eliciting a transgene specific immune response leading to the elimination of transduced cells. Furthermore, conventional *ex vivo* T cell or HSC gene therapy remains a patient-specific treatment since it requires large amounts of clinical grade vectors and *ex vivo* production of gene modified autologous cells. Therefore, *in vivo* administration of the vector omits this labor-intensive and costly *ex vivo* process. Lastly, *ex vivo* HSC-based gene therapy approaches require conditioning of the patient with chemotherapy to make space in the bone marrow niche and allow engraftment of gene modified cells. Conditioning carries multiple

life-threatening risks and requires a sophisticated and expensive infrastructure which could be bypassed by *in vivo* gene delivery.

In vivo gene therapy might, in the future, consist of a single injection of this vectorized medicine into the blood stream, thus making this therapy more broadly available to patients while reducing costs, risks for the patients and potentially improving therapeutic outcomes.

5.2 The humanized mice: an indispensable model for in vivo studies

The development of mice humanized by xenotransplantation of human HSCs does not only provide a unique system to study differentiation, function and interaction of human blood cells, but also to evaluate the genetic modification by vectors targeted to specific cell types *in vivo*.

Two models, the NSG mouse [12, 13] and BALB/c Rag $2^{-/-}$, $\gamma c^{-/-}$ (BALB/c RAGA) [164] mouse demonstrate engraftment of hHSCs that is consistently higher than in all previously developed humanized mouse strains [165]. Meanwhile, improved humanized mouse models have been reported ([17, 20] and see introduction). In the future, these will be the better models to evaluate specificity of targeted vectors for transduction of specific cell types upon *in vivo* administration. But study of *in vivo* transduction of HSC and T cells for the moment mostly relies on the well characterized NSG and BALB/c RAGA mouse models.

5.3 Upgraded cytokine-displaying LVs allow in vivo gene transfer into hHSCs (Fig. 5A)

Classical VSV-G-LVs are not suited for *in vivo* HSC gene transfer. Firstly, they are highly sensitive to the human complement system and are rapidly degraded *in vivo*. Moreover, VSVG-LVs can transduce almost any kind of cell with the risk of transducing off-target cells such as APCs. In addition, the vast majority of HSCs is in a resting state *in vivo* [166] and VSV-G-LVs are not able to govern efficient gene transfer into unstimulated HSCs [146].

Therefore, a unique LV was designed displaying ‘early-acting-cytokines’ such as stem cell factor (SCF) at the surface to allow a mild and transient stimulation via ligation to its c-Kit receptor on HSCs and thereby preserving their stemness. Together with SCF, these authors displayed a fusion envelope, the RD114 envelope glycoprotein of an endogenous retroviral cat virus, which is highly resistant to human complement degradation [167]. More importantly, *in vivo* injection of the RD114/SCF-LVs into the BM cavity of humanized HSC-BALB/c RAGA mice resulted in augmented selective transduction efficacy in the CD34⁺-cells in the marrow of these humanized mice. Therefore, direct *in vivo* inoculation of the SCF/RD114-LVs might omit *ex vivo* handling, thus simplifying HSC-based gene therapy in the future, especially for bone marrow failures such as Fanconi Anemia [74]. Another study actually mobilized HSCs from the BM of humanized mice to perform *in vivo* HSC transduction in the blood stream. To reveal the low transduction levels of HSCs hidden in the BM, a chemio-selection marker was introduced in the HSCs, which allowed for the selective survival of the transduced cell progeny *in vivo* upon chemotherapeutic treatment [168].

5.4 Evaluation of in vivo CAR T cell gene therapy in humanized mice (Fig. 5B)

CAR modified T cells are potent tools for the treatment of cancer (see Point 2). As mentioned above, it would be of great benefit if the CAR-encoding vector could be injected directly *in vivo* to the target cell of choice, e.g. hCD4⁺ or hCD8⁺ T cells. LVs were engineered to specifically target hCD4⁺ or hCD8⁺ T cells through introduction of a scFv or a Designed Ankyrin repeat protein (DARPIN) directed against CD4 or CD8 epitopes into the measles virus glycoprotein H. These CD4-MV and CD8-MV retargeted vectors showed, respectively, exclusive transduction into the CD4⁺ or CD8⁺ subset of hT cells *in vitro* in PBMCs and *in vivo* in NSG mice humanized with human peripheral blood mononuclear cells (PBMCs) [169,

170]. Additionally, transductional targeting of B cells was achieved by direct inoculation of CD19-MV LVs into the bloodstream of hPBMC engrafted NSG mice [171, 172].

More recently, LVs were pseudotyped with receptor-retargeted Nipah virus glycoproteins (NiV-LVs) using a similar strategy [173]. Importantly, this research team has very recently taken a first step toward *in vivo* reprogramming of CAR T cells using CD8NiV-LVs retargeted to CD8⁺ T cells. A single administration of CD8NiV-LVs encoding a CD19-CAR in the blood stream of HSC-humanized NSG mice generated CD19-CAR expressing CD8 T cells *in vivo*, which led to the depletion of the CD19⁺ B cells from all hematopoietic tissues (Fig. 5B; [174]. Surprisingly, similar to patients that were reinfused with autologous CD19-CAR T cells generated *ex vivo*, the CD8NiV-LV injected humanized mice developed a cytokine storm [174]. Although the humanized mice in this context are extremely useful as a preclinical model for *in vivo* evaluation of vectors targeted to specific immune cells, prudence is warranted since findings cannot be directly extrapolated to patients since these mice do not recapitulate a fully functional human immune system. Clearly, further testing in non-human primates is required before moving to a clinical trial with *in vivo* CAR T cell gene therapy[137].

6. Conclusion

Humanized mice have been instrumental in evaluating safety, efficacy and specificity of hematopoietic gene therapy technologies of various nature and applications. What's more, they often provide the missing link between the proof of concept of novel gene therapy tools and their translation into the clinic. The improved mouse models that mimic human hematopoiesis and immune response even closer, will help the gene therapy field to address questions otherwise still out of reach and help the novel complex gene therapies under development to get translated to the clinic.

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Figure Legends

Fig. 1: Preclinical modeling of HSC gene therapy in humanized mice

A. Different monogeneic or infectious diseases and cancers for which hematopoietic stem cell gene therapies have been developed and evaluated in humanized mice.

B. The relevant preclinical data obtained for the different therapies indicated in (A).

Fig. 2: Recapitulation of anti-HIV HSC-based gene therapy in humanized mice

Cord blood or fetal liver human CD34⁺ cells are transduced with lentiviral vectors encoding for the different therapeutic approaches indicated, after which they are injected into NSG or BLT mice. Upon reconstitution with human blood system, the mice are challenged by HIV. Anti-HIV efficacy and possible toxicity of the gene therapy is evaluated over time as indicated.

Fig. 3: Different humanized mouse models for evaluation of CAR T-cell therapies

A. Xenograft mouse model, in which a human tumor cell line is engrafted in immunocompromised mice (NOD)/SCID γ c^{-/-} (NSG)), followed by infusion of human CAR T cells.

B. Patient derived xenograft (PDX) model consist of injection of a primary tumor biopsy from a patient, followed by infusion of human CAR T cells. In PDX mice, the tumor cells as well as the tumor microenvironment (e.g. immune cells) are present *in vivo*.

C. An autologous mouse model for human B-ALL. Two humanized mice using the hCD34⁺ progenitor cells and tissue from the same donor are produced. hCD34⁺ cells are engrafted into one NSG recipient, together with fetal thymic tissue to develop a humanized mouse with human immune-competence. In parallel, a second mouse is engrafted with CD34⁺ cells from the same human donor transduced with a B-ALL relevant oncogenic fusion gene, MLLAF9. When the second mouse develops hB-ALL, these autologous B-ALL cells are injected into the first immune-competent humanized mouse, thus generating a valid B-ALL model similar to the setting in patients. In this latter model, autologous antiCD19-CAR T are infused.

Fig. 4: Human B cell maturation into antibody secreting plasma cells after engraftment into NSG mice.

CD4⁺ T cells and B cell are isolated from the same healthy donor. B-cell are transduced with BaEV-LVs encoding for coagulation Factor IX (A) or encoding for a broadly neutralizing antibody against hepatitis C virus (B). Transduced B cells and CD4⁺ T cells are co-engrafted into NSG mice by IP injection. Sampling of blood is performed to evaluate the human chimerism in the blood of these mice. Once 50% of human cell engraftment is reached, the mice are sacrificed and the FIX concentration or the anti-HCV IgGs are determined in the serum of the humanized mice. The B-cell differentiation process toward plasmablast and plasma cells in the spleen of these mice is shown.

Fig. 5: Evaluation of gene therapy vectors *in vivo* in humanized mice

A. Cord blood CD34⁺ cells were injected into the Balbc-RAGA mouse model and humanized for 10-12 weeks before intrafemoral injection of the stem cell factor (SCF)/RD114-displaying lentivector encoding for GFP. 6-8 weeks later, the bone marrow from the injected femurs was isolated and evaluated for transduction of CD34⁺ cells (GFP⁺) by flow cytometry. The isolated CD34⁺ cells were then reinjected into secondary recipient Balbc-RAGA mice to confirm transduction of HSCs with serial engraftment potential.

B. Cord blood CD34⁺ cells were injected into the NSG mice and maintained for 10-12 weeks before injection of the CD8-targeted (CD8NiV)-LV encoding for a CAR directed against CD19 present on B cells. Mice were sacrificed at 5-12 weeks for FACS analysis of the immune cells. The CAR expression was revealed exclusively in hCD8 T cells, which were amplified *in vivo* through contact with CD19⁺ B cells. Upon contact these gene-modified CD8 T cells were able to eliminate the human B cells in various hematopoietic tissues (blood, spleen, bone marrow).

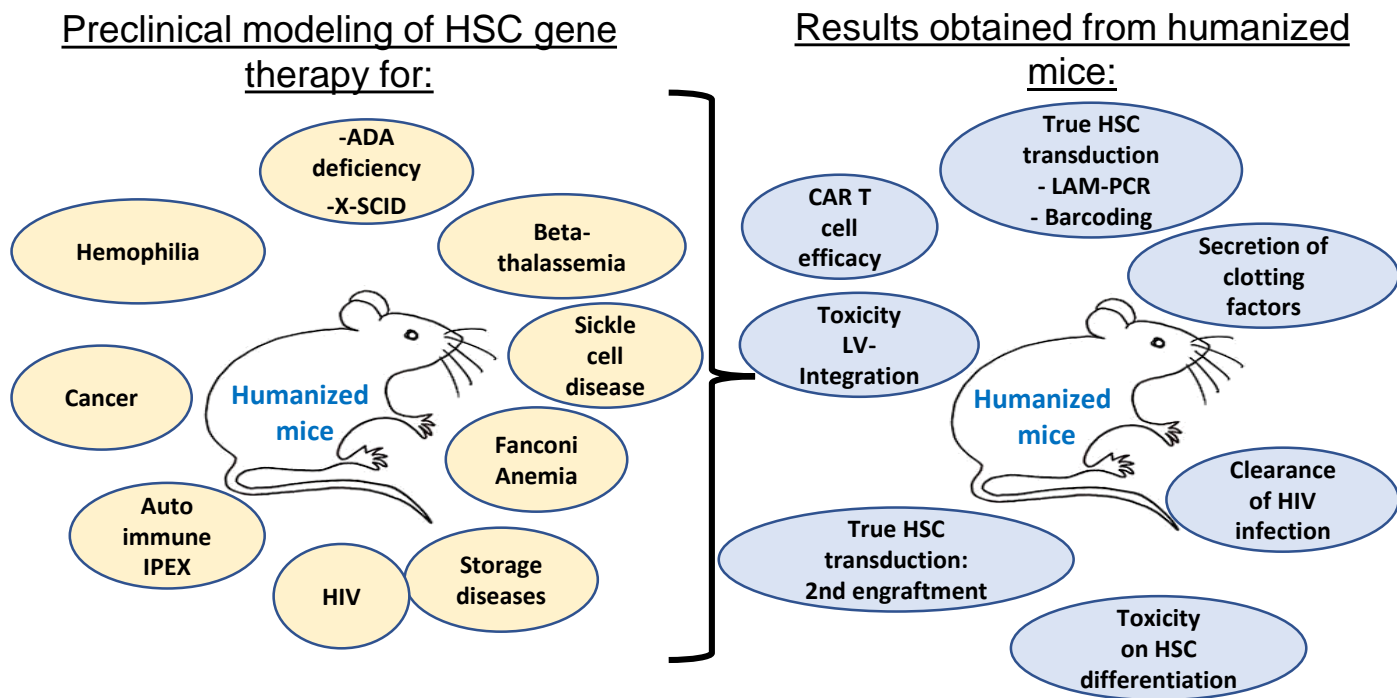


Figure 1

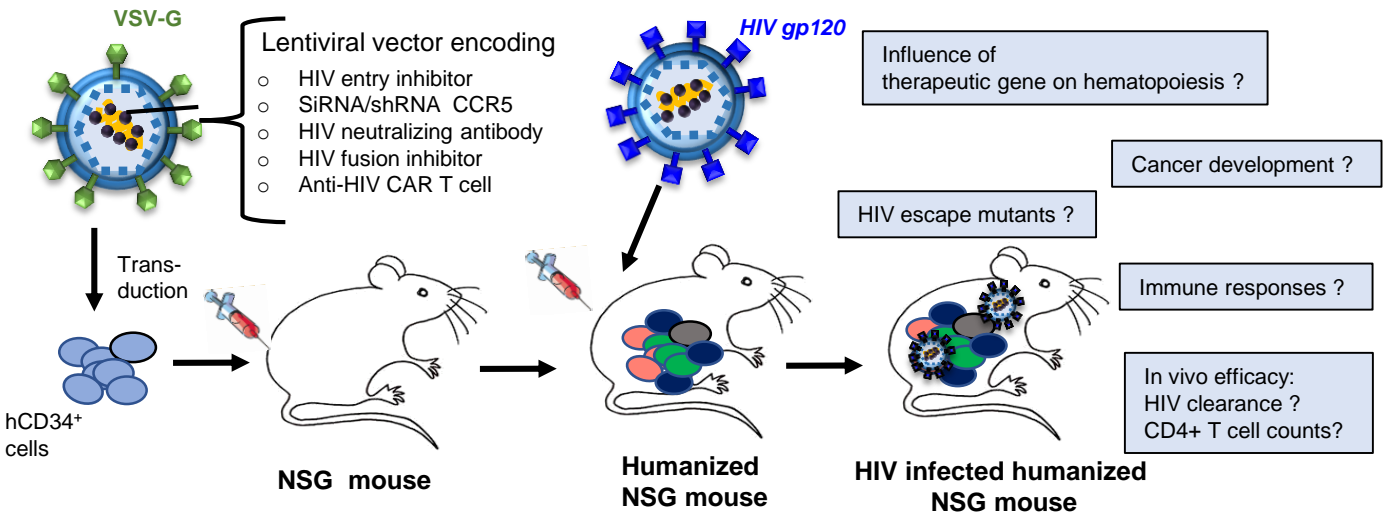
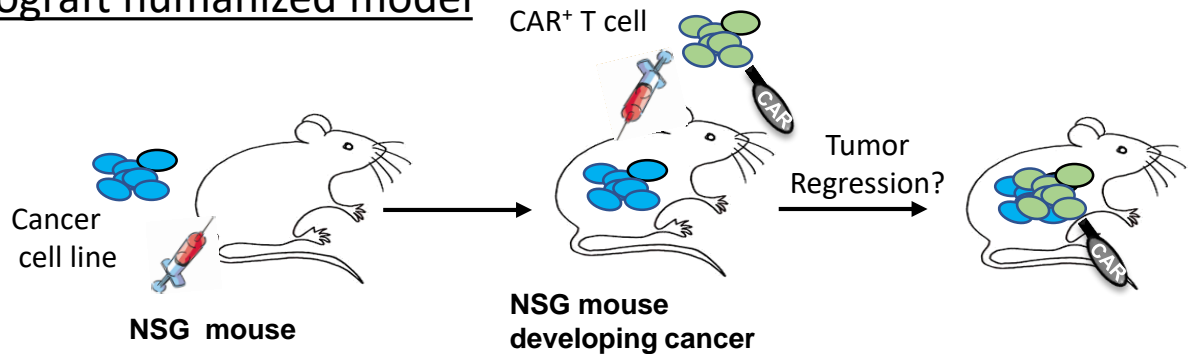
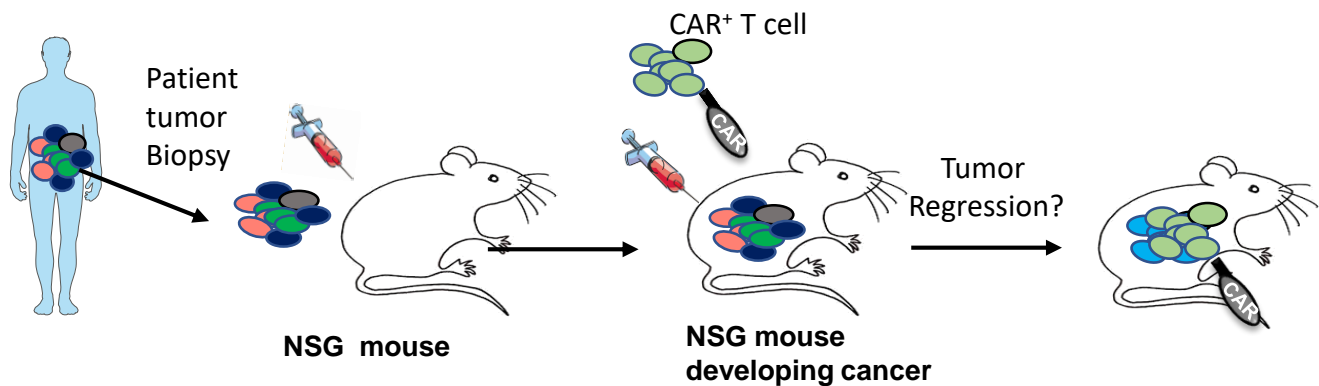


Figure 2

A. Xenograft humanized model



B. PDX humanized model



C. Fully autologous humanized tumor mouse model

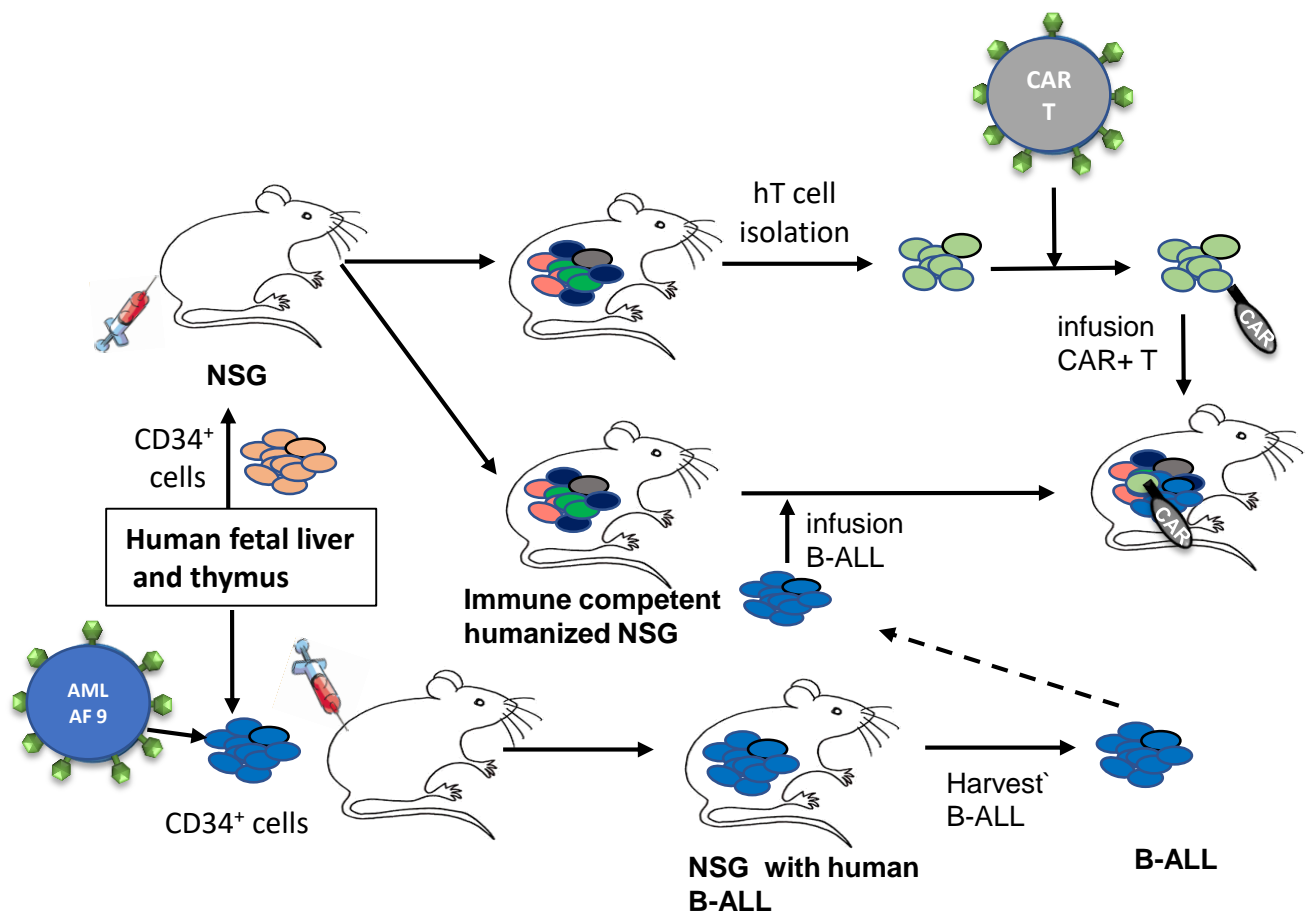


Figure 3

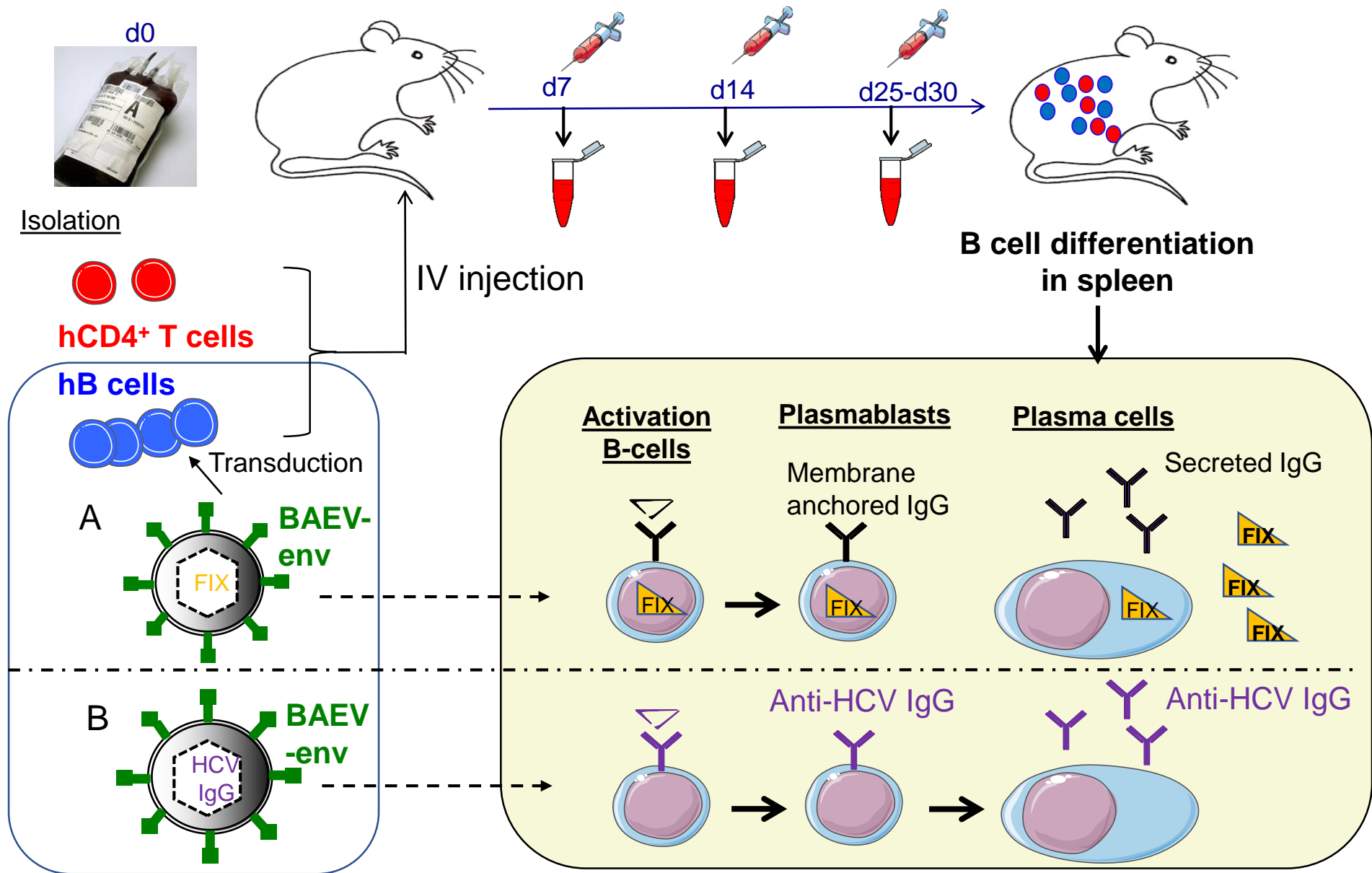
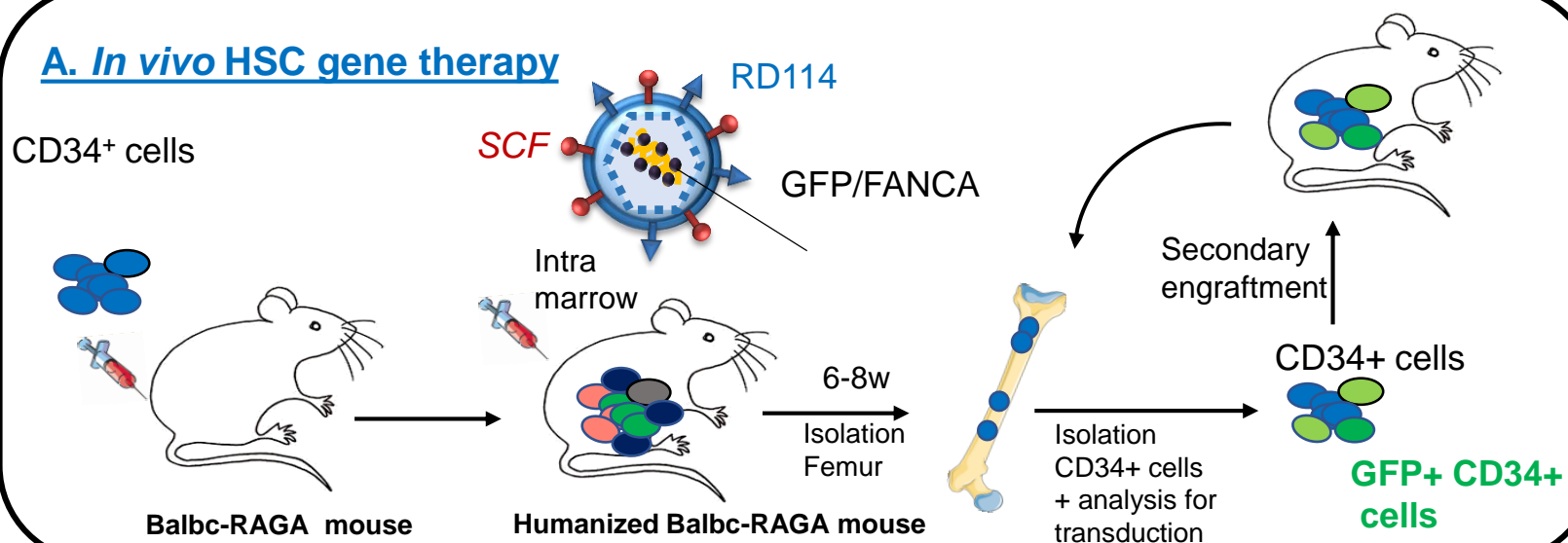


Figure 4

A. In vivo HSC gene therapy

CD34⁺ cells



B. in vivo T cell gene therapy

CD34⁺ cells

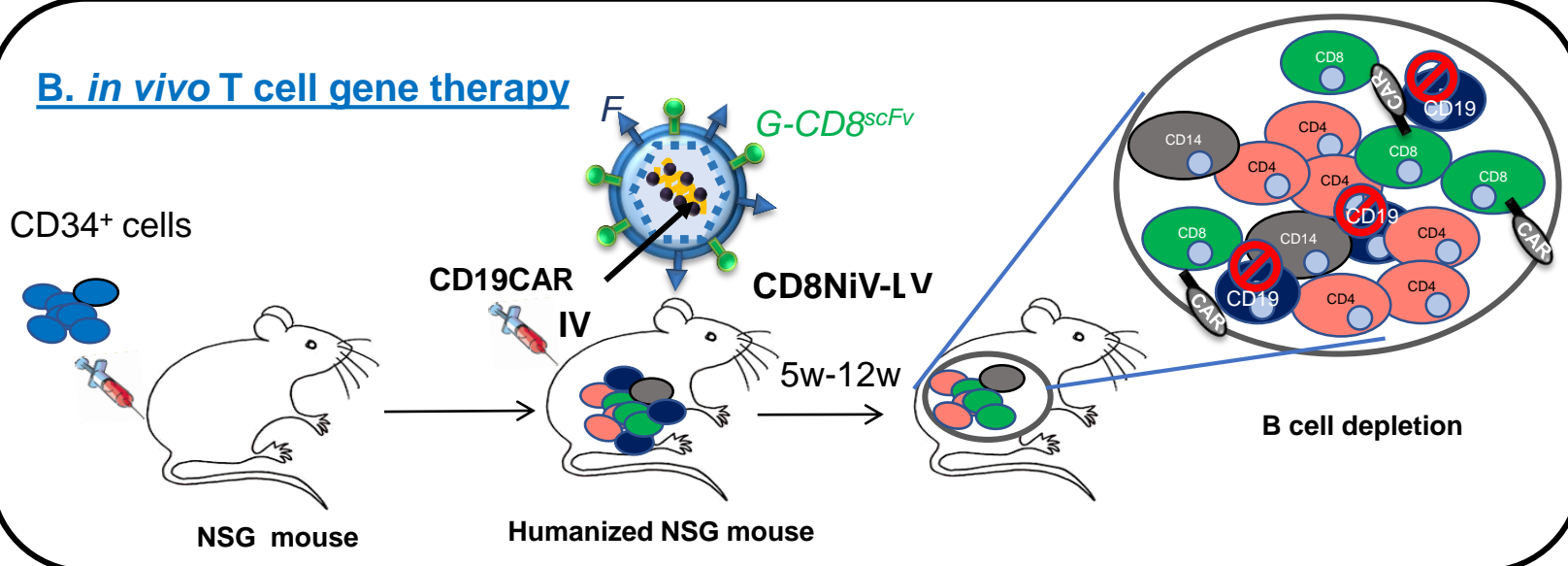


Figure 5

Table 1: Preclinical evaluation of HSC-based gene therapy in humanized mice models

Disease	Mouse model	Human cells infused	Results	REF
ADA-SCID	NSG	Healthy donor (HD) CD34 ⁺ cells transduced with ADA gRV	Efficient engraftment and safety pattern of genomic integration	31
SCID-X1	NSG	CD34 ⁺ cells transduced with IL2RG SIN-gRV	Long term hematopoietic reconstitution	38
	NSG	CD34 ⁺ cells transduced with IL2RG SIN-LV	- long term hematopoietic reconstitution - insertion site analysis showed correction in HSCs	40
	NSG	CD34 ⁺ cells corrected by gene editing	Efficient hematopoietic reconstitution	41,42
IPEX syndrome	NSG	CD34 ⁺ cells + FOXP3 LV controlled by constitutive promoter	- improved maintenance of most primitive HSCs - impaired T cell differentiation and responses	47
	NSG	CD34 ⁺ cells + FOXP3 LV controlled by FOPX3 regulatory elements	- correct differentiation of HSCs into Tregs	48
X-CGD	NSG	CD34 ⁺ cells transduced with gp91phox LV controlled by a chimeric promoter	- high levels of O ₂ · ⁻ production - higher myeloid engraftment levels	51
	NSG	CD34 ⁺ cells transduced with a gp91phox LV containing a synthetic promoter + miR126 target element	Myeloid lineage specific expression of gp91	52
	NSG	HSCs from X-CGD patient corrected by gene editing	Reversion of the mutation in 14% of long-term engrafted cells	53
	NSG deficient for CYBB	Disruption of the murine CYBB gene in CD34 ⁺ cells	- abrogates O ₂ · ⁻ production - resembles the disease in patients: higher sensitivity to infections so ideal as preclinical model	54
SCD	c-Kit mutant NSG	SCD-HSCs, in which the erythroid specific enhancer BCL11A is disrupted by gene editing	- induction of fetal hemoglobin - downregulation of adult sickling globin	65
Thalassemia	c-Kit mutant NSG	Thalassemia-HSCs, in which the erythroid specific enhancer BCL11A is disrupted by gene editing	- improved red blood cell hemoglobinization - red blood cell normalization	66
Fanconi anemia	NSG	PB CD34 ⁺ cells from FA patients corrected with a FANCA LV containing a constitutive promoter	- engraftment of corrected cells and proliferative advantage in vivo - similar myeloid and lymphoid differentiation capacity to HD	85
	NSG	HD CD34 ⁺ cells corrected by gene editing	Long term engraftment capacity in NSG mice	91

		targeting the safe harbor locus AAVS1		
	NSG	HD CD34 ⁺ cells targeted by NHEJ mediated gene editing	Efficient targeting of HSCs by NHEJ mediated gene editing	92
	NSG	FA PB CD34 ⁺ cells targeted by NHEJ mediated gene editing	Proliferative advantage of therapeutic indels	92
HIV	NSG	CD34 ⁺ cells transduced with LV expressing human neutralizing antibodies	- secretion of anti-HIV antibodies - delayed in HIV-1 viremia and hCD4 ⁺ cell maintenance in vivo	97
	NSG	CD34 ⁺ cells transduced with LV expressing soluble CD4 receptor	Reduction of HIV titer over time in vivo	98
	NSG and BLT mice	HD CD34 ⁺ cells transduced with LV downregulating HIV co-receptor CCR5	Generation of HIV resistant cell progeny in vivo	102-104
	NSG	HD CD34 ⁺ cells in which CCR5 is abrogated by gene editing	Normal in vivo differentiation capacity	105-108
	NSG	Modified CD34 ⁺ cells with a CAR-T directed against HIV	- differentiation into functional T and NK cells resistant to HIV - suppressed HIV replication	112
	NSG	CD34 ⁺ cells expressing 3 different anti-HIV genes	Multi-lineage hematopoiesis and CD4 T cells resistance to HIV in vivo	115-116

