



# The Pre-Clinical Development of Gene Therapy for DOCK8 Deficiency

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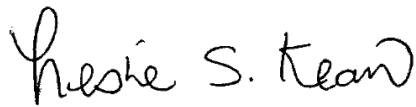
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**This Thesis, THE PRE-CLINICAL DEVELOPMENT OF GENE THERAPY FOR DOCK8 DEFICIENCY, presented by Roxane Labrosse and Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology has been read and approved by:**



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**Date: 04-21-2020**



THE PRE-CLINICAL DEVELOPMENT OF GENE THERAPY FOR DOCK8 DEFICIENCY

by ROXANE LABROSSE

A Thesis Submitted to the Faculty of

Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of *Master of Medical Sciences in Immunology*

in the Department of *Pediatric Hematology-Oncology*

Harvard University

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## ABSTRACT

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Roxane Labrosse

The pre-clinical development of gene therapy for DOCK8 deficiency

### Background

DOCK8 deficiency is a combined immunodeficiency leading to recurrent infections and severe allergic inflammation. If left untreated, survival rates into adulthood are low. While allogeneic hematopoietic stem cell transplant (HSCT) can cure this disease, suitable donors are not universally available, and morbidity such as graft-versus-host disease (GvHD) remains problematic. The development of novel curative therapies for these patients is therefore considered a high-priority.

### Objective

To develop a pre-clinical model of gene therapy (GT) for DOCK8 deficiency.

### Methods

To determine which viral vector would be best suited for in vivo transplantation experiments in Dock8<sup>-/-</sup> mice, we first generated and compared the expression of a codon-optimized cDNA of DOCK8 in a lentiviral (LV) versus alpharetroviral ( $\alpha$ RV) backbone by transducing the human Jurkat T cell line (DOCK8 null), and comparing DOCK8 expression of both vectors by flow cytometry analysis. We next evaluated the efficacy of various ubiquitous promoters (SFFV, MND, EFS) at driving DOCK8 expression using the same methods. To test whether these vectors could reconstitute DOCK8 function, we stimulated transduced Jurkat T cells with IL-21 and assessed restoration of STAT3 phosphorylation via flow cytometry. Vector copy numbers (VCN) were determined by qPCR.

## Results

The lentiviral construct consistently drove better DOCK8 expression (% DOCK8+ cells: 16.7% vs 2.0%,  $p < 0.01$ ; MFI: 256.7 vs 233.7,  $p = 0.02$ ) and better DOCK8 function (pSTAT3+ cells: 16.4% vs 3.1%). When compared to MND, the EFS promoter required a higher VCN for the same transduction efficiency (% DOCK8+ cells 35.6% vs 35.2%,  $p = 0.9$ ; MFI 275.3 vs 221.7,  $p = 0.005$ , mean VCN 5.3 vs 3.4 copies/cell,  $p = 0.02$ ). Finally, we discovered significant toxicity from overexpression of DOCK8 after transduction of murine HSCs.

## Conclusion

Taken together, these data indicate the potential of lentiviral vectors at driving high DOCK8 expression with restoration of function in the context of strong nonselective promoters, highlighting the feasibility of their use in gene therapy for DOCK8-deficient patients. However, we also uncovered unexpected toxicity from DOCK8 overexpression in HSCs. A vector with a more tightly regulated pattern of DOCK8 expression is therefore required to pursue in vivo GT experiments in a *Dock8*<sup>-/-</sup> mouse model.

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## CHAPTER 1: BACKGROUND

### What are primary immune deficiencies

Primary immune deficiencies (PIDs) are a group of rare yet life-threatening inborn diseases that originate from defects in genes that regulate the development or function of immune cells and proteins (1). To date, over 350 such monogenic diseases have been reported and typically present with an increased susceptibility to severe infections (2), although other features such as a predisposition to autoimmunity (3, 4), atopy (5) and cancer (6) are now better appreciated as being part of the clinical spectrum of PIDs. If left untreated, PIDs often lead to premature death secondary to fulminant or chronic infections (7).

### Current treatments for severe primary immune deficiencies

Supportive treatments such as prophylactic antimicrobial therapy and immunoglobulin replacement therapy are commonly used as preventive measures to preclude serious infections from occurring in these patients. In their most severe forms however, PIDs require a functional replacement of immune cells via allogeneic hematopoietic stem cell transplant (HSCT), commonly referred to as bone marrow transplant (BMT).

Although HSCT has made tremendous advancements in the past two decades and can effectively cure many PIDs (8-10), some limitations remain. One such obstacle is the requirement of an HLA-matched donor, of whom HLA-matched related donors (MRD) yield the best clinical outcomes. However, these optimal donors are only available in approximately 20-30% of cases (11). The remaining affected patients rely on worldwide donor registries, which do not universally find suitable donors and disfavour minority groups especially (12). Furthermore, the search of an appropriate donor can result in significant delays during which time immunocompromised patients remain vulnerable to acquiring serious infections. This is

of particular importance in PID patients, in whom the presence of active infections at the time of HSCT highly increases post-transplant related mortality (13).

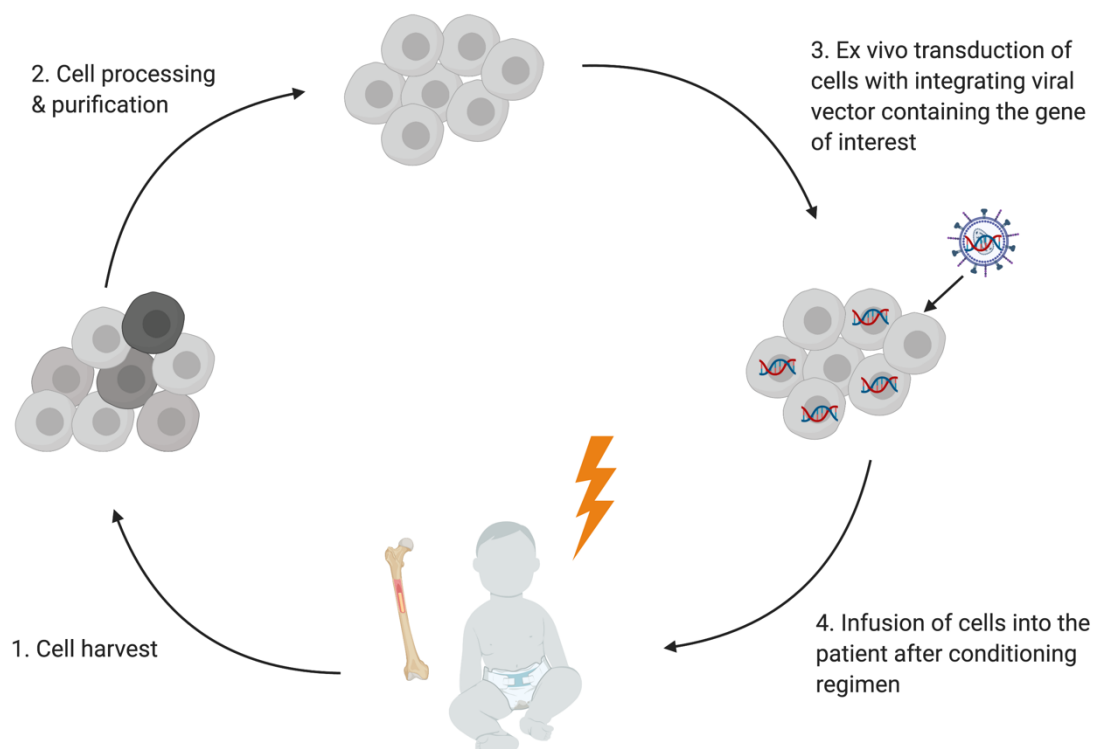
Haploidentical related donors (i.e. half-matched donors) have been used in some circumstances, but with much less success. Indeed, a greater mismatch between HLA alleles increases the risk of graft rejection and of graft-versus-host disease (GvHD). GvHD, perhaps the most devastating complication of HSCT, occurs when healthy donor immune cells recognize host MHC antigens as foreign and subsequently target the recipients organs, causing tissue damage and inflammation (14). This can lead to significant morbidity and mortality, and requires heavy immunosuppression which further compromises the immune system in this already vulnerable population (15).

Finally, myeloablative conditioning regimens are often required for successful engraftment of hematopoietic stem cells (HSCs), and act by creating bone marrow niches for donor cells to engraft and by preventing their rejection by host immune cells. However, the agents used for conditioning can cause significant acute toxicity such as profound immunosuppression, temporarily leaving the patients at risk for life-threatening infections. Long-term sequelae caused by these agents, such as infertility, pulmonary fibrosis, and secondary malignancies, are also of concern (16-19).

### Gene therapy for PIDs – an emerging curative alternative

The emergence of new molecular diagnostic tools in the past decade has led to a rapid expansion of known genetic causes for PIDs, which in turn has allowed the development of novel therapeutic opportunities that target their underlying defect (20-23). One such therapy is gene therapy (GT), in which autologous hematopoietic stem cells are manipulated ex vivo with integrating viral vectors expressing gene of interest and infused back into the patient after appropriate preparative conditioning (Figure 1) (24-28). Gene therapy is an exciting emerging

alternative to HSCT for many reasons. First, the use of autologous cells obviates the need to find a matched donor, since the patient himself acts as both donor and recipient. Moreover, the use of autologous cells effectively eliminates the risk of GvHD and significantly reduces the risk of graft rejection. Finally, the attenuated conditioning regimens used in GT could lower the risks of immediate and long-term toxicity.



*Figure 1. Ex vivo gene therapy for primary immunodeficiencies. Hematopoietic stem cells (HSCs) are collected from the patient either via bone marrow harvest or mobilization of peripheral blood HSCs. Cells are then processed and enriched for the CD34+ fraction and transduced with viral vectors that integrate a healthy copy of the defective gene. After conditioning regimen (used in most cases), corrected cells are reinfused into the patient for long-term engraftment.*

Clinical trials have already shown efficacy of GT in a variety of non-malignant diseases typically treated with HSCT, including PIDs such as X-linked and adenosine deaminase severe combined immunodeficiency (X-SCID and ADA-SCID), Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD) (29, 30) (Table 1).

Table 1. Past and current gene therapy trials for primary immunodeficiencies

Disease (gene)	Vector	Trial center(s) (ref)	N (total)	Efficacy	Genotoxicity	Oncogenic events
<b>X-SCID (IL2RG)</b>	$\gamma$ -retrovirus	France (31) UK (32) USA (33)	15	YES	YES	T-ALL (n=6, including 1 death)
	SIN $\gamma$ -retrovirus	USA, France, UK (34)	9	YES	NO	None to date
	SIN lentivirus	USA (35, 36)	14	YES	NO	None to date
<b>ADA-SCID (ADA)</b>	$\gamma$ -retrovirus	Italy USA UK (37)	55	YES	NO	None to date
	SIN lentivirus	UK & USA (37)	53	YES	NO	None to date
<b>X-CGD (CYBB)</b>	$\gamma$ -retrovirus	USA Germany & Switzerland UK (38) Korea (39)	23	NO/ Transient	YES	MDS (n=4, including 2 deaths)
	SIN $\gamma$ -retrovirus	Germany (NCT01906541)	-	Ongoing trial	Ongoing trial	Ongoing trial
	SIN lentivirus	UK, Switzerland, Germany & USA (40) France (NCT02757911) China (NCT03645486)	7	YES/ Ongoing trials	NO/ Ongoing trials	None to date
<b>WAS (WASP)</b>	$\gamma$ -retrovirus	Germany (41)	10	YES	YES	T-ALL (n=6, including 2 with secondary AML) AML (n=1) None to date
	SIN lentivirus	Italy (42) UK & France (43) USA (NCT01410825)	23	YES/ Ongoing trials	NO/ Ongoing trials	None to date
<b>LAD 1 (ITGB2)</b>	$\gamma$ -retrovirus	USA (44)	2	NO	NO	None to date
	SIN lentivirus	USA (NCT03812263), Spain (NCT03825783)	-	Ongoing trials	Ongoing trials	Ongoing trial
<b>ART-SCID (DCLRE1C)</b>	SIN lentivirus	USA (NCT03538899)	-	Ongoing trial	Ongoing trial	Ongoing trial

Abbreviations: ADA-SCID, adenosine deaminase-deficient severe combined immunodeficiency; AML, acute myeloid leukemia; ART-SCID, Artemis-deficient severe combined immunodeficiency; LAD 1, leukocyte adhesion deficiency type 1; MDS, myelodysplastic syndrome; ref, reference; SIN, self-inactivating configuration; T-ALL, T-cell acute lymphoblastic leukemia; WAS, Wiskott-Aldrich syndrome, X-CGD, X-linked chronic granulomatous disease, X-SCID, X-linked severe combined immunodeficiency.

Although GT appears to be a promising alternative to cure most if not all PIDs, it is imperative for each disease to be individually studied in pre-clinical models to confirm safety and efficacy before becoming readily available to humans. The importance of thoroughly studying the impact of gene correction in a gene-specific manner is distinctively illustrated by the results of pre-clinical studies evaluating the feasibility of GT for X-linked hyper-IgM syndrome (X-HIM) caused by mutations in the *CD40L* gene. In these studies, the replacement of *CD40L* using a gamma-retroviral ( $\gamma$ RV) vector in a murine *CD40L*<sup>-/-</sup> model showed restoration of class switching of B cells, thus effectively correcting the underlying immune defect. However, the constitutive expression of CD40L in T cells in the absence of strict physiologic regulation led to a severe T-lymphoproliferative disorder in the majority of the treated mice (45, 46). This phenomenon had not been reported in previous GT preclinical studies for other diseases and was an apparent consequence of the distinct role played by CD40L on immune cell activation. Studies are now underway to attempt using site-specific gene editing techniques that preserve the endogenous regulatory elements surrounding the *CD40L* gene with encouraging preliminary results (47, 48), further confirming the importance of performing these gene-specific studies.

To this end, and because of an unmet medical need for alternative curative therapies for patients suffering from DOCK8 deficiency, a life-threatening PID described in detail below, we sought to develop a pre-clinical model of GT for this devastating disease using both in vitro and in vivo models to demonstrate the feasibility, safety and efficacy of GT to cure DOCK8-deficient patients.

#### [DOCK8 deficiency – an orphan disease with poor prognosis](#)

Dedicator of cytokinesis 8 (DOCK8) deficiency is a rare combined immunodeficiency resulting from biallelic mutations in the *DOCK8* gene and leading to an atypical autosomal recessive form of the hyper-immunoglobulin E (IgE) syndrome (49). In addition to the inherent

vulnerability to infections, this disease is characterized by severe allergic inflammation, recurrent autoimmunity, and a predisposition to developing early-onset malignancies (50).

The DOCK8 gene was first identified in 2004 (51), and disease-causing loss-of-function mutations in the DOCK8 gene were soon after recognized in 2009 (52). This very large gene, located on chromosome 9, encompasses 48 exons that span over 200 kilobases (kB), and encodes the DOCK8 protein (molecular weight: 190 kDa). The majority of mutations associated with DOCK8 deficiency are deletions, which can be very large and often result in complete absence of protein expression (53, 54). The DOCK8 protein is part of the DOCK-C subfamily of DOCK proteins and acts as a guanine exchange factor (GEF), which can activate small guanine triphosphate binding proteins (GTPases) that integrate cell membrane signals and mediate actin cytoskeleton remodelling. DOCK protein family members each contain two essential domains (Figure 2): the Dock-homology region 1 (DHR-1) promotes localization to the cell membrane and subsequent signaling events, while the DHR-2 domain mediates the GEF activity responsible for the exchange of GDP for GTP (51, 55-57).

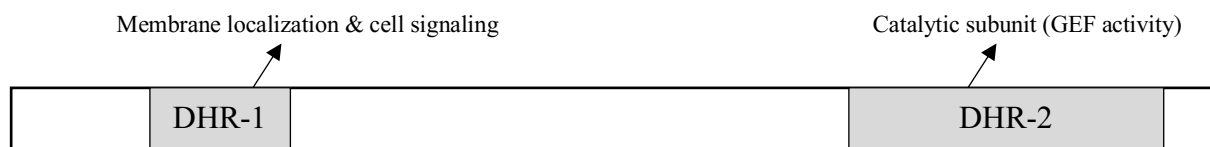


Figure 2. DOCK8 gene structure and protein functions. DOCK8-DHR1: amino acid residues 560-729; DOCK8-DHR2: amino acid residues 1632-2066.

DOCK8 is predominantly expressed in hematopoietic cells, where it serves many roles both in innate and adaptative immune cells. Indeed, DOCK8 deficiency results in defective innate immune responses due to impaired macrophage and dendritic cell migration (58, 59), decreased plasmacytoid dendritic cell number and function (60), low ILC3s (61), and defective NK cell cytotoxicity (62). The adaptive arm of the immune system is also broadly impaired in these patients, with a decrease in thymic T cell output resulting in low naïve T cells and low T-cell receptor excision circles (TRECs). Survival of CD8<sup>+</sup> T cells is severely compromised,

as well as memory responses of this immune compartment (63, 64). CD4<sup>+</sup> T cells are highly polarized towards a T<sub>H</sub>2 phenotype, leading to eosinophilia and high IgE levels (65). Interestingly, DOCK8 has recently been implicated in regulating signal transducer and activator of transcription 3 (STAT3) phosphorylation in T cells in a GEF-dependent manner and in facilitating the translocation of STAT3 into the nucleus (66). The importance of DOCK8 in enhancing the transcription of STAT3-dependent genes and consequent development of T<sub>H</sub>17 cellular immunity may help explain some of the overlapping clinical features seen in DOCK8 deficiency and in Job syndrome, the autosomal dominant hyper-IgE syndrome resulting from a loss-of-function mutation of *STAT3* (67-69). Finally, humoral immunity is also affected in DOCK8 deficiency, with patients displaying low memory B cells and variable levels of hypogammaglobulinemia in all antibody isotypes with the notable exception of IgE, which is almost universally elevated (70, 71).

With the many immunological compartments affected, it is therefore unsurprising that the clinical spectrum associated with DOCK8 deficiency is so broad and severe (50, 52, 65, 72-74). The profound immunodeficiency leads to recurrent sinopulmonary bacterial infections, severe viral infections that classically target the skin (such as human papilloma virus, herpes simplex virus, varicella zoster virus, molluscum contagiosum virus) and chronic mucocutaneous candidiasis. The severe allergic inflammation seen in DOCK8 deficiency includes a refractory atopic dermatitis, asthma, and IgE-mediated food allergies, a distinctive feature not seen in Job syndrome. The consequent immune dysregulation is also manifest by patients presenting autoimmune diseases such as autoimmune cytopenias, vasculitis and inflammatory bowel disease (75, 76). Finally, patients are also susceptible to developing early-onset cancers, specifically virally-driven neoplastic diseases such as squamous cell carcinomas (often human papilloma virus-driven) and lymphomas (often Epstein-Barr virus-driven). The paradoxical hypo- and hyperresponsiveness of the immune system to both internal and external antigens found in these patients highlights the intricate role of DOCK8 on regulating immune cells (Figure 3).

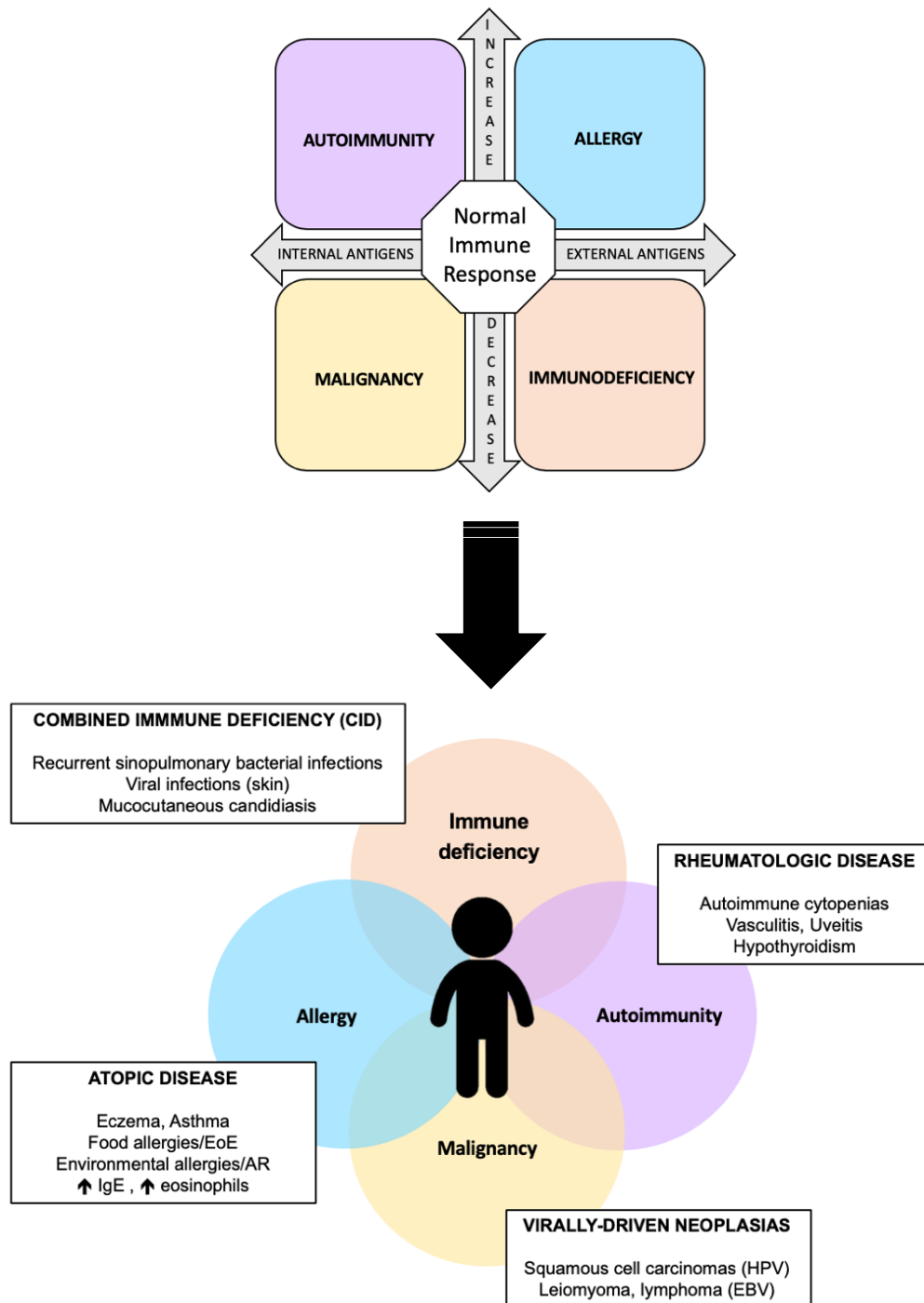


Figure 3. Clinical manifestations of *DOCK8* deficiency. AR, allergic rhinitis; EBV, Epstein-Barr virus; EoE, eosinophilic esophagitis; HPV, human papilloma virus.

If left untreated, the clinical consequences of this disease are devastating, with less than half of patients surviving beyond the second decade of life (50). Moreover, 80% of patients reaching adulthood will suffer major health events, defined as either life-threatening infections,

cerebral events (stroke, vasculitis, encephalitis), malignancy, or death, significantly affecting the quality of life of those few survivors.

Allogeneic hematopoietic stem cell transplantation has been shown to improve considerably DOCK8-associated immunodeficiency and atopic disease. In a case-series of 11 patients with DOCK8 deficiency who underwent HSCT, resolution of opportunistic infection and eczema occurred in all patients due to a robust reconstitution of cellular immunity with a predominance of healthy donor-derived T cells (77). In this study, patients had normalization of T cell numbers and phenotype, and 6 of 7 patients tested had restoration of antibody responses to vaccination (77). Similarly, in the largest retrospective analysis to date evaluating the outcomes of 81 DOCK8-deficient patients post-HSCT, the overwhelming majority of patients also showed improvement or resolution of their disease-related symptoms and immune defects (78).

While efficient, HSCT associates with potential morbidity and mortality as discussed above, hence the need for more specific approaches. Specifically, some high-risk DOCK8-deficient patients have suboptimal outcomes: survival is particularly poor after mismatched related (haploidentical) transplant, with only 66% overall survival at 2 years post-HSCT (78). Furthermore, GvHD remains one of the leading causes of mortality following transplant in this population. Curative therapy for DOCK8-deficient patients therefore remains a high-priority for future development, especially for those who lack well-matched donors.

#### GT for DOCK8 deficiency – Feasibility

DOCK8 deficiency is an ideal candidate for gene therapy development. Indeed, this large protein is primarily expressed in hematopoietic cells, which makes it a perfect target for correction through genetic manipulation of the hematopoietic stem cell compartment.

As previously mentioned, it has been well established that HSCT can correct the clinical and laboratory manifestations of immune dysfunction seen in patients with DOCK8 deficiency (77-80). Because improvements in both infection susceptibility and atopic disease have been described after successful allogeneic HSCT, clinical improvement is therefore also likely after gene therapy. Importantly, DOCK8-deficient patients with mixed chimerism also show clinical improvement following allogeneic HSCT (77). Data in murine models and in patients indicate a strong selective advantage for DOCK8 expressing cells in the T cell and memory B cell lineages, leading to a functional restoration of these important immunological compartments despite incomplete quantitative correction (70, 77). Moreover, patients with spontaneous somatic reversion with wild type levels of DOCK8 being expressed in T cell clones also exhibit an attenuated allergic phenotype (81, 82). Taken together, these observations suggest that transduction of only a portion of HSCs by means of GT could be sufficient to provide clinical benefit by rescuing T cell and B cell function beyond the clinically relevant threshold.

Finally, because of the success of GT trials for Wiskott-Aldrich syndrome (WAS), a closely related PID, GT also appears promising for DOCK8 deficiency. Indeed, trials of GT conducted in three Europe centers (Paris, London and Milan) have consistently shown sustained benefits in patients suffering from WAS (42, 43, 83). In our own experience, 5 patients with severe WAS treated with GT at the Boston Children's Hospital dramatically improved both cellular and humoral defects and clinical features associated with WAS such as eczema and infections (manuscript in preparation). In addition to the susceptibility to severe infections, DOCK8 and WAS share many clinical features including severe allergic inflammation, recurrent autoimmunity, and early-onset malignancies, perhaps due to their interactive role in regulating cellular cytoskeletal function in immune cells (84, 85). These trials therefore predict a potential similar benefit of GT for DOCK8-deficient patients than what has been observed in WAS GT trials.

## GT for DOCK8 deficiency – Unanswered questions

While GT appears to be a promising cure for DOCK8 deficiency, there are still many unknowns that need to be addressed in order to maximise efficiency and ensure long-term safety. Because GT's success is largely attributable to the use of optimal vectors, we focused our attention on determining which viral backbone in combination with which promoter would work best in vitro, to then pursue in vivo GT experiments in a Dock8<sup>-/-</sup> mouse model.

Indeed, although the efficacy of GT in PIDs was first demonstrated over 20 years ago, first-generation  $\gamma$ -retroviral ( $\gamma$ RV) vectors used in those initial clinical trials unfortunately had a high incidence of insertional oncogenesis, which led to the development of secondary hematological cancers in many patients (41, 86, 87). This unforeseen complication arose because of the transactivation of adjacent genes caused by the strong viral long terminal repeat (LTR) elements, which served as a strong enhancers to mediate transgene expression. Furthermore, the “semi-random” integration pattern seen with  $\gamma$ RV vectors, which preferentially targets transcriptional start site (TSS) regions, led to vector integration occurring near proto-oncogenes such as LMO2, ultimately leading to clonal cell proliferation and hematological cancers (41, 88-90). These genotoxic events were obviously deemed an unacceptable level of risk, and trials were globally put on hold for over a decade until safer, alternative vectors were developed.

Gene therapy clinical trials are now being conducted for these same PIDs (and more) using the next generation of vectors, which improve safety by deleting the enhancer elements in the U3 region of the LTR, termed self-inactivating (SIN) configuration, and by driving transgene expression with a weaker internal promoter. So far, no oncogenic event has been reported with these second-generation vectors, with now more than a decade of follow-up in some. Moreover,  $\gamma$ RV vectors have now largely been replaced by lentiviral (LV) vectors due to their more neutral insertion pattern. However, LV vectors also have some limitations. Although their

integration pattern show less skewing of integration near TSSs compared to  $\gamma$ RV, they pose a greater risk of disrupting the transcription units of active genes. Furthermore, since the theoretical packaging capacity of LV vectors does not exceed 7.8 kB, these vectors might be insufficient to accommodate large genes such as DOCK8 (6.4 kB).

Other than choosing an appropriate viral backbone, the use of the right promoter is also of crucial importance in GT. Indeed, although the replacement of LTRs by a weaker internal promoter is certainly safer, it also poses some challenges. In recent GT clinical trials for WAS, the  $\gamma$ RV vector initially used was replaced by a second generation SIN-LV vector. In lieu of intact LTRs, a 1.6kB fragment of the endogenous WAS promoter was used to drive expression of the human WAS cDNA (42, 43, 83). Although evidently safer, the weaker internal promoter used in these studies did nonetheless drive suboptimal WAS protein (WASp) compared to healthy controls, which is also in accordance with our own experience using this same vector (Supplement 1). Another example of the importance of choosing the right promoter is highlighted by initial GT studies conducted for chronic granulomatous disease (CGD), in which the spleen focus-forming virus (SFFV) LTR element was used as a potent promoter with initial success (38, 87). The clinical benefits were however not sustained over time due to methylation of the viral promoter which silenced transgene expression. This was further aggravated by the development of insertional mutagenesis leading to myelodysplasia in four treated patients (38, 87).

With this in mind, some of the questions that we wish to address with our pre-clinical GT studies are the following:

- 1) Can a gene as large as DOCK8 be properly expressed with current GT techniques?
- 2) What viral backbone is best suited for DOCK8 deficiency GT?
- 3) What promoter can drive optimal DOCK8 expression while limiting genotoxicity?
- 4) Can DOCK8 function be restored with GT?
- 5) Is there toxicity from overexpression of DOCK8 in hematopoietic compartments?

## CHAPTER 2: DATA

### Introduction

The main objective of these preclinical studies is to establish the safety and efficacy of GT for DOCK8 deficiency in a *Dock8*<sup>-/-</sup> murine model, and pave the way for future human applications. Our collaborators in the Geha lab have developed a *Dock8*<sup>-/-</sup> mouse model that exhibit many of the immunological features seen in patients with DOCK8 deficiency, including quantitative defects in total CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells, in naïve CD4 (CD44<sup>lo</sup>) T cells, in naïve CD8 (CD44<sup>lo</sup>) T cells and in marginal zone (MZ)-like B cells (CD23<sup>-</sup> CD21<sup>hi</sup> IgM<sup>hi</sup>) (Figure 4A-B). Moreover, these mice also replicate qualitative immune defects seen in humans such as impaired T cell proliferation after antigen challenge, defective germinal center formation and low antibody production after vaccination with KLH antigen (Figure 4C-D). These *Dock8*<sup>-/-</sup> mice were generated by introducing a premature stop codon in exon 9 of the *Dock8* gene, resulting in complete abrogation of DOCK8 protein expression (85). This is in contrast to a previously published *Dock8*<sup>-/-</sup> mouse model (*pri/pri* homozygous mutants) in which the animals inherit a Ser>Pro substitution in the critical DHR2 domain, predicted to disrupt important protein function (63, 70). Although this model also recapitulates the disease phenotype seen in humans, the vast majority of human DOCK8 mutations result from large deletions and complete lack of protein expression rather than protein dysfunction as per the *pri/pri* mutants' model. We thus plan to move forward with *in vivo* transplantation experiments using the *Dock8*<sup>-/-</sup> mouse model from the Geha lab, which best phenocopies the genetic anomalies seen in humans with this disease.

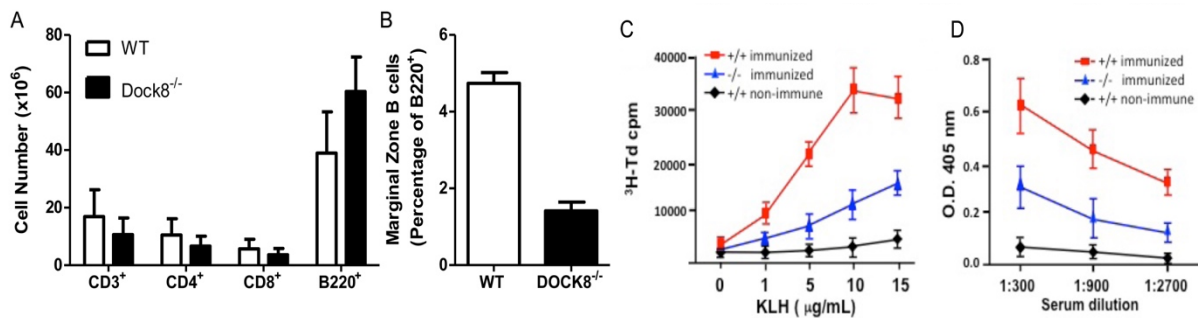


Figure 4. The immunological anomalies seen in *Dock8*<sup>-/-</sup> mice phenocopy those seen in humans. A) Comparison of T cell subset and B cell numbers in the spleen of *Dock8*<sup>-/-</sup> mice versus wild type (WT) mice. B) Comparison of marginal zone B cell levels in *Dock8*<sup>-/-</sup> mice versus WT mice. C) In vitro KLH-specific T cell proliferation following vaccination. D) Serum KLH-specific IgG following vaccination.

To evaluate the efficacy of GT at rescuing the immunological phenotype in *DOCK8* deficiency, we will use cohorts of lethally irradiated *Dock8*<sup>-/-</sup> mice, and inject them with WT lineage negative (LIN-) cells (recapitulating allogeneic HSCT), or with LIN- cells transduced with either a mock vector containing only a dTomato fluorescent protein (control mice), or with a *DOCK8*-dTomato vector (GT-treated mice). At 16-20 weeks post-GT, we will evaluate the immune reconstitution provided by each set of experiments (Figure 5). To test the hypothesis that both cellular and humoral immunity will be improved after GT, we will assess: i) improvement in T cell defects by determining total thymocyte number and distribution of CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup> and single positive CD4<sup>+</sup> or CD8<sup>+</sup> thymocytes, total CD3/CD4/CD8 numbers in spleen/lymph nodes, percentage of CD44<sup>lo</sup> naïve CD4 and CD8 T cells in spleen, lymph nodes, and blood and ii) improvement in B cell defects by determining numbers of transitional (CD93<sup>+</sup>), follicular (IgM<sup>int</sup> CD21<sup>int</sup>), MZ progenitor (IgM<sup>hi</sup> CD21<sup>hi</sup> CD23<sup>+</sup>) and MZB (IgM<sup>hi</sup> CD21<sup>hi</sup> CD23<sup>-</sup>) cells within CD19<sup>+</sup> splenocytes, and vaccine response to KLH antigen. We will also establish whether germinal center formation can be restored, and confirm the presence of fluorescent (transduced) cells at these sites.

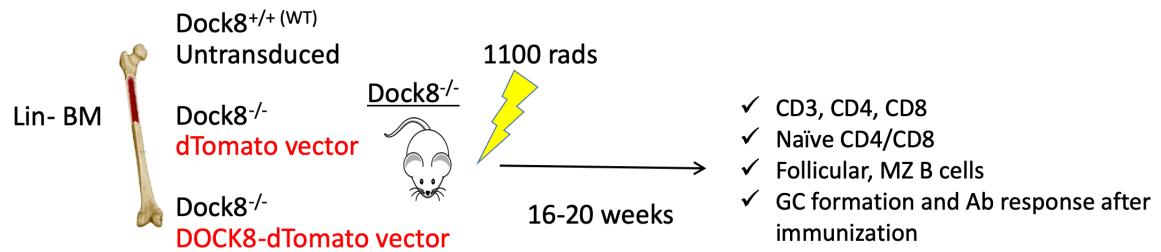


Figure 5. Schematic representation of planned GT in vivo experiments in a Dock8<sup>-/-</sup> mouse model

However, before moving forward with these in vivo experiments, many questions need to be addressed in order to determine which vector will be best suited for GT with thorough in vitro studies. To answer these questions, we have generated and tested a series of codon optimized DOCK8 cDNA-containing vectors with two different backbones and various promoters in order to assess which vector would drive the highest expression of DOCK8.

## Materials and Equipment

1. Plasmids – bacterial transformation & cloning
  - a. Lentiviral backbone (courtesy of Axel Schambach, Hannover, Germany)
  - b. Alpharetroviral backbone (courtesy of Axel Schambach, Hannover, Germany)
  - c. Linearized viral vectors containing the following genes: DOCK8, SFFV, MND, EFS, IRES, T2A (courtesy of Axel Schambach, Hannover, Germany)
  - d. One Shot™ Stbl3™ Chemically Competent E. coli (ThermoFisher, Catalog #C737303)
  - e. Difco LB Broth, Lennox (BD Biosciences, #240230)
  - f. LB Broth with agar, Lennox (Sigma, #L2897)
  - g. Ampicillin Sodium Salt (Fisher, # 69-52-3)
  - h. 14 mL round-bottom tubes
  - i. Erlenmeyer flasks
  - j. 42 °C water bath
  - k. Shaking incubator at 37 °C
  - l. PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Catalog #K210006)
  - m. PureLink™ HiPure Plasmid Miniprep Kit (Invitrogen, Catalog #K210003)
  - n. 50% glycerol solution
  - o. EcoDry Cloning Systems (Clontech, #638913)
  - p. PCR Thermocycler
2. Cell culture and primary cells
  - a. PBMC, fresh or thawed frozen
  - b. Ficoll-Paque PLUS (Sigma, #45001750LR)
  - c. HEK293T cell line
  - d. HT1080 fibrosarcoma cell line
  - e. Leukemic Jurkat T cell line
  - f. Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs)

- g. Advanced Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies, #12633-020)
  - h. Dulbecco's modified Eagle's medium (DMEM) media (ThermoFisher Scientific, #11995073)
  - i. Iscove's Modified Dulbecco's Media Formulation (IMDM) media (ThermoFisher Scientific, # SH3022801)
  - j. Fetal Bovine Serum (FBS)
  - k. Penicillin-streptomycin (Sigma, # P0781)
  - l. L-glutamine (Sigma, # G7513)
  - m. HEPES buffer 1M solution pH 7.3 (Fisher Scientific, Catalog #15630080)
  - n. 0.25% Trypsin-EDTA (Life technologies, #25200056)
  - o. Phosphate-buffered saline (PBS) 7.4 1X (Fisher Scientific, #10010-049)
  - p. T25 flasks (Corning, #430639)
  - q. Cell culture dish 10mm (Corning, # 430167)
  - r. 1000 mL 0.22um vacuum filter system (Corning, # 430517)
  - s. Cryotubes (ThermoFisher Scientific, # 377267)
3. Transfection and transduction reagents
- a. Calcium Phosphate Transfection Kit (Invitrogen, Catalog #K2780-01)
  - b. Chloroquine Diphosphate Salt (Fisher Scientific, #ICN19391910)
  - c. 0.45 um syringe filter (Fisher Scientific, # 09-720-005)
  - d. 20 mL Syringe (ThermoFisher Scientific, # 14555560)
  - e. 20% sucrose solution
  - f. SW28.1 Swinging-Bucket Rotors
  - g. Ultracentrifuge
  - h. Polybrene (Sigma, #TR1003G)
  - i. LentiBOOST (courtesy of Myriam Armant, TransLab)
4. Flow cytometry, FACS antibodies & phosphoflow assay
- a. BD LSRFortessa™ cell analyzer

- b. BD FACSDiva™ Software
- c. FlowJo™ Software Version 8.8.7
- d. Falcon round-bottom FACS tubes (Falcon, #352054)
- e. FACS buffer (PBS with 1% FBS)
- f. BD Fixation/Permeabilization Solution Kit (BD Biosciences, #554714)
- g. BD Phosphoflow Perm Buffer III (BD Biosciences, #558050)
- h. BD Cytofix Fixation Buffer (BD Biosciences, #554655)
- i. Annexin V Binding Buffer (Biologend, # 422201)
- j. Recombinant human IL-21 (Peprotech, #200-21-10ug)
- k. FACS antibodies (see list below)

Target	Manufacturer	Identifier	Clone	Conjugated dye	Dilution
<b>Human CD3</b>	BD	#555340	HIT3a	PE	1:5
<b>Human CD20</b>	Biologend	#302324	2H7	PerCP	1:20
<b>Human CD14</b>	BD	#557831	MφP9	APC-Cy7	1:20
<b>DOCK8 mouse monoclonal IgG1</b>	Santa Cruz Biotech	sc-376911	G-2	-	1:50
<b>Mouse IgG1 isotype</b>	Biologend	#400102	MPOC-21	-	1:125
<b>Anti-mouse IgG1</b>	Biologend	#406606	RMG1-1	FITC	1:500
<b>Mouse anti-STAT3 (pY705)</b>	BD	#557817	4/P-STAT3	Alexa Fluor 647	1:10
<b>Annexin V</b>	Biologend	640919		APC	1:20
<b>Mouse Ly-6G</b>	StemCell Technologies	60031Fl.1	1A8	FITC	1:100
<b>Mouse CD11b</b>	StemCell Technologies	60001Fl.1	M1/70	FITC	1:100
<b>Mouse CD45R</b>	StemCell Technologies	60019Fl.1	RA3-6B2	FITC	1:100
<b>Mouse CD3e</b>	StemCell Technologies	60015Fl.1	145-2C11	FITC	1:100
<b>Mouse TER119</b>	StemCell Technologies	60033Fl.1	TER-119	FITC	1:100
<b>Mouse CD19</b>	StemCell Technologies	60006Fl.1	6D5	FITC	1:100
<b>Mouse CD177</b>	StemCell Technologies	60025AZ.1	2B8	FITC	1:100
<b>Mouse Sca1</b>	StemCell Technologies	60032PE.1	E13-161.7	PE	1:100

## 5. qPCR

- a. Absolute qPCR mix, ROX (ThermoFisher, # AB1139A)
- b. Microamp Optical 96-well Rxn Plate (Life Technologies, # N8010560)
- c. ThermalSeal RT2RR™ Sealing Films (Excel Scientific, # TS-RT2RR-100)
- d. DNeasy Blood & Tissue Kit (Qiagen, # 69504)
- e. Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument
- f. Oligonucleotides

Oligonucleotide	Sequence	5' modification
<b>233 LentiPsi Fwd</b>	CAGGACTCGGCTTGCTGAAG	
<b>233 LentiPsi Rev</b>	TCCCCGCTTAATACTGACG	
<b>233 LentiPsi Probe</b>	CGCACGGCAAGAGGCGAGG	FAM
<b>GTDC1 Fwd</b>	GAAGTTCAGGTAAATTAGCTGCTG	
<b>GTDC1 Rev</b>	GGCACCTTAACATTTGGTTCTG	VIC
<b>GTDC1 Probe</b>	ACGAACTTCTTGGAGTTGTTTGCT	

## 6. Mouse procedures

- a. C57BL/6 mice
- b. Scalpel & scissors
- c. Autoclaved mortar
- d. 40 um cell strainer
- e. 1X RBC lysis buffer (BD Biosciences, #555899)
- f. EasySep™ Mouse Hem Prog Isolation Kit (StemCell Technologies, #19856)
- g. EasySep™ Magnet (StemCell Technologies, #18000)
- h. EasySep™ Buffer (StemCell Technologies, #20144)

## 7. Other

- a. Biosafety cabinet
- b. Centrifuge
- c. 37 °C water bath
- d. Stationary CO<sup>2</sup> incubator at 37 °C
- e. Calibrated pipettes
- f. Autoclave

## Methods

### Plasmids

For biosafety reasons, we utilized a third generation split-packaging system for vector manipulation consisting of i) the self-inactivating lentiviral vector (SIN-LV-promoter-insert), ii) the lentiviral gag/pol (LV-GP) helper plasmid and iii) the RSV-REV helper plasmid, and iv) the VSVg envelope. Similarly, for the  $\alpha$ RV, a i) self-inactivating alpharetroviral vector (SIN- $\alpha$ RV-promoter-insert) was used in combination with ii) the alpharetroviral gag-pol ( $\alpha$ GP) helper plasmid and with iii) either the VSVg or the RD114/TR envelope.

### Cloning of vectors

Plasmid cloning was performed by PCR method using the In-Fusion cloning system (Takara Bio). Briefly, the viral backbone (either lentivirus or alpharetrovirus, courtesy of A. Schambach) were first linearized using restriction enzymes, followed by agarose gel separation and gel purification to isolate the linear vector backbone. In parallel, we designed gene-specific primers with 15 bp overhangs complimentary to the vector ends flanking the insert gene of interest using the In-Fusion Cloning primer design tool (Takara Bio). The DNA insert was amplified via PCR using the CloneAmp HiFi PCR pre-mix, and the PCR product was ran on gel and purified using the Nucleospin Gel & PCR clean-up kit. Ligation of the insert DNA to the viral backbone was accomplished with the In-Fusion cloning reaction (Takara Bio), as per the manufacturer's instructions. Competent *E. coli* were then transformed with the reaction mixture, and individual clones were screened for confirmation of the desired sequence via Sanger sequencing.

### Production of viral supernatant

The human embryonic kidney cell line 293T (HEK 293T) was used for viral production. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Approximately  $5 \times 10^6$  HEK 293T cells were plated 24h prior to transfection, to obtain 70% cell confluency the following day. Four

hours prior to transfection, chloroquine (25  $\mu$ M) was added to the culture media. Standard calcium phosphate methods were used for co-transfection with the following lentivirus plasmid proportions: SIN-LV-prom-DOCK8-marker (12  $\mu$ g), LV-GP (12  $\mu$ g), RSV-REV (5  $\mu$ g) and VSVg (2  $\mu$ g). For the alpharetrovirus, the following proportions were used: SIN- $\alpha$ RV-prom-marker-DOCK8 (10  $\mu$ g),  $\alpha$ GP (2.5  $\mu$ g), VSVg (1.5  $\mu$ g). Forty-eight hours after transfection, viral supernatant was collected, filtered through 0.45  $\mu$ m pore filters, and centrifuged at 10,000 RPM for 12h for virus concentration, before storage at -80  $^{\circ}$ C.

#### Viral titting

The day before transduction, a total of  $1 \times 10^5$  HT1080 cells were plated in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Prior to transduction, polybrene (8  $\mu$ g/mL) was added to the media. Supernatant containing viral particles was added at the following dilutions: 1:100, 1:1000, and 1:10,000. Transduction was aided by centrifugation of cells (1500g x 60 minutes at room temperature). Cells were harvested for flow cytometry analysis 48h after transduction, and viral titers were calculated based on the percentage of cells carrying the fluorescent marker of interest (GFP or dTomato).

#### Transduction of Jurkat cells

The day of transduction, Jurkat cells were resuspended at a concentration of  $2 \times 10^5$  cells/mL in Advanced Roswell Park Memorial Institute (aRPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine and 2.5% HEPES buffer. Polybrene was also added to the media at a concentration of 8  $\mu$ g/mL. Supernatant containing viral particles was added a various multiplicity of infection (MOI), ranging from 1 to 50. Transduction was aided by centrifugation of cells (1500g x 60 minutes at room temperature). Cells were collected and analyzed by flow cytometry 48h after transduction.

#### Transduction of murine LIN- cells

Bone marrow cells were harvested from wild type (WT) C57BL/6 mice and murine LIN- were isolated by magnetic separation using the EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell Technologies). Purified LIN- cells were then resuspended at a concentration of  $2 \times 10^6$  cells/mL in media containing Iscove's Modified Dulbecco's Media Formulation (IMDM) supplemented with 10% FBS and 1% Penicillin-Streptomycin. The following cytokines were also added to the media for pre-stimulation: MGDF (100 ng/mL), hGCSF (100 ng/mL), and mSCF (100 ng/mL). Cells were cultured in this cytokine-rich media for 48h, after which time polybrene was added to the media (8  $\mu$ g/mL) and two transductions were performed 8-12h apart using viral supernatant. Both transductions were aided by centrifugation of cells (1500 rpm x 60 minutes at room temperature). Cells were collected and analyzed by flow cytometry 48h after transduction.

#### DOCK8 staining

Cells were washed with FACS PBS (PBS with 1% BSA) and  $5 \times 10^5$  cells were resuspended in 100  $\mu$ L of FACS PBS in round bottom FACS tubes. When relevant, cells were incubated for 30 min at 4 °C with external surface staining antibodies, then washed twice with FACS PBS. Cells were then resuspended in 250  $\mu$ L of Fixation/Permeabilization Solution (BD Biosciences) for 20 minutes at 4 °C. Cells were then washed twice with the BD Perm/Wash™ Buffer (BD Biosciences). This was followed by a first step of internal staining with the primary DOCK8 antibody (or a mouse IgG1 isotype control) for 30 minutes at 4 °C, with cells again being washed twice with the BD Perm/Wash™ Buffer before being stained with the secondary anti-mouse IgG1 FITC-conjugated antibody for 30 minutes at 4 °C. A final washing step was performed with the BD Perm/Wash™ Buffer before cells were resuspended and analyzed by flow cytometry on the BD LSRFortessa™ cell analyzer.

#### Annexin V staining

Cells were washed with FACS PBS (PBS with 1% PBS) and resuspended in Annexin V Binding Buffer (Biolegend) at a concentration of  $1 \times 10^6$  cells/mL. Then, 100  $\mu$ L of this solution was transferred to a round-bottom FACS tube. APC-conjugated Annexin V antibody was then added to the cells and incubated at room temperature for 15 min. Finally, 400  $\mu$ L of Annexin V Buffer was added to the cells prior to flow cytometry analysis.

#### Determination of vector copy number by qPCR

Genomic DNA was first extracted from cells using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Quantification of vector copy number (VCN) of transduced cells was obtained using a TaqMan real time PCR assay with primers and probes targeting i) the vector-specific lenti-psi region and ii) the house-keeping gene GTCD1. A reference sample containing 1 integration site was used as a control for the assay.

#### STAT3 phosphorylation assay

A total of  $1 \times 10^5$  cells were plated in a 96-well plate in aRPMI 1640 media without any FBS supplementation (serum starvation) and rested for 2-6 hours prior to stimulation. Cells were then stimulated with IL-21 (100 ng/mL) for 30 min, and then immediately fixed in BD Cytfix™ Fixation Buffer (BD biosciences) for 20 min at 4 °C. After centrifugation and removal of supernatant, cells were resuspended in BD Phosphoflow Perm Buffer III (BD biosciences) for 30 min at 4 °C. Cells were then washed twice with FACS buffer, and stained with the mouse anti-STAT3 (pY705) AF647-conjugated antibody (BD biosciences) for 60 minutes at room temperature. Finally, cells were washed again and resuspended for flow cytometry analysis.

## Results

DOCK8 expression can be determined by flow cytometry

Because DOCK8 is an intracellular protein located within the cell cytoplasm, its detection has typically been achieved with immunoblotting assays of cell lysates. Dr Pai and colleagues have however developed an assay that allows for the rapid detection of DOCK8 via flow cytometry (91). This technique offers many advantages over immunoblotting, such as the ability to simultaneously stain for cell-specific surface markers (Figure 6A) and to precisely determine protein expression levels via the mean fluorescent intensity (MFI). Moreover, DOCK8 intracellular staining in EBV LCL lines derived from healthy donors and DOCK8-deficient patients demonstrate the highly specific quality of this assay (Figure 6B). We therefore used this technique to determine levels of DOCK8 expression following transduction of primary cells and cell lines using our different vectors.

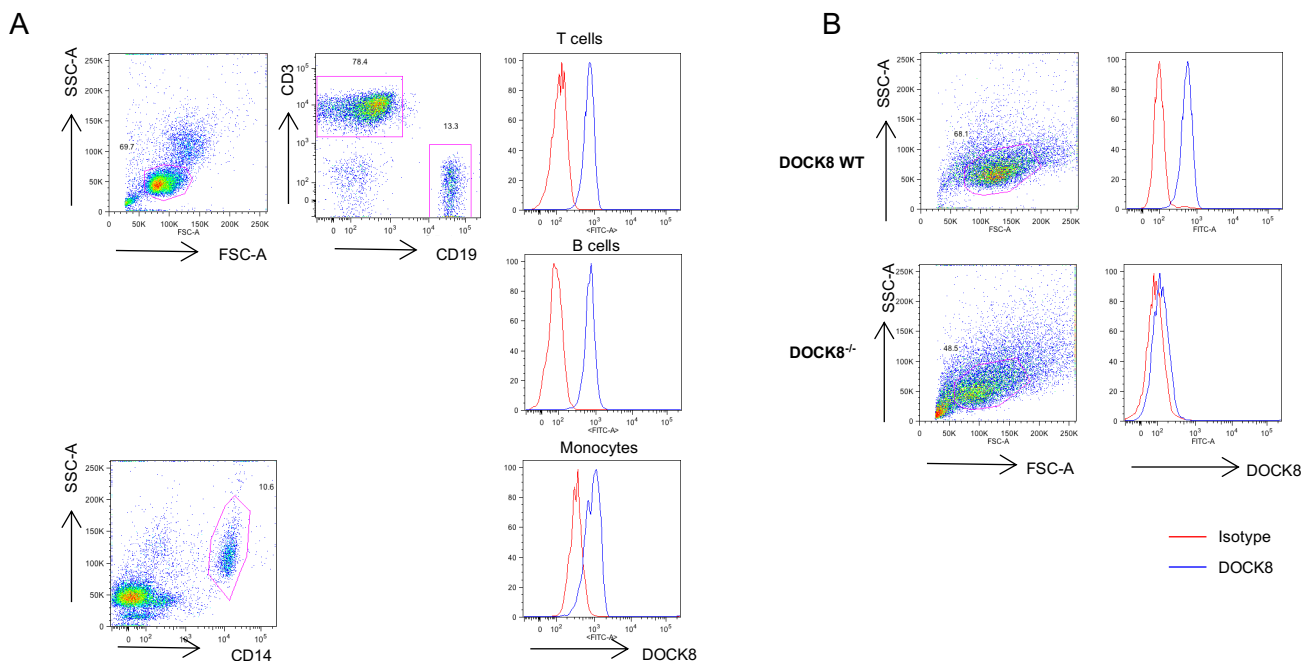


Figure 6. Detection of DOCK8 protein via intracellular staining and flow cytometry analysis in A) Peripheral blood mononuclear cells (PBMCs) from healthy donors, specifically in T cells (CD3+), B cells (CD19+) and monocytes (CD14+) and B) EBV LCLs derived from a healthy donor (WT) and a patient with DOCK8 deficiency (DOCK8<sup>-/-</sup>).

Co-expression of DOCK8 and a fluorescent marker is achieved using bicistronic vectors

Because of the intracellular localization of the DOCK8 protein, its detection with flow cytometry methods requires cell fixation and permeabilization prior to staining, thus rendering any downstream functional assay with live cells impossible. We therefore generated multicistronic vectors containing either the green fluorescent protein (GFP) or the dTomato (dT) fluorescent protein as markers to better track transduced cells. The use of a fluorescent marker to identify transduced cells also allowed for rapid assessment of transduction efficiency and facilitated any experiments that required cell sorting.

Presumably due to the very large DOCK8 insert (6.4 kB), the co-expression of DOCK8 and fluorescent markers was initially inconsistent, which prompted us to design vectors in both the DOCK8-marker and marker-DOCK8 configurations within lentivirus (LV) and alpharetrovirus ( $\alpha$ RV) backbones using the strong SFFV promoter (Figure 7). In addition, we generated these bicistronic vectors using both the IRES element and the T2A peptide to separate these inserts. The T2A peptide offers the advantage of being significantly shorter than the IRES element (approximately 60 bp versus 600 bp), which is particularly relevant in the context of our already large insert (Figure 7).

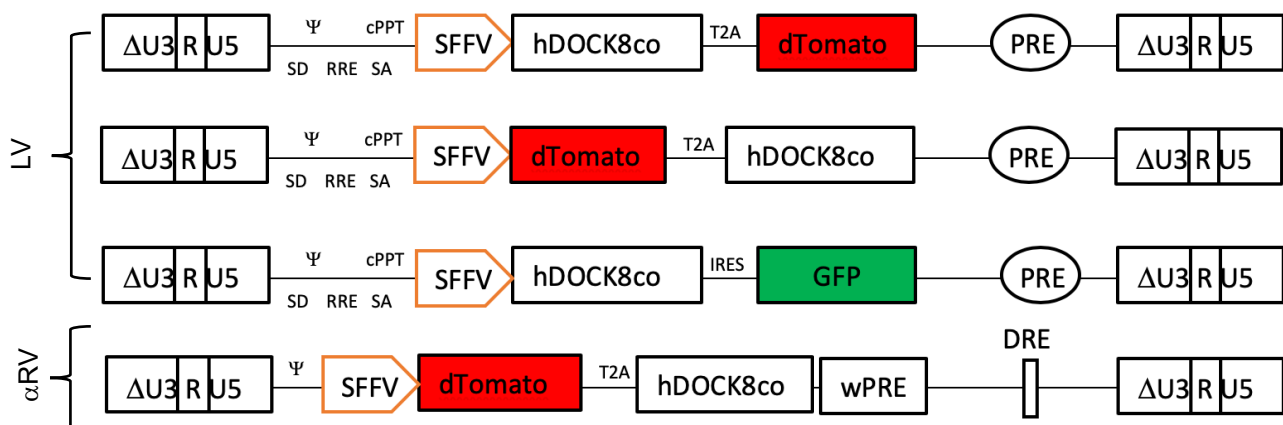


Figure 7. Design of various configurations of bicistronic lentiviral (LV) and alpharetroviral ( $\alpha$ RV) vectors generated for transduction experiments

The optimization of transfection and transduction techniques finally allowed us to obtain simultaneous expression of both DOCK8 and fluorescent markers in all of the vectors tested, independently of the gene order (DOCK8-marker vs marker-DOCK8), of the gene separating element used (IRES vs T2A) or of the viral backbone (LV vs  $\alpha$ RV) used when transducing either HT1080 cells (Figure 8) or Jurkat cells (data not shown), two human cell lines that do not express DOCK8. We consequently concluded that, moving forward, we could reliably use dTomato or GFP fluorescence as a surrogate for DOCK8 expression when using these vectors.

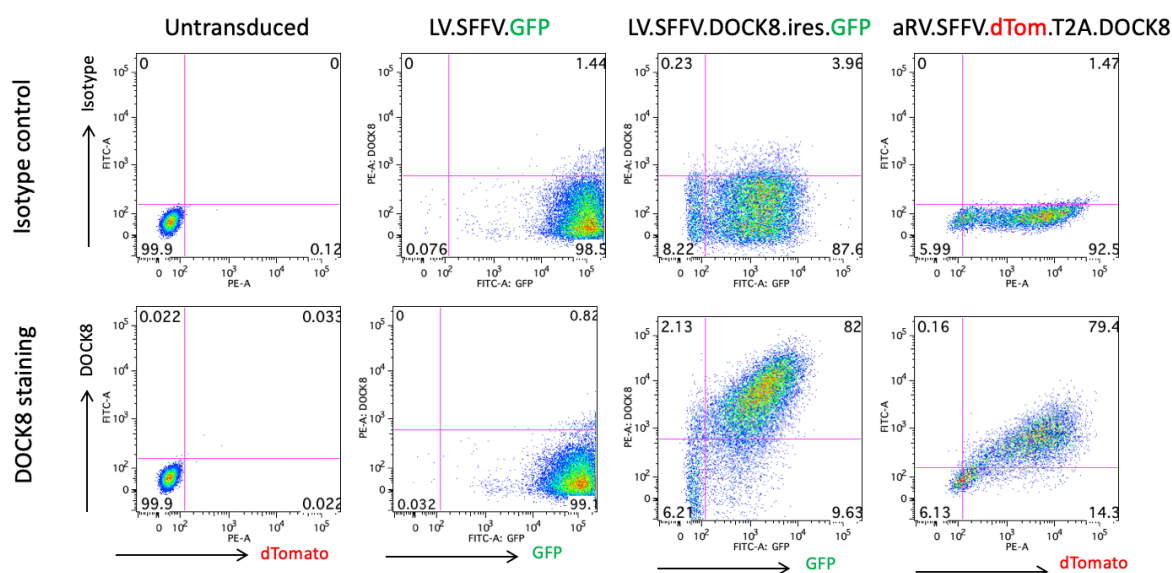


Figure 8. Representative FACS plots of HT1080 cells transduced with various vectors

The lentiviral backbone leads to superior DOCK8 expression compared to the alpharetroviral backbone

We then sought to determine which viral backbone would lead to greater expression of DOCK8. To this end, we compared the efficacy of a self-inactivating lentiviral backbone (SIN-LV) with that of a self-inactivating alpharetroviral backbone (SIN- $\alpha$ RV). As mentioned above, lentiviruses have become the norm in terms of clinical use for GT due to their well-studied safety profile. Indeed, the relatively neutral insertion pattern seen with LV compared to  $\gamma$ RV,

paired with the SIN configuration, has led to many successful GT clinical trials in which no oncogenic events have occurred to date. Moreover, lentiviruses do not require cell mitosis in order to integrate their genetic material into a host's genome, making them potent vectors for transducing HSCs in which cell division and potential differentiation is undesirable (92). However, because of the size of the DOCK8 gene and their maximal packaging capacity of 7.8 kB, these vectors might not be optimal to hold such a large insert. Alpharetroviruses are another type of integrating retroviruses with a number of advantages for potential clinical use, including a larger theoretical packaging capacity reaching 8.8 kB, a complete lack of viral elements which could potentially be immunogenic, and the possibility of developing a stable packaging line for commercial vector generation (92, 93). Moreover, although their safety has not yet been established in a clinical setting, their low transformative capacity in in vitro immortalization assays and their relatively neutral insertion pattern are all indicators that these vectors should also be safe for clinical use (92, 93).

In collaboration with Axel Schambach (Hannover, Germany), we generated both lentiviral and alpharetroviral vectors containing a codon optimized version of the human DOCK8 cDNA (hDOCK8co) (Figure 9). Codon optimization is an experimental approach designed to maximize protein production by creating targeted synonymous codon changes without changing the underlying amino acid sequence (94). This allowed us to further improve the expression of DOCK8 in both vector settings.

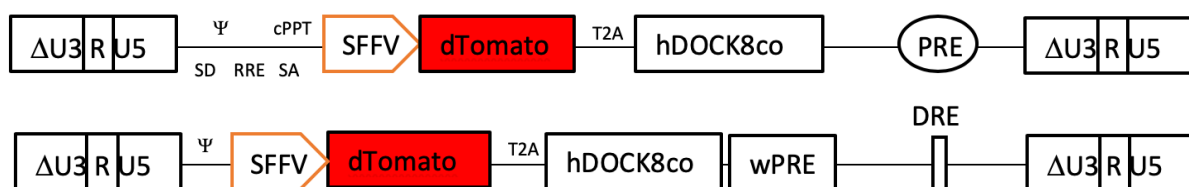


Figure 9. Representation of a SIN-lentiviral vector (top) and a SIN-alpharetrovirus vector (bottom) generated for comparative studies

We then used human Jurkat T cells, a leukemic T cell line that does not express DOCK8 (Supplement 3) (66), for comparative transduction studies of these two backbones. At the same multiplicity of infection (MOI), the lentiviral vector consistently led to a higher number of transduced cells compared to the alpharetroviral vector (Figure 10A-B). Moreover, the LV also led to a higher expression of DOCK8 protein in those cells that were successfully transduced (Figure 10C).

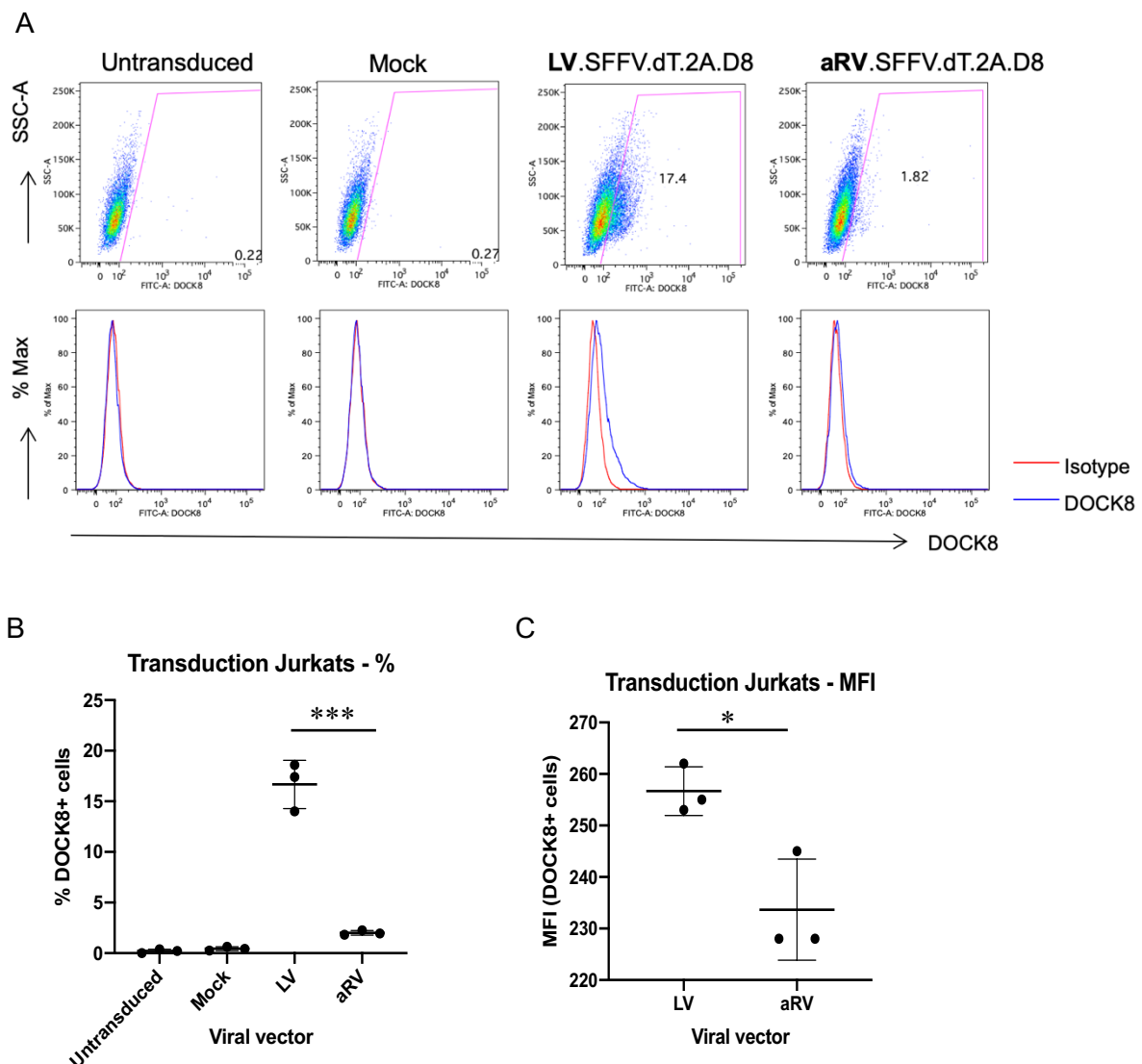


Figure 10. Comparative transduction experiments using a lentiviral vector (LV.SFFV.dT.2A.D8) versus an alpharetroviral vector (aRV.SFFV.dT.2A.dT). A) Representative FACS plots of Jurkats transduced with the different viral backbones. B) Transduction efficiency comparison of LV-transduced vs  $\alpha$ RV-transduced cells. C) DOCK8 MFI comparison of LV-transduced vs  $\alpha$ RV-transduced cells.

## MND promoter drives more efficient DOCK8 expression than EFS

We therefore opted to move forward with experiments using the LV vectors to analyze which promoter would provide the most efficient DOCK8 expression. To engineer these various vectors, we used PCR-generated inserts of the promoters of interest, and then fused these to a linearized LV vector backbone in which the promoter region had been removed using restriction enzyme digestion (Figure 11).

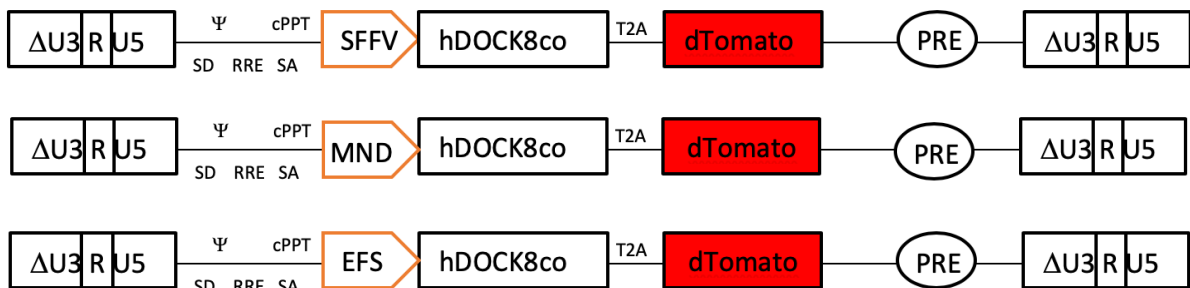


Figure 11. Designs of various LV vectors using different promoters

As candidates, we chose two promoters already in clinical use for GT that both display constitutive expression of transgene and are therefore active in hematopoietic cells. The elongation factor 1 $\alpha$  short (EFS) promoter leads to sustained gene expression in mammalian cells independently of cell type and has been safely and successfully used in GT trials for X-SCID (43), a severe disease caused by mutations in the *IL2RG* gene encoding the common gamma chain ( $\gamma$ c), a structural component of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 interleukin receptors (95). However, similarly to what was seen in WAS GT studies, the EFS promoter drove weaker  $\gamma$ c protein expression compared to wild type levels (Supplement 2), raising the concern that weak promoters might drive insufficient expression to restore immunological function unless compensated by higher VCN. In fact, the majority of the patients in this X-SCID study had a VCN above 1 in their graft product, all of which saw benefit from GT. Conversely, both patients who failed GT (1 death, 1 rescued with HSCT) had a VCN below 1. This promoter might therefore drive suboptimal DOCK8 expression at low VCN.

We therefore decided to test a second promoter, the myeloproliferative sarcoma virus enhancer, negative control region deleted, *d1587rev* primer-binding site substituted (MND) promoter. This synthetic promoter uses a modified LTR U3 region of the Moloney Murine Leukemia Virus (MoMuLV) in which the enhancer sequence is replaced by the myeloproliferative sarcoma virus enhancer and where the negative control region has been permanently deleted (96). The MND promoter drives ubiquitous transgene expression and has already been successfully used in a clinical trial for ADA-SCID (97). Interestingly, despite the vector used in this trial being a  $\gamma$ RV with intact LTRs, no oncogenic events were noted in any of the patients. This may however be due to the specific metabolic anomalies seen in ADA-SCID which differ from X-SCID and other PIDs, and may not reflect an intrinsic safety profile from this  $\gamma$ RV specific vector that would be applicable to other diseases. In the non-PID setting however, a SIN-LV using the MND promoter has also been shown to be safe in GT trials for adrenoleukodystrophy, a rare genetic condition resulting from very long chain fatty acid buildup in the central nervous system (98). Since we also plan on using a LV in the SIN configuration, this trial provides evidence for safety and efficacy of the MND promoter in the context of ex vivo HSC GT.

Finally, as previously mentioned, the silencing-prone spleen focus-forming virus (SFFV) promoter is a very strong promoter not suitable for clinical use, and served as a positive control to monitor transduction efficiency in our experiments.

We performed side-by-side transduction experiments to determine which promoter would be most efficient at driving DOCK8 expression. In the exact same transfection setting, the EFS promoter consistently yielded lower titers of viral supernatant when transduction of HT1080 cells were used for viral titrating in three independent experiments (Figure 12). This was not due to a larger insert of the EFS promoter (243 bp) compared to the MND promoter (383 bp).

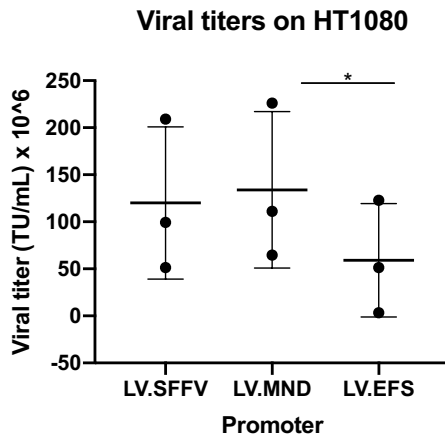


Figure 12. Viral titers as determined by transduction of HT1080 cells

We then again used the Jurkat T cell line to compare transduction efficiency of the various vectors. Again, the untransduced cells and the cells transduced with a mock vector (LV.SFFV.GFP or LV.MND.dTom) did not express any DOCK8 protein. Using the same MOI for transduction (based on HT1080 titring), the percentage of DOCK8-expressing cells was similar between both the LV.MND.DOCK8 and the LV.EFS.DOCK8 vectors (Figure 13A-B). Surprisingly, the LV.EFS.DOCK8 vector led to a slightly higher level of DOCK8 expression per cell (Figure 13C). However, when examining VCN in both sets of transduced cells, we found a significantly higher VCN in the LV.EFS.DOCK8-transduced cells (mean VCN value of 5.3 vs 3.4 copies/cell,  $p=0.02$ ) (Figure 13D). To compensate for significantly lower titers, a considerably greater volume of viral supernatant had to be used when transducing cells with the LV.EFS.DOCK8 vectors, which may explain the higher VCN and consequent higher MFI found with this vector. A higher VCN is therefore required with the LV.EFS.DOCK8 vector to transduce the same percentage of cells as the LV.MND.DOCK8 vector, which also translates into slightly higher expression.

To obtain the ideal vector for clinical use, there is an exquisite balance between finding a promoter that is strong enough to drive sufficient gene expression without risking transactivation of neighbouring genes, while taking into account the fact that weaker promoters might require a higher VCN, which in turn may also cause genotoxicity. In our

experiments, the LV.MND.DOCK8 vector appeared to be an attractive choice because of its high transduction efficiency at low VCN.

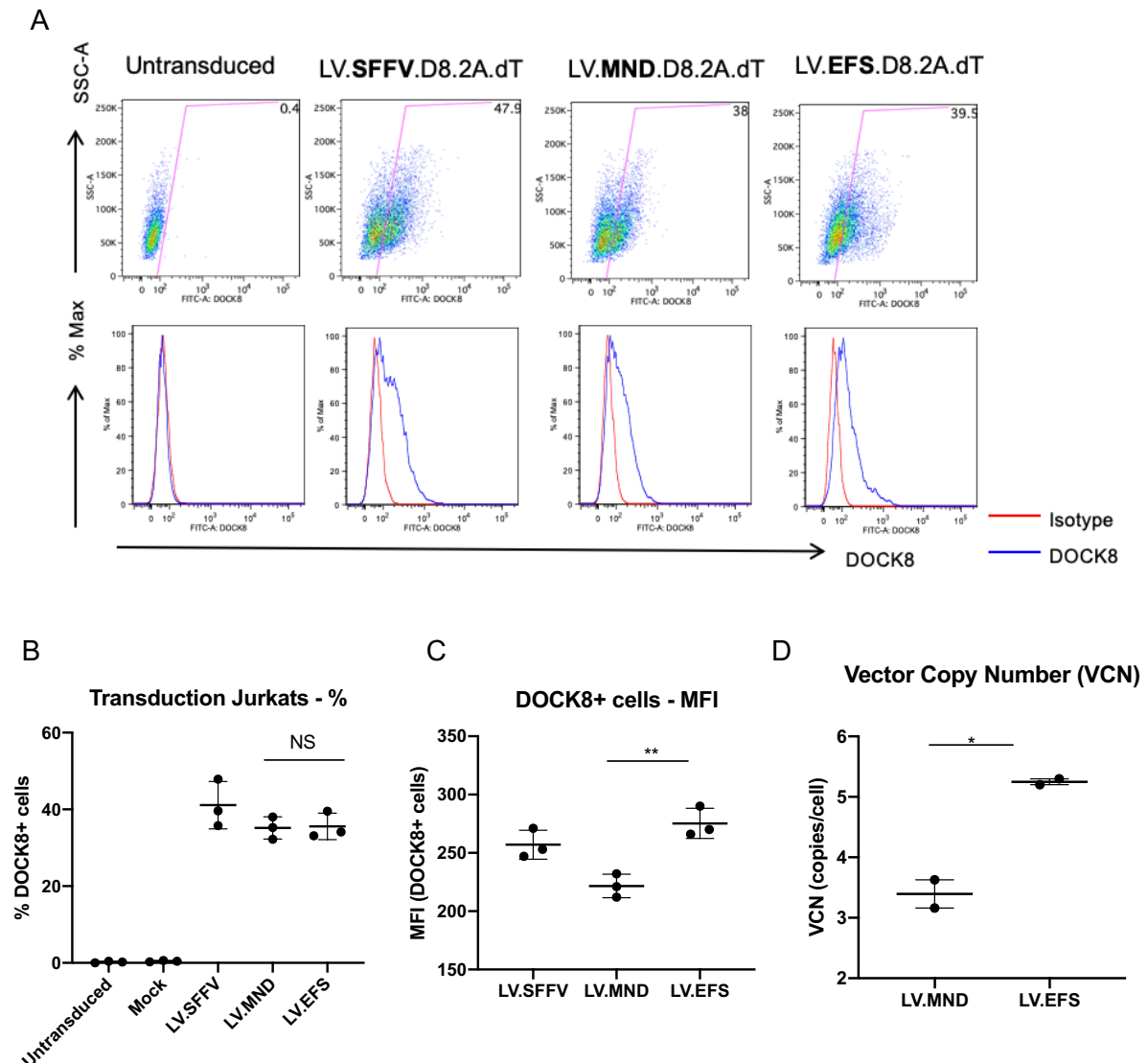


Figure 13. Comparative transduction experiments using the MND vs EFS promoters in a lentiviral backbone. A) Representative FACS plots of Jurkats transduced with the different viral vectors. B) Transduction efficiency comparison of MND-transduced vs EFS-transduced cells. C) DOCK8 MFI comparison of MND-transduced vs EFS-transduced cells. D) VCN comparison of MND-transduced vs EFS-transduced cells.

DOCK8 function can be restored with lentiviral vectors

As previously discussed, recent studies have identified a role of DOCK8 in mediating T<sub>H</sub>17 differentiation via the activation and phosphorylation of STAT3 (pSTAT3) downstream of IL-6 and IL-21 signaling in T cells, facilitating the translocation of this transcription factor into the

nucleus (66). We therefore leveraged these recent findings to see if DOCK8 function could be restored with GT using the Jurkat T cell line that does not express DOCK8. After IL-21 stimulation, STAT3 phosphorylation was completely absent in DOCK8-null untransduced Jurkat cells (Figure 14). When transducing these cells with a LV vector in which DOCK8 levels were high and correlated with dTomato expression, phosphorylation of STAT3 was restored to WT levels (pSTAT3+ cells: 16.4%). However, when transducing Jurkat cells with an  $\alpha$ RV vector in which DOCK8 expression was poor, the phosphorylation of STAT3 remained impaired (pSTAT3+ cells: 3.1%). Of note, in vectors where we experienced initial difficulties in achieving co-expression of both DOCK8 and fluorescent markers (see section above), phosphorylation of STAT3 was near-absent in dTomato+DOCK8- cells, indicating a DOCK8-specific phenomenon (as shown in Figure 14).

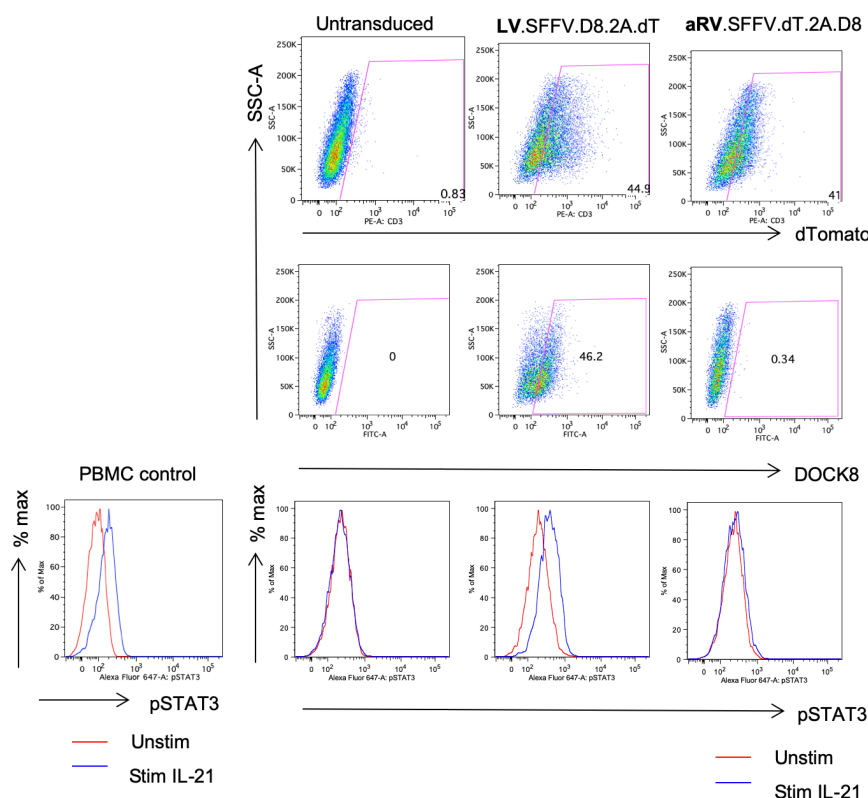


Figure 14. Restoration of DOCK8 function after transduction of Jurkat cells with a LV vector expressing high levels of DOCK8. STAT3 phosphorylation events are gated on dTomato+ cells only. Stim, stimulated with IL-21 (100 ng/mL) for 30 min; unstim, unstimulated.

## DOCK8 expression is not sustained in the Jurkat T cell line

To assess whether overexpression of DOCK8 would lead to persistent protein expression without signs of toxicity in a T cell model, we first transduced Jurkat T cells with either the mock LV.MND.dT vector or the DOCK8-containing LV.MND.D8.2A.dT vector, and serially followed the percentage of transduced cells over a 30 day period (Figure 15A). Over time, there was a significant decline in the mean percentage of DOCK8-transduced cells only, with initially a mean percentage of 75.9% transduced cells at day 2, which progressively declined to 18.2% at day 30. Similarly, the MFI of transduced cells also decreased from 438 to 239 at those same timepoints. In contrast, the percentage of mock-transduced cells was maintained (88.5 to 87.4%), as well as the MFI (2956 to 3396) at those same timepoints. To help distinguish whether this decline was due to loss of transduced cells versus loss of protein expression, we determined the bulk VCN (using total cells) at various timepoints post-transduction: day 6, day 13 and day 20 (Figure 15A). Although we saw an initial decline of VCN from day 6 to day 13, the VCN remained stable thereafter.

To exclude any potential variable in culture conditions that could explain this decrease in transduced cells over time, we transduced Jurkat cells with either a LV.SFFV.GFP (mock) vector or a LV.SFFV.D8.2A.dT (DOCK8) vector separately, then mixed these cells at a 1:1 ratio of transduced cells 48h after the initial transduction, and co-cultured them for 30 days. Again, we saw a DOCK8-specific effect where DOCK8-transduced cells declined over time, while mock-transduced cells remained stable (Figure 15B).

Finally, we performed Annexin V staining at day 2 post-transduction to determine whether DOCK8-transduced cells were more likely than mock-transduced cells to express apoptotic markers (99). Annexin V is a cellular protein that binds strongly to phosphatidylserine residues expressed on the outer leaflet of the cell membrane of cells committed to death by apoptosis (100). Annexin V staining revealed a substantially higher proportion of apoptotic cells in the

DOCK8-transduced Jurkat cells compared to those transduced with a mock-vector (16.1% vs 1.0%) (Figure 15C), indicative of some toxicity.

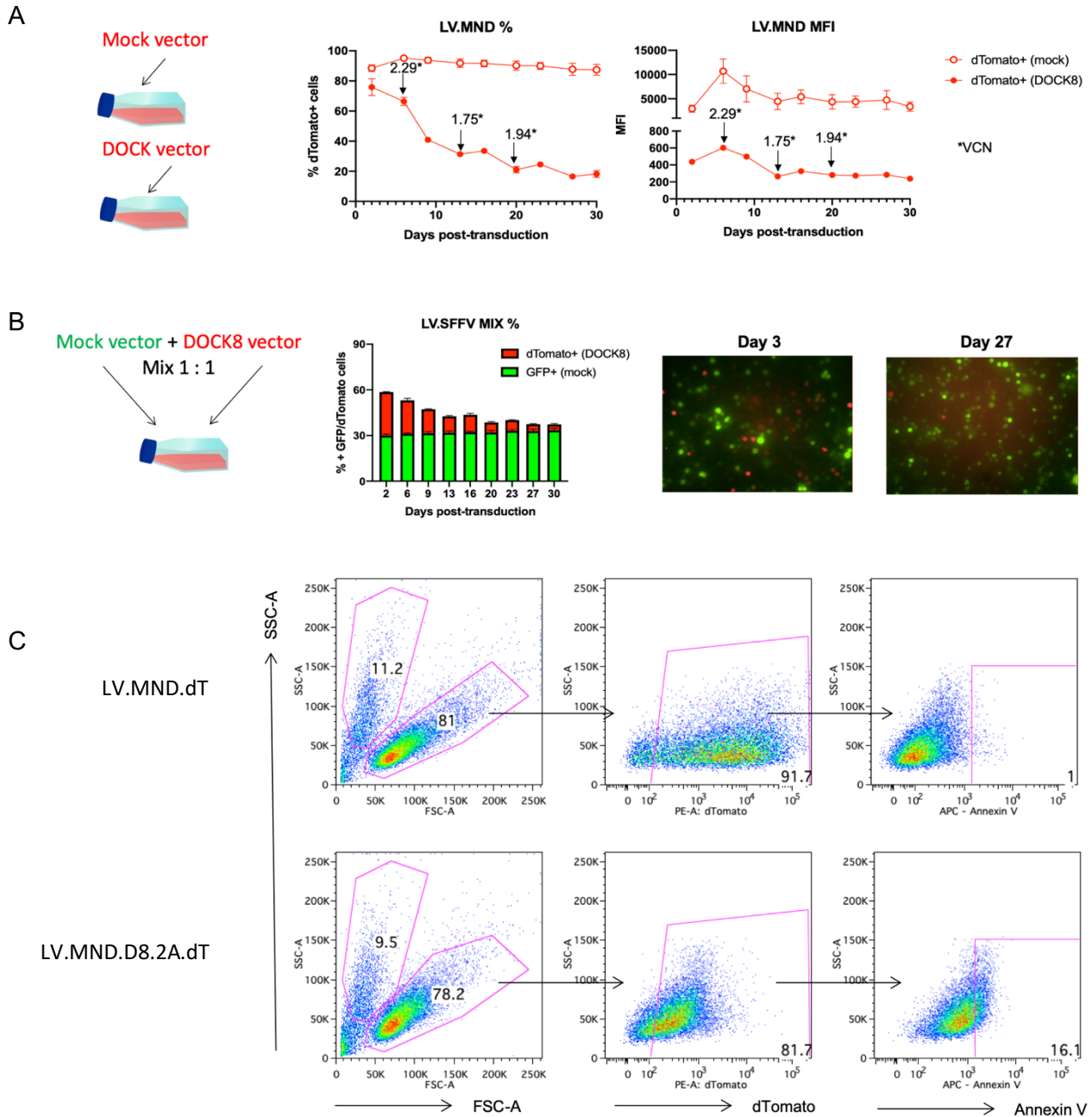


Figure 15. Transduction experiment of Jurkat cells with serial follow-up of transduced cells. A) Transduction of cells with either the LV.MND.dT (mock) vector or the LV.MND.D8.2A.dT (DOCK8) vector with serial follow-up of transduced cells over 30 days by flow cytometry B) Transduction of cells with either LV.SFFV.GFP (mock) vector or LV.SFFV.D8.2A.dT (DOCK8) vector, with co-culture at a 1:1 mix ratio at 48h post-transduction with serial follow-up of transduced cells over 30 days by flow cytometry and fluorescent microscopy C) Annexin V staining done 48h after initial transduction.

Indeed, a likely explanation for the phenomenon of gradual decrease in DOCK8-transduced Jurkat cells is toxicity due to DOCK8 overexpression in this T cell line. This is in part supported by the increase in Annexin V staining in the DOCK8-expressing cells only. Moreover, the initial decrease in the MFI within the first two weeks after transduction could be related to a dose-

dependent toxic effect where high DOCK8-expressing cells die first, although the serial MFI values do follow the same pattern of mock-transduced cells. Another possible explanation could be methylation of the MND promoter leading to silencing, as is seen with some other promoters. The stable VCN over time despite the decreasing percentage of gene-expressing cells argues in favor of this hypothesis. However, with promoter-specific silencing, we would also expect the LV.MND.dT-transduced cells to decrease their dTomato expression over time, which was not the case. Moreover, the MND promoter has been used in clinical settings before and silencing events have not yet been reported. Furthermore, we also performed experiments with vectors containing the SFFV promoter (Figure 15B), which is known to cause silencing with time, and saw a similar pattern as the one seen with the MND promoter where the mock-transduced cells remained stable over a period of 4 weeks, further demonstrating that silencing is unlikely to explain our findings. Finally, we cannot exclude that overexpression of DOCK8 led to a decrease in cell proliferation, which could explain why the untransduced and mock-transduced cells outcompete the DOCK8-transduced cells because of impaired cell growth.

#### DOCK8 overexpression leads to significant toxicity in the hematopoietic stem cell compartment

Because of our previous experiments demonstrating the superior efficacy of using a lentiviral backbone and the MND promoter, we decided to use these in combination to pursue in vivo experiments. However, we first sought to determine whether overexpression of human DOCK8 would lead to toxicity in murine hematopoietic stem cells. To this end, four attempts were made to transduce murine lineage negative (mLIN-) hematopoietic stem cells with a DOCK8-expressing LV vector. Each time, we noticed significant toxicity compared to mock vectors despite using variable levels of MOI. Moreover, the vast majority of apparent dTomato+ cells seemed to rather represent autofluorescence, a phenomenon likely occurring from dying cells (Figure 16). To confirm whether or not the cells had in fact been transduced,

we performed VCN assays on total cells, and found a very high VCN in both mock-transduced (mean VCN: 13.5 copies/cell) and DOCK8-transduced (mean VCN: 8.5 copies/cell) cells, indicating that cells had indeed integrated the vector into their genome. The very high VCN found in this case likely also partially represents episomal DNA that may have been picked-up by the qPCR assay, which was performed early post-transduction of these primary cells (36h after the last transduction).

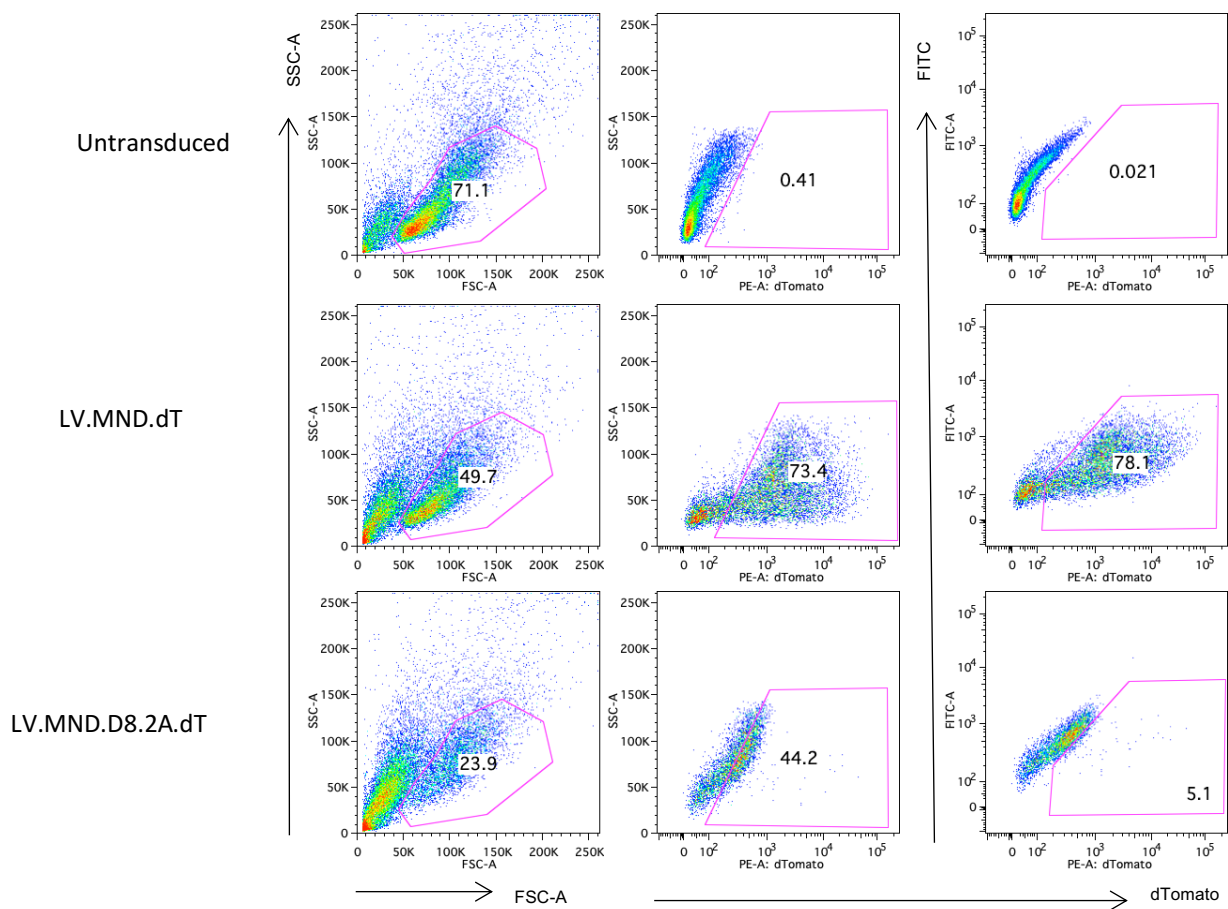


Figure 16. Representative FACS plot of murine LIN- transduced with a LV.MND.D8 vector and a LV.MND.D8.2A.dT

## CHAPTER 3: DISCUSSION AND PERSPECTIVES

### Discussion

To our knowledge, this is the first attempt at developing GT for DOCK8 deficient patients. One of the major difficulties of this task lies in the very large size of the DOCK8 gene, significantly impeding the transduction efficiency of these sizeable vectors.

Nevertheless, we were able to show that, despite the anticipated advantage of alpharetroviral vectors due their larger packaging capacity, the lentiviral vectors were in fact superior at driving high expression of DOCK8 protein. These results can partially be explained by differences in vector-specific requirements for genome integration. Similar to  $\gamma$ -retroviruses, the nuclear import of  $\alpha$ -retroviruses and subsequent chromosomal contact is dependent upon cell mitosis (92). Conversely, since lentiviruses can directly penetrate the nuclear membrane via nuclear pores, they can freely transduce non-dividing cells, which may help explain our higher transduction efficiency with these vectors. Another important consideration is that, while  $\alpha$ RV vectors do offer the potential advantage of stable packaging cell lines for large scale production, these are still being developed and it could be years before they are available for clinical use. Furthermore, their safety has not yet been established in human clinical trials. On the other hand, LV vectors have now already been used in various clinical trials, and their well-studied safety profile make them a dependable choice for timely clinical use. For all these reasons, we elected the lentiviral backbone as a superior choice over the alpharetrovirus backbone.

We next determined which promoter performed best by comparing the MND promoter to the EFS promoter. Our rationale behind choosing these two promoters was the fact that they had already been shown to be safe and efficient in previous clinical trials, with the intent of safely

accelerating the translation of our findings into clinical trials. When evaluating which promoter was the most efficient at driving DOCK8 expression, we found that the MND promoter consistently did best in comparison to the EFS promoter. Indeed, we found that a higher VCN was required with the EFS promoter to transduce the same percentage of cells as the MND promoter, which unsurprisingly translated into slightly higher levels of DOCK8 expression per transduced cells.

The transduction efficiency (overall percentage of transduced cells) is an important consideration in GT, especially when taking into account compartments that may not provide a survival advantage for gene marked cells. Indeed, because of the strong selective advantage of DOCK8-expressing T cells and memory B cells, we would expect these cells to thrive despite a lower number of transduced cells (77). However, both conventional (cDC) and plasmacytoid (pDC) dendritic cells are also severely impaired in DOCK8 deficiency (60, 101, 102), and these myeloid cells may require a minimal level of corrected cells in order to restore their unique functions. Indeed, cDCs play an important role as professional antigen presenting cells and are crucial for proper activation of the adaptive arm of the immune system. Recent work has shown that DOCK8-sufficient cDCs are required for priming of T<sub>FH</sub> cells and effective antibody-mediated protection (102). Similarly, the susceptibility to severe and refractory viral cutaneous infections seen in DOCK8 deficiency might in part be attributable to the profound defect in pDCs, which are the major source of IFN $\alpha$  production responsible for the antiviral response (60). A certain threshold of correction of both these compartments would therefore be desirable in order to restore normal antibody levels and help with clearance of cutaneous viral infections in these patients.

Another aspect to consider when transducing cells for clinical purposes is the careful balance between a low enough VCN to limit potential genotoxicity while maintaining sufficient levels of protein expression to correct the underlying disease manifestations. The EFS promoter has

already been shown to drive suboptimal common gamma chain ( $\gamma_c$ ) expression in the X-SCID trial compared to healthy controls (95), which is in line with our findings. However, the use of a potent promoter such as MND may also increase the risk of transactivating unrelated contiguous genes. One potential way of getting around this caveat is with the use of insulators, which could further improve safety by shielding adjacent genes from cis activation (103, 104). Trials are now underway to evaluate the safety of this method in gene therapy for X-SCID (35, 36). Of note, insulators such as the chicken  $\beta$ -globin insulator (cHS4) have shown however to decrease lentiviral-mediated transduction efficiency (105), which may be problematic with DOCK8 vectors, in which getting high viral titers is already challenging due to the very large size of the gene insert.

For all the reasons listed above, we chose the lentiviral vector in combination with the MND promoter as our front-runner candidate for use in in vivo experiments. An important step before performing GT transplantation experiments in our murine Dock8-deficient model was to demonstrate the absence of toxicity from overexpression of human DOCK8 in the HSC compartment. Furthermore, we initially attempted to evaluate if cell differentiation was affected by transduction of mLIN<sup>-</sup> cells with a DOCK8-bearing vector with the use of a colony-forming unit (CFU) assay. However, we were not able to perform the CFU assay because of the important and immediate toxicity noted in DOCK8-transduced mLIN<sup>-</sup> cells.

Taken together, these data demonstrate the feasibility of using SIN-LV vectors to drive efficient expression of DOCK8 for GT clinical use. However, the loss of DOCK8 expression over time in our T cell model and the significant toxicity seen after transduction of murine HSCs warrant further study.

## Future perspectives

While these experiments are certainly encouraging, more work is required in order to validate our findings and to determine whether or not GT is a viable therapeutic option for DOCK8-deficient patients.

Some of our preliminary data indicate that DOCK8 function can be restored in vitro in DOCK8-deficient lines using GT techniques, although more data is needed to corroborate these findings. Indeed, we found that phosphorylation of STAT3, a DOCK8-dependent event, was reinstated in the Jurkat T cell line only when DOCK8 was expressed at sufficient levels. A potential way to confirm these experiments in another T cell model would be with the use of a Herpesvirus saimiri (HVS)-immortalized T cell line originating from a DOCK8-deficient patient (106). Indeed, infection of human primary T cells with HVS leads to the immortalization of T lymphocytes while preserving the phenotypic and functional characteristics of the parental cells (107). This model could be leveraged to both confirm the role of DOCK8 in STAT3 signaling in T cell in response to IL-6 and IL-21 (66), and to demonstrate the use of GT in restoring this important pathway. Another important feature of DOCK8 recently discovered in T cells is its ability to co-localize with WASp via the WASp-interacting protein (WIP) to help mediate TCR-driven WASp activation and cytoskeletal reorganization of immune cells (85). While our Jurkat T cell line does not express DOCK8, it does express low levels of WASp (Supplement 3). We plan to utilize Jurkat cells as a T cell model once again, and to show via confocal imaging that DOCK8 expression resulting from a LV-transduced vector can physiologically co-localize with WASp.

While T cell dysfunction is a cardinal feature of combined immune deficiencies such as DOCK8 deficiency, a B cell intrinsic role of DOCK8 has also been recently uncovered and likely contributes to disease phenotype (108). Indeed, DOCK8 mediates a MyD88 signaling cascade in TLR9-activated B cells, a crucial pathway for B cell proliferation and

immunoglobulin production (108). Upon TLR9 ligation, DOCK8 is phosphorylated by Pyk2, allowing its binding to Lyn, a Src kinase. This event is then followed by Syk activation, which in turn promotes STAT3 activation and translocation into the nucleus. Interestingly, the authors in this study also convincingly demonstrated that IL-6 and IL-21-driven activation of STAT3 was not impaired in B cells of DOCK8-deficient patients, indicating a divergent role of DOCK8 in B cells compared to T cells in response to the same external stimuli (108). To establish whether this pathway can be functionally restored in GT, we plan to use Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs) that we already generated with primary B cells from three different DOCK8-deficient patients. Similar to HVS T cell lines, EBV LCLs retain features present in the parental cells from which they originated, making them an ideal B cell GT model. After transduction with either a mock or a DOCK8-containing LV vector, we will stimulate these cells with the TLR9 agonist CpG, and evaluate DOCK8-dependent downstream events such as Pyk2 and STAT3 phosphorylation. One potential obstacle that we expect with this experiment however is the difficulty to obtain a high transduction efficiency in these cell lines. Indeed, EBV LCLs are notoriously very difficult to transduce, and this has been our experience as well. Their transduction is further complicated by the very large DOCK8 insert of our vectors which does not allow for high viral titers.

The question of transduction efficiency will likely also be important to address when trying to transduce human HSCs with DOCK8-containing vectors, since these cells are also difficult targets to infect (109). Transduction enhancers, such as lentiBOOST and protamine sulfate, have been successfully used to increase transduction efficiency in this context (110), and will certainly be helpful here. Another ingenious approach has been studied in the context of developing GT for Duchenne muscular dystrophy, an inherited muscle degenerative disease associated with hemizygous mutations of the dystrophin gene, the largest known human gene (79 exons spanning over 2,200 kB) (111). To circumvent the limiting packaging capacity from current viral vectors to accommodate this massive gene, researchers have used an *exon skipping* approach, in which non-essential exons are deleted, resulting in an internally

truncated protein that remains nonetheless functional (112). While more studies are needed to determine if the DOCK8 gene contains non-essential coding regions and what these are (Figure 2), this approach could be an interesting alternative to increase transduction efficiency with the use of a shorter DOCK8 cDNA.

Finally, an important yet unexpected finding from our experiments was the significant toxicity unveiled from DOCK8 overexpression in murine LIN<sup>-</sup> cells transduced with our vectors. First, we will confirm that the toxicity seen is a direct result of DOCK8 expression, and not due to the incapacity of cells to accommodate such a large vector, by transducing Jurkat cells with an equally large vector in which the functional domains of DOCK8 will be mutated such that protein function would be impaired. Furthermore, before moving forward, it will be imperative to determine whether this toxicity is also present in human CD34<sup>+</sup> cells, that only express low levels of DOCK8 in normal circumstances (91). If we confirm toxicity from DOCK8 expression in human HSCs, this would preclude the use of a promoter that drives ubiquitous protein expression such as MND. Several alternatives would be possible. First, we could perform studies to identify the native DOCK8 promoter. By using techniques such as the transient transfection promoter activity assay, a series of putative promoter sequences can be functionally tested and characterized in mammalian tissue culture cells (113, 114). The discovery and use of the endogenous DOCK8 promoter would allow the retention of physiological DOCK8 expression patterns, therefore minimizing toxicity. However, these studies are time-consuming, and it could be long before such a vector is available. Furthermore, if the native promoter is large, this would further increase the length of the already large DOCK8 insert currently in use. Another approach to consider would be the use of an already established constitutive promoter driving expression of a protein with a similar expression pattern in hematopoietic cells, such as WASp. As previously mentioned, DOCK8 and WASp both play important roles in regulating the actin cytoskeleton of immune cells, and these two proteins interact together in both T cells and in B cells (85, 115). These proteins also both act interactively in innate immune cells such as NK cells (62), further suggesting that

their expression patterns are likely very similar. Furthermore, the use of a 1.6 kB fraction of the human WAS promoter has already been used safely in various clinical trials including the ongoing trial at Boston Children's Hospital, although this promoter might also drive weak DOCK8 expression (42, 43). A third option would be to create a vector in which a mature microRNA (miRNA) would suppress mRNA expression of DOCK8 at the HSC stage only (116). With this approach, DOCK8 would only be expressed in differentiated cells, adopting a more physiological expression pattern. Finally, an alternative solution that would allow all the intrinsic regulatory elements to remain intact would be the development of site-specific gene editing approaches with novel tools such as the CRISPR-Cas9 system (117). Although this last approach is very promising in the field of PID therapeutics, correction efficiency and off-target effects remain problematic (26, 118, 119). In addition, because the majority of mutations associated with DOCK8 deficiency are large deletions, a very long template would have to be introduced within a homology-directed repair event, further impeding correction efficiency; in most cases, precise single nucleotide or small gene segment correction using homology-directed repair techniques would consequently not be feasible.

With the many unresolved questions remaining, it is therefore apparent that more studies are needed before GT can be clinically available for DOCK8 deficient patients. However, these studies provide an initial proof of concept for the use of GT in this context. The forthcoming steps will include the confirmation of toxicity of DOCK8 in human HSCs, the design of a vector that preserves the physiological expression pattern of DOCK8, and performance of in vivo GT transplantation experiments in a *Dock8*<sup>-/-</sup> murine model. The ultimate goal of these experiments is to demonstrate safety and efficacy of GT for DOCK8 deficiency to enable the development of first-in-human clinical trials for patients suffering from this devastating disease.

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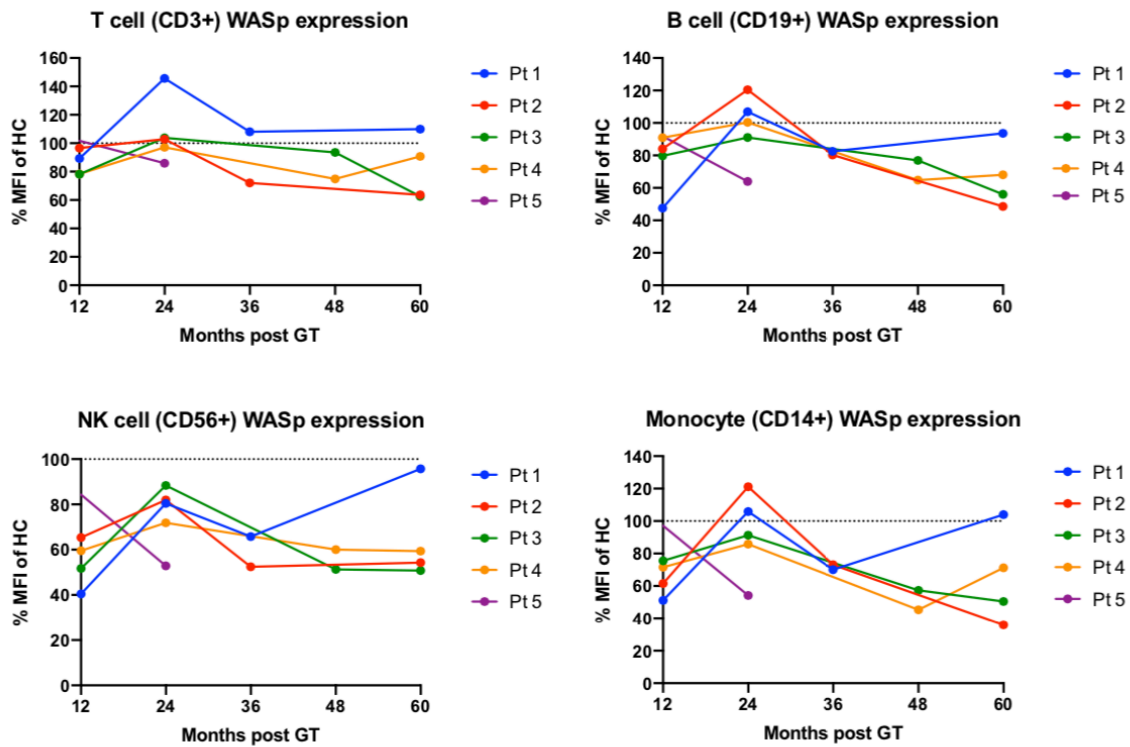
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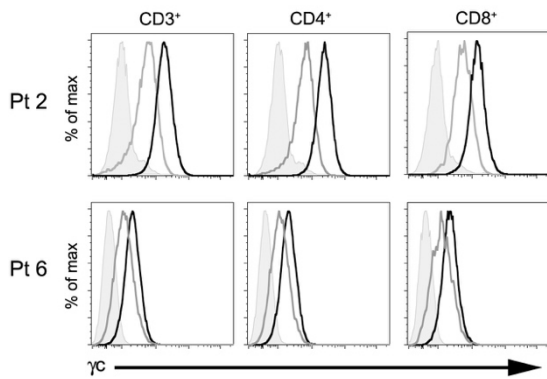
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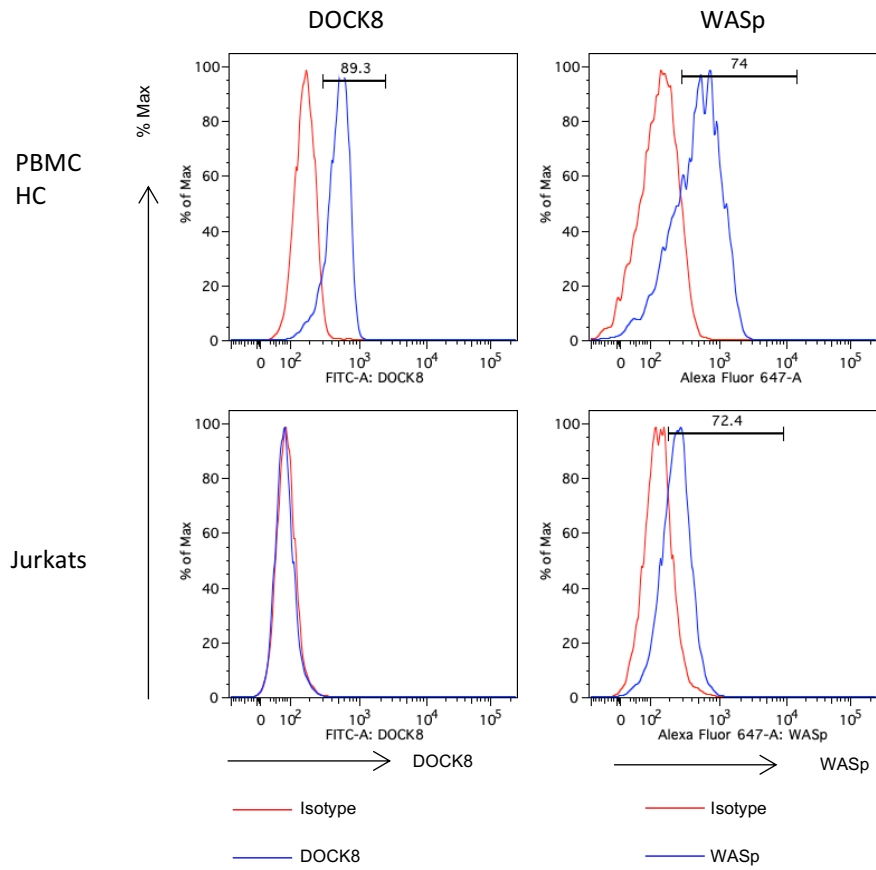
## SUPPLEMENTAL MATERIAL



Supplement 1. WASp expression within WASp+ cells following GT measured by flow cytometry



Supplement 2. Common gamma chain ( $\gamma$ C) transgene expression in lymphocytes of two patients post-GT is subnormal. VCN of graft product for patient 2 is 2.82 and for patient 6 is 2.92 copies/cell. Healthy controls (black open histogram), patient (gray open histogram) and isotype control (gray shaded histogram).



Supplement 3. *DOCK8* and *WASp* staining in the Jurkat T cells. Abbreviations: HC, healthy control; PBMC, peripheral blood mononucleated cells; WASp, WAS protein.

## LIST OF ABBREVIATIONS

$\alpha$ RV	Alpharetrovirus
$\gamma$ RV	Gammaretrovirus
ADA-SCID	Adenosine deaminase-deficient severe combined immunodeficiency
AR	Allergic rhinitis
aRPMI	Advanced Roswell Park Memorial Institute
BMT	Bone marrow transplant
cDC	Conventional dendritic cell
CFU	Colony-forming unit
CGD	Chronic granulomatous disease
DHR-1	Dock-homology region 1
DHR-2	Dock-homology region 2
DMEM	Dulbecco's modified Eagle's medium
DOCK8	Deducator of cytokines 8
dT	dTomato fluorescent protein
EBV	Epstein-Barr virus
EFS	Elongation factor 1 $\alpha$ short
EoE	Eosinophilic esophagitis
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GT	Gene therapy
GTP	Guanosine triphosphate
GvHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HSV	Herpes simplex virus
HVS	Herpesvirus saimiri
IgE	Immunoglobulin E
IMDM	Iscoe's Modified Dulbecco's Media Formulation
LCL	Lymphoblastoid cell line
LIN-	Lineage negative
LTR	Long terminal repeat
LV	Lentivirus
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mLIN-	Murine lineage negative
MND	Myeloproliferative sarcoma virus enhancer, negative control region deleted, dI587rev primer-binding site substituted
MOI	Multiplicity of infection
MoMuLV	Moloney Murine Leukemia Virus
MRD	Matched related donor
NK cell	Natural killer cell
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PID	Primary immune deficiency

RPM	Rotation per minute
T <sub>FH</sub>	Follicular helper T cell
T <sub>H</sub>	Helper T cell
TSS	Transcriptional start site
SCID	Severe combined immunodeficiency
SFFV	Spleen focus-forming virus
SIN	Self-inactivating configuration
STAT3	Signal transducer and activator of transcription 3
TRECs	T-cell receptor excision circles
VCN	Vector copy number
VZV	Varicella zoster virus
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WIP	WASp-interacting protein
WT	Wild type
X-SCID	X-linked severe combined immunodeficiency