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Optimization and Validation of Lentiviral Based Gene Therapy for FHL3

By

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Optimization and Validation of Lentiviral Based Gene Therapy for FHL3

By

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B.S., Illinois Wesleyan University, 2013

Advisor: H. Trent Spencer, PhD

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Division of Biological and Biomedical Science, Immunology and Molecular Pathogenesis Program

2020

Abstract

Optimization and Validation of Lentiviral Based Gene Therapy for FHL3

"Gene therapy" is the introduction, removal, or alteration of a person's genetic code in order to treat or prevent disease. Many different sub-types of gene therapy exist. Some therapies can modify specific cell types for transient expression of foreign DNA, and others integrate permanently into the genomes of stem cells and create lasting change across all the subsequently generated daughter cells. While originally envisioned to correct diseases that were caused by a single defective gene, researchers are now developing gene therapy products to combat complex diseases and disorders such as cancer, arthritis, and HIV. Compared to other disease interventions such as drug based therapies or vaccines, gene therapy is still in the early phases of development. The first successful report of human gene therapy was made less than thirty years ago, and the field has experienced distinct periods of rapid growth, setback, and re-acceleration. Excitingly, within the last three years the FDA approved the first gene therapy for use in the USA, signaling that the translational potential of this field will be actualized.

This dissertation focuses on the creation, validation, and optimization of a lentiviral based gene therapy for treating the primary immune disorder familial hemophagocytic lymphohistiocytosis Type III (FHL3). This disease, which is characterized by fever, hepatosplenomegaly, cytopenias, and, if left untreated, organ failure and death, is caused by a mutation to the *UNC13D* gene which renders cytotoxic cells incapable of degranulating cytolytic vesicles. Here we evaluated two lentiviral vector gene therapy approaches. The first approach seeks to transduce hematopoietic stem and progenitor cells (HSPCs) for autologous hematopoietic stem cell transplant (HSCT). The second approach seeks to resolve the hyperinflammation common to most FHL3 patients by transducing the patient's own T cells and re-infusing them back into the patient such that the underlying cause of inflammation could be resolved. Furthermore, in order to maximize the chances of this gene therapy making it to clinical trial, we have sought to optimize transduction efficiency by altering our protocols for lentiviral vector production, isolation of target cells, and transduction. Our hope is that this work provides insight into optimization strategies broadly used throughout the field of lentiviral gene therapy, and that our work pertaining specifically to FHL3 gene therapy might one day benefit patients.

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List of Abbreviations

2B4	CD244
A-HLH	Autoimmune hemophagocytic lymphohistiocytosis
AAV	Adeno-associated viruses
ADA	Adenosine deaminase
ADA-SCID	Adenosine deaminase severe combined immunodeficiency
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency
ALC	Absolute lymphocyte counts
ALL	Acute lymphoblastic leukemia
ALPS	Autoimmune lymphoproliferative syndrome
ANA	Antinuclear antibody
ANOVA	ANalysis Of VAriance
APC	Antigen presenting cell
ASGCT	American Society of Gene and Cell Therapy
AT	Antithrombin
ATTC	American Type Culture Collection
ATG	Anti-thymocyte globulin
Atg9b	Autophagy Related 9B
ATP	Adenosine triphosphate
B-LCL	B cell large cell lymphoma
Ba	Barium
BLAST	Basic local alignment search tool.
BSA	Bovine serum albumin
BV	Brilliant violet
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CAR	Chimeric antigen receptor
CBC	Complete blood count
CD	Cluster differentiation factor
CdDNA	Coding DNA

CECAM1A	Carcinoembryonic antigen-related cell adhesion molecule 1
CFU	Colony forming units
cGMP	Current Good Manufacturing Practice
CHOA	Children's Healthcare of Atlanta
CHS	Chediak-Higashi Syndrome
Cl	Chlorine
CNS	Central nervous system
COVID-19	Corona VIrus Disease
CpG	5'—C—phosphate—G—3'
CQ	Chloroquine
CSA	Cyclosporine A
CSF1R	Colony stimulating factor 1 receptor
CTL	Cytotoxic T lymphocytes
CypA	Cyclophilin A
DC	Dendritic cell
DMEM	Dulbecco’s modified eagle media
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor 1 alpha
EFS	Ef1α Short promoter
EKCOU	Ef1α_Kozak_CodonOptimizedUNC13D
ELF	E74 Like ETS
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope protein
ER	Endoplasmic reticulum
FA	Filterable agent
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor
FDA	(US) Food and Drug Administration

FHL	Familial Hemophagocytic Lymphohistiocytosis
FITC	Fluorescein isothiocyanate
Flt3	Fetal liver kinase 2
fMLF	N-Formylmethionine-leucyl-phenylalanine
FVIIa	Factor VIIa
Gag	Group specific antigen
GCSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GMCSF	Granulocyte colony stimulating factor
GP	Glycoprotein
GR-1	Granulocyte marker 1
GRIM	Granule release, immune-regulatory function, metabolic activities
GS	Griscelli syndrome
GTP	Guanosine triphosphate
H/F	Hemagglutinin and Fusion proteins
HBSS	Hank's balanced salt solution
HClO	Hypochlorous acid
HD	Healthy donor
HEK-293T	Human embryonic kidney 293T
hFLt3	Human flt3 ligand
hIL-11	Human interleukin 11
HIV	Human immunodeficiency virus
HLH	Hemophagocytic Lymphohistiocytosis
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HSPC	Hematopoietic stem and progenitor cells
HTLV(-1)	Human T-lymphotropic virus (1)
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon gamma
IFNγR1/2	Interferon gamma receptor R1/R2
IFITM3	Interferon induced transmembrane protein 3
Ig	Immunoglobulin

I-HLH	Infection associated hemophagocytic lymphohistiocytosis
IL	Interleukin
IPP	Inosinic pyrophosphrylase
IRAK-1	Interleukin 1 receptor associated kinase 1
IRB	Institutional Review Board
IRF2	Interferon regulatory factor 2
ITR	Inverted terminal repeat
IvIGg	Intravenous immunoglobulin
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
kb	Kilobase
LAH4-L1	Vectofusion-1
Lamp	Lysosome associated protein
LCMV	Lymphocytic choriomeningitis virus
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LE	Late endosome
LFA-1	Lymphocyte function-associated antigen 1
LG	Lytic granule
LPS	Lipopolysaccharide
LTR	Long terminal repeat
LV	Lentiviral vector
LYST	*Lysosomal trafficking regulator*
µg	Microgram
µL	Microliter
M-HLH	Malignancy associated hemophagocytic lymphohistiocytosis
MAC	Myeloablative conditioning
MAS	Macrophage activation syndrome
MCMV	Murine Cytomegalovirus
MCSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
Mg	Magnesium

MHD1/2	Munc homology domain ½
mIL-3	Murine interleukin 3
MIT	Massachusetts Institute of Technology
mL	Milliliter
MMP	Matrix metalloproteinases
MND	Myeloproliferative sarcoma virus enhancer, negative control region deleted, *d*l587rev primer-binding site substituted)
MOI	Multiplicity of infection
MPL	Myeloproliferative leukemia virus
MPO	Myeloperoxidases
mRNA	Messenger ribonucleic acid
mSCF	Murine stem cell factor
MTOC	Microtubule-organizing center
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming
MUN	Mammalian uncoordinated
Munc13-4	Mammalian uncoordinated family 14, protein 4
MVB	Multivesicular body
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
Nef	Negative regulatory factor
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NK	Natural killer
NKG2D	Natural Killer Group 2D
NSF	National science foundation
Ψ	Psi packaging signal
PAN	Polyarteritis nodosa
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy-7	Phycoerythrin-Cy7
PFU	Plaque forming units
PGE2	Prostaglandin E2, dinoprostone
PHA	Phytohemagglutinin
PI	Propidium iodide
PIC	Pre-integration complex
PIP2	Phosphatidylinositol(4,5)-biphosphate
PIP5K	Phosphatidylinositol-4-phosphate 5-kinases
PMN	Polymorphonuclear leukocytes
PRF	Perforin
PRT	Phosphoribosyltransferase
Pol	Polymerase
polyA	Polyadenylation
PS	Phosphatidylserine
PS	PROMATINE SULFATE
PVDF	Polyvinylidene fluoride
qPCR	Quantitiative polymerase chain reaction
Rab	Ras-associated binding
RAC	Recombinant DNA Advisory Committee
Rac2	Rac Family Small GTPase 2
Rap1a	Ras-proximate-1 or Ras-related protein 1
RBC	Red blood cells
Rev	Reverse transcriptase
RIC	Reduced intensity conditioning regiment
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RRE	Rev reverse element
Sca-1	Stemcell antigen 1, aka “Ly-6a” or “lymphocyte activation protein-6A”)
SCID	Severe combined immunodeficiency

SCID-X1	X-linked severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sHLH	Secondary hemophagocytic lymphohistiocytosis
SIN	Self-inactivating
siRNA	Small interfering ribonucleic acid
SIRs	Sustained inflammatory response syndrome
SLE	Systemic lupus erythematosus
SLP	Surface layer protein
SNARE	SNAP receptor
SNAP	Soluble NSF attachment protein
Sr	Strontium
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
Stk	Serine/threonine kinase
STX	Syntaxin
STXBP	Syntaxin-binding protein
t-SNARE	Target SNARE
Tat	Trans-Activator of Transcription
TC	T cell
TCR	T cell receptor
TFE(B/3)	Transcription factor E(B/3)
TFF	Tangential flow filtration
TH1	T helper 1
TIRF	Total internal reflection fluorescence
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
TPO	Thrombopoietin
Tregs	Regulatory T cells
TRIM	Tripartite motif family
TRM	Transplant related mortality
TSCM	T stem cell memory

TVS	Tubulovesicular structures
U	Units
U3	Unique 3 region
U5	Unique 5 region
Ulk1	Unc-51 like autophagy activating kinase
UNC13D	Uncoordinated 13 homologue D
USA	United States of America
v-SNARE	Vesicle-SNARE
VAMP	Vesicle associated membrane proteins
VCN	Vector copy number
VLA	Very late antigen
Vif	Viral infectivity factor
VOD	Veno-occlusive disease
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus
WBC	White blood cell
WPB	Weibel Palade Bodies
WT	Wild type
XLP	X-linked lymphoproliferative disorder

Chapter 1

Introduction

1.1 A History of Gene Therapy and Retroviral Biology

In this section of the introduction we will describe the evolution of modern gene therapy. This section of the introduction is meant to give the reader a basic understanding of the theory and techniques that were applied in the studies conducted in later chapters of this dissertation.

1.1.1 What is Gene Therapy?

"Gene therapy" is the introduction, removal, or alteration of a person's genetic code in order to treat or prevent disease [1]. Delivering a gene or the tools for alteration or removal of a gene can be done using either viral vector or non-vector methods. Non-vector delivery methods involve either 1) forcing naked strands of DNA or RNA into target cells using electroporation, passive, or ballistic delivery systems, or 2) packaging DNA or RNA into liposomes (small membrane-bound vesicles) and delivering them to target cells. In contrast, viral vector mediated gene therapies involve using modified viruses to infect target cells and deliver the gene or tools for gene modification.

Some viral vectors such as those derived from adenovirus and herpes simplex virus, have only a few modification made to them, thus allowing this viruses to replicate and cause multiple rounds of infection in target cells. Such therapies are most useful for treating cancer [2, 3]. However most viral vectors, such as those created from modified adeno-associated virus (AAV), gamma-retroviruses, and lentiviruses, are modified to such a great extent that these viral vectors cannot replicate, and therefore can only infect the first round of target cells that they encounter.

Within this latter category of non-replicating viral vectors, some, such as AAV, are non-integrating. This means that while these infect a cell and can deliver genetic material, that genetic material does not stably incorporate itself into the DNA of the host cell. As such, every time that gene modified cell divides, the subsequently generated daughter cells only inherit a fraction of the therapeutic genetic material, and with time the effects of the gene therapy fade. In contrast, an integrating or retroviral vector stably integrates transgenic material into the genome of the infected host cell. This means that even after many rounds of cell division, every daughter cell will have an intact copy of the original gene modification. The focus of this dissertation will be on integrating viral vectors and their adaptation towards creating a gene therapy for familial hemophagocytic lymphohistiocytosis type III (FHL3).

1.1.2 Discovery of Viral Mediated Gene Transfer

The discovery of viral mediated gene transfer dates back to 1952, when Dr. Lederburg and his graduate student Norten Zinder showed that Salmonella nutritional mutants could exchange genetic material despite be separated by a filter that was impenetrable to bacteria. The two hypothesized that this agent—termed "filterable agent" or "FA" was likely passively carried by bacteriophages (viruses which infect bacteria) which could pass through the filter [4]. Bacteriophages had been acknowledged in the scientific community since the 1910s when they were independently discovered by Fredrick W. Twort [5, 6] and Félix d'Hérelle [7], but it had never been shown that these viruses could mediate the swapping of bacterial genes. Lederberg and Zinder named this new mode of gene transfer "transduction" from the Latin translation of "to lead across."

1.1.3 Nascent Gene Transfer Studies

Moving genetic material from one strain of bacteria to another is quite easy compared to moving DNA into mammalian cells. After Lederberg and Zinder's first transduction experiments, it would take many years of developing cell culture techniques and broadening human understanding of genetics before similar results could be obtained in human cells. The first report of transferring functional DNA into human cells came in 1961 when Lorraine Kraus from the University of Tennessee obtained normal hemoglobin expression in sickle-cell anemia patient bone marrow cells after incubating those cells in media with DNA from a healthy human [8]. Seven years, later, a similar result was reported by researchers at the National Institutes of Health (NIH), Bethesda that added healthy DNA to cultures of Lesch-Nyhan syndrome patient cells, correcting for deficiencies in inosinic pyrophosphorylase (IPP) (aka hypoxanthine -guanine phosphoribosyltransferase (PRT)) [9]. While neither of these methods constitutes transduction, these experiments foreshadowed a future in which foreign DNA would be introduced into human cells in order to treat disease.

The first experiments that examined the use of a viral mediated gene transfer into human cells came from Stanfield Rogers at the Oak Ridge National Laboratory in 1966. Rogers published observations that rabbits infected with Shope papillomavirus—a DNA virus which normally infects but does not integrate into the DNA of basal epithelial cells – had low levels of arginine in their blood as a result of viral encoded arginase. Even more interestingly, some of the humans who handled this virus and viral infected animals in the laboratory also had low levels of arginine their blood [10]. This observation that human DNA could be modified by a virus led researchers to speculate if such a virus could be

deployed as a type of therapy. This line of inquiry ultimately resulted in the first gene therapy trial. In the 1970s, researchers from the Salk Institute intravenously injected Shope papillomavirus into three very young German sisters suffering from hyperargininemia. The idea was that patient cells would become infected with the virus and would benefit from viral arginase expression. However, none of the patients experienced a reduction in blood arginine levels, and the lack of success of this first gene therapy trial has since been attributed to instability of the injected virus [11-13].

1.1.4 Early Gene Therapy Successes (1970s – 1990s)

Creating safe, stable viral vectors for the purpose of gene transfer into human cells was the major hurdle confronting gene therapy researchers throughout the rest of the 1970s and into the 1980s. Moreover, if the field of gene therapy was going to be used to treat the vast multitudes of human genetic disease, it would be necessary to create a system for incorporating any desired transgene into the viral vector, thus eliminating the need to rely on viral encoded enzymes. The breakthrough came in 1984, when researchers from the Massachusetts Institute of Technology (MIT) published a report on the creation of the first retroviral vector suited for gene transfer into murine cells. This viral vector was derived from a Moloney murine sarcoma virus (a gammaretrovirus) that had been altered to incorporate genes for dihydrofolate reductase and neomycin and G418 resistance. Primary and even secondary transplantation studies in mice showed that this viral vector stably induced transgene expression [14]. This experiment put retroviral vectors at the forefront of viral vector mediated gene therapy.

Gammaretroviral vectors would be used in some of the important "firsts" in gene therapy history. They were used in the first gene therapy trial to test an engineered viral vector for cancer research. This came in the form of the 1989 study conducted by the National Cancer Institute (NCI) by a team led by Steven Rosenberg. Five patients suffering from malignant melanoma received tumor-infiltrating lymphocytes which had been transduced with a retroviral vector that encoded for neomycin resistance [15]. While only a third of the patients responded positively to treatment, all the patients absorbed the transduced cells without adverse side effects, thus demonstrating that retroviral gene transduction was feasible, safe, and that a substantial number of the transduced cells could express the gene and persist for extended periods of time.

Hot on the heels of the 1989 NCI study came additional studies in which researchers began transducing human hematopoietic stem cells with gamma-retroviruses and showing stable transduction in these cells [16-19]. These culminated in 1991 when geneticist French Anderson and immunologist Michael Blease from the National Heart, Lung, and Blood Institute published the first gene therapy clinical trial to be approved by the NIH's Recombinant DNA Advisory Committee (RAC). In this trial, MIT's originally published gammaretroviral vector (aka "Mulligan's vector") was adapted to express adenosine deaminase (ADA)—the gene lacking in patients with the disease ADA-severe combined immunodeficiency (SCID). Popularized in the public imagination as "bubble boy disease," patients with SCID (which can be divided into different sub-types including ADA-SCID) are extremely vulnerable to infectious disease, and require enzyme supplementation and eventually hematopoietic stem cell transplant. In this first approved gene therapy clinical trial, peripheral CD34+ cells from ADA-SCID patients were

transduced with a gamma-retroviral vector which encoded for the sequence for adenosine deaminase expression [20, 21]. The first person to receive this treatment was four year old Ashanti DeSilva, who famously recovered so well after therapy that she was able to start school [11]. As of the writing of this dissertation, Ashanti is 33 years old and is an editor specializing in rare diseases at *The Mighty*—and online community hub for people facing challenges relating to health and disabilities [22]. Other patients who received treatment from that same trial also responded well, maintaining measurable levels of ADA enzyme, normal white blood cell counts, and stronger immune systems years after the initial therapy. Furthermore no immediate negative reactions took place as a result of the gene therapy [23]. History books glowing refer to this trial as the first "successful" human gene therapy trial, and it set expectations high for future gene therapy studies [11].

Bolstered by the success achieved in the aforementioned trial, hundreds of gene therapy clinical trials were launched throughout the rest of the 1990s and into the early 2000s. While some of these trials were also designed to treat rare genetic disorders [24-26], others trials targeted cancer [27, 28], and even infectious diseases like HIV/AIDS [29]. In this time period, additional viral vectors such as polyoma virus [30] and lentiviral vectors were describe and used in clinical trials [31-33]. Furthermore, the FDA published its first guidelines for governing gene therapy in the USA [34].

1.1.5 Gene Therapy Setbacks (1999 – early 2000s)

Unfortunately, in the fever to make gene therapy products a reality, some important shortcuts were taken that would ultimately lead to some disastrous results. The first of these tragedies occurred with the death of Jesse Gelsinger, an 18 year old American,

who had been taking part in a gene therapy clinical trial to treat his ornithine transcarbamylase deficiency. The trial was originally a safety study designed to treat afflicted newborn infants using an adenoviral vector—a vector adapted from a non-integrating DNA virus [35]. In later litigation and in the subsequent FDA/NIH investigation, the issue was raised as to whether Gelsinger should have ever been enrolled in the trial owing to his age and pretrial tests which indicated that he had poor liver function. Furthermore, other patients had experienced un-reported flu-like symptoms as a reaction to the gene therapy [35]. Finally, and perhaps most tragically, despite having been born with ornithine transcarbamylase deficiency, Gelsinger and his family had learned to manage his disease through a restricted diet and medications, allowing him to live into adulthood and virtually making him a healthy volunteer. Despite this, Gelsinger was enrolled in the final treatment group of the clinical trial as the 18th patient to receive the gene therapy. Within 24 hours Gelsinger started experiencing an intense inflammation reaction which escalated into multiple organ failure and brain death. Four days after receiving the gene therapy the decision was made to take him off of life support [35].

Another blow to the gene therapy field came several years later when it was found that gene therapy could not only cause fatal inflammation, but also cancer. In the early 2000s, follow-up with five of the twenty boys who had received gene therapy for X-linked SCID were found to have developed T cell leukemia as a result of receiving gammaretroviral vector based gene therapy [36]. The vectors had integrated into the host genomes at an oncogenic site, causing cancer. This pushed the field of gene therapy to further refine viral vectors, as well as develop techniques to assess where within the genome the vector was integrating [36].

Aside from curbed enthusiasm, major changes were made in the fields of gene therapy as a result of these setbacks. To begin, both the US and France suspended further gene therapy trials that sought to treat patients with SCID [37]. Furthermore the FDA and the NIH launched an investigation into the University of Pennsylvania in Philadelphia's Institute for Human Gene Therapy and eventually fined the university $514,000. The NIH tightened and clarified its guidelines for monitoring and reporting on gene therapy trials, and new reporting methods were specifically created for the documentation of gene therapy patients suffering from adverse side effects as a result of their treatment. Beyond that, individual institutions imposed their own tightened restrictions on gene therapy research including putting in new protections for patients, creating stricter institutional review boards, and prohibiting financial conflicts of interest for gene therapy researchers involved in clinical trials [35].

Beyond matters of regulation, researchers recognized that advancements in viral vector design were necessary to create safe gene therapy products. Studies into adenoviral vectors found that these vectors can have an integration frequency as high as one percent of all transduction events [38]. Furthermore, many people have pre-existing immunity to adenoviral vectors from previous exposures to WT adenoviruses. This puts patients at an increased risk for an adverse immune response upon receiving adenoviral vector gene therapy. Finally, patients injected with adenoviral vectors can rapidly develop immunity to these vectors, leading to increased clearance and lessened potency of adenoviral gene therapies over time [39]. Consequently, over the years these vectors have fallen out of favor in exchange for adeno associated vectors (AAVs) and retroviral vectors. Further

discussion of retroviral vectors, specifically the gammaretroviral vectors and lentiviral vectors, will be made in the subsequent sections.

1.1.6 Recovery of Gene Therapy Research and the Beginning of Commercialization (2000s – present)

Despite the setbacks of the late 1990s and early 2000s, steady, incremental developments in the design and production of vectors has allowed the field of gene therapy to recover. Successful clinical trial results have been obtained with regards to the safety and efficacy of gene therapy products designed to treat patients with hemophilia, inherited retinal disease, thalassemia, and cerebral adrenoleukodystrophy, to name only a few [40].

The first gene therapy to be licensed was Gendicine, a recombinant human p53 adenovirus therapy for treating neck and head cancer. This therapy was approved by the China Food and Drug Administration in 2003 [41]. However, it would take another nine years before a second gene therapy would be licensed, this time in Europe. The therapy in question, "Glybera," was an AAV vector designed by the Dutch company UniQure for the treatment of lipoprotein lipase deficiency [42]. However, too few patients needed the drug, and even though the drug was a success from the standpoint of gene therapy research, it was a commercial flop. By 2017 UniQure withdrew marketing authorization [43]. In 2016 Europe licensed their second gene therapy, the gammaretroviral gene therapy product "Strimvelis" which was developed by GlaxoSmithKline for the treatment of children with ADA-SCID [44]. In 2017, the FDA approved several gene therapies. First, Luxturna, a drug developed by Spark Therapeutics and Children's Hospital of Philadelphia for treating Leber's congenital amaurosis, became the first FDA approved *in vivo* gene therapy [45].

That same year two cell based gene therapies for treating acute lymphoblastic leukemia (ALL), Kymriah and Yeskarta, were also FDA approved [45, 46]. To date, the FDA lists seventeen different approved cell and gene therapy products [47], and many other gene therapy products are in the pipeline. All this will hopefully lead the reader to conclude that despite some early setbacks, gene therapy has gained a firm foothold in modern medicine.

1.1.7 Retroviruses and Applications towards *Ex Vivo* Gene Transfer

1.1.7a The Discovery of Retroviruses

The Central Dogma of biology is that the flow of genetic information is one-directional, involving first the transcription of DNA into RNA and then the translation of that RNA into protein. This keystone tenant held fast in biology until the late 1960s/early 1970s, when virologists became increasingly interested in the theoretical existence of "retroviruses" or viruses that could transcribed RNA back into DNA. The discovery of reverse transcriptase, the enzyme which could facilitate the conversion of RNA to DNA, was announced in 1970 from two separate studies and helped affirm the probable existence of retroviruses. However it would take another full decade before Gallo and coworkers would publish the first confirmed case of a retrovirus—human T-lymphotropic virus 1 (HTLV-1) [48, 49].

1.1.7b Characterization of retroviruses

To date, many different retroviruses have been described in chicken, fish, cattle, mice, cats, non-human primates, and humans. Some of these viruses, such as human

immunodeficiency virus (HIV) or human T-lymphotropic virus (HTLV) cause disease or cancer as a result of integrating their viral genomes into host cell DNA. However, there are also many retroviruses, such as spumaviruses (aka "foamy viruses") which are not linked to any specific pathogenic condition [50]. Interestingly, human genomes are marked with the signs of many different retrovirus integration events which have been passed down through the generations and as far as anyone can tell are not linked to disease [50, 51].

Retroviruses are classified in the family "Retroviridae" and further divided into the sub-families of *Orthoretrovirinae* and *Spumaretrovirinae* [51]. The most famous retroviruses, including HIV, are in the *Orthoretrovirinae* sub-family, and for the purposes of this introduction we will confine ourselves to a discussion of this sub-family. *Orthoretrovirinae* consists of five genuses: Lentiviruses, Alpharetrovirus, Betaretrovirus, Gammaretrovirus, and Epsilonretrovirus [52]. All of the members of the Retroviridae family are characterized by reverse transcription of their twin single-stranded positive-sense RNA genome into cDNA, integration of that cDNA into the host genome (aka the creation of a "provirus"), and the subsequent expression of that cDNA to produce more RNA genomes and viral proteins which can be assembled to yield new virions.

In terms of function, retroviruses can be divided into two types: simple or complex. Retroviruses in the alpha-, beta- and gammaretrovirus genuses are considered "simple." These retroviruses encode for only the bare necessities for viral reproduction: the Gag, Pol, and Env proteins. In contrast, complex retroviruses from either the deltaretrovirus, epsilonretrovirus, or lentivirus genuses or from the *Spumaretrovirinae* sub-family, carry genes for additional accessory proteins which can help in the virus enhance its replication and avoid immune detection.

1.1.7c Retroviral Vectors in Gene Therapy

As noted previously, gammaretroviral vectors played a prominent role in the early days of gene therapy. Despite this, the integration profile of these vectors makes them liable for oncogenic insertion. Specifically, gammaretroviral vectors have a higher risk of integrating near the transcription start sites of actively transcribed genes, sometimes even inserting themselves within the promoter site. This can lead to oncogenic transformation, and consequently gammaretroviral vectors are sometimes called "onco-viral vectors." As such, interest in gammaretroviral vectors has largely given way to a preference for lentiviral vectors [53-55].

Like gammaretroviral vectors, lentiviral vectors have a preference for integrating in actively transcribed DNA. But unlike gammaretroviral vectors, lentiviral vectors integrate away from the transcription start sites, preferring instead to integrate downstream of the promoter region [53, 56]. This makes oncogenic transformation less likely. Furthermore, lentiviral vectors can transduce non-dividing cells, whilst gamma retroviral vectors are severely limited in this ability. This is thought to result from how the pre-integration complex (PIC) from a lentiviral vector can more easily be transported across nuclear envelopes [57].

Since their initial debut into the world of gene therapy research, lentiviral vectors have undergone several rounds of refinement. First generation LVs were produced by transfecting producer cells with three separate plasmids: one that encoded for HIV packaging (Gag and Pol) and accessory proteins (Vif, Vpu, Vpr, Nef, Tat, and Rev), another that encoded for an envelope protein, and one that encoded for a transgene. Using

three separate plasmids helped reduce the risk of producer cells from generating replication competent virus. To aid in this, long-terminal repeats (which coordinate the integration of viral genes into host DNA) and the ψ region (a packaging signal sequence) were removed from the Gag/Pol and Env plasmids, and instead only included in the transgene plasmids [58].

Despite these precautions, first generation LVs still imposed substantial risks of generating recombinant, replication competent LVs. Consequently, second generation LVs were created. The preparation of these LVs was almost identical to that in first generation LVs, but the sequences for the accessory proteins Vif, Vpu, Vpr, and Nef were removed. Consequently, only four of the nine HIV genes are expressed in second generation lentiviral vectors, thus reducing the chances of producing replication competent virus.

Even with these safety measures, there was still anxiety about the role that the LTRs could play in producing replication-competent LVs in the event of co-infection of a cell with an LV and a WT lentiviral vector. Furthermore, because LTRs have their own promoter and enhancer regions, there was worry that these cells could act to enhance the expression of proto-oncogenes. To remedy these concerns, third generation LVs were designed such that mutation to the 3'U3 region eliminated the promoter and enhancer functionality of the LTR viral promoter, rendering them self-inactivating (SIN). Specifically, during reverse transcription this mutated 3'U3 region is copied into the 5'LTR, and consequently the LV DNA can integrate but it is incapable of producing additional replication competent RNA genomes. This modification necessitates the inclusion of another promoter downstream of the 5' LTR in order to induce transgene transcription. Inclusion of an additional promoter negates the need for Tat—an HIV gene

which normally enhances viral transcription [58-60]. Consequently, Tat was also deleted from third generation LVs. Finally, as an added safety measure, Rev, the HIV accessory protein that facilitates nuclear export of un-spliced/minimally spliced transgene RNA has been integrated into its own separate vector. This further reduces the chances of a newly budding lentiviral vector from obtaining plasmid DNA for all the genes necessary for its stable reproduction. The overall effect of this is that the risk of producing replication competent virus is mitigated [58].

1.2 An Introduction to HLH

Having discussed the evolution and current state of gene therapy research, we now turn our discussion to describing the different forms of hemophagocytic lymphohistiocytosis (HLH) disease. The final section of this introduction will focus specifically on familial hemophagocytic lymphohistiocytosis type III (FHL3), but an understanding the broad scope of HLH presentation and treatment will help the reader appreciate the disease from a clinical perspective. Furthermore, no clinical research study to date has focused exclusively on FHL3 patients, and therefore it is necessary to begin our discussion with what is broadly known about HLH before focusing on the specific nuances of the FHL3 subtype of disease.

1.2.1 Prevalence

Estimates for the prevalence of FHL vary widely between different sources. Two often-quoted figures is that the incidence of FHL is 1.2/1,000,000 children per year, or one

out of every 50,000 live births [61]. However, these figures are from a study published in 1991 and the study population was restricted to Sweden, and therefore caution should be used when extrapolating these figures to other populations. One research group from Japan estimated a similar annual incidence of HLH (1/800,000 people per year) [62], but another group found nearly double the number of cases in children under 15 years old (0.342 cases per 100,000) [63]. Turkey, with a comparatively high number of consanguineous couples, tends to have a higher reported incidence: 7.5/10,000 people in one report, or one in every 1,418 patients omitted to the Ihssan Dogramaci Children's Hospital [64]. In contrast, Allen et al estimated from their study at Texas Children's Hospital that tertiary care pediatric hospitals in the United States should expect 1 case of HLH per 3000 inpatient admissions [65].

One strong factor in estimating the incidence of HLH within a population is the incidence of consanguineous couples. Because FHL can have an autosomal recessive pattern of inheritance, it stands to reason that in communities with weaker social taboos against producing consanguineous offspring would have a higher incidence of HLH, specifically of FHL. This phenomena has been reported in communities found in Saudi Arabia [66], Pakistan [67, 68], France, Portugal, Australia, Mali, Germany, Turkey, northern Africa [69], Iran [70], and Sweden [71].

A second reason to doubt the often-cited 1.2/1,000,000 children per year statistic on incidence is the developments within the field of HLH research which have allowed doctors to identify HLH patients faster and more decisively than in the past. In particular, the diagnostic guidelines laid down by the HLH-94 guidelines have been particularly essential for the identification of HLH patients, leading to an increasing number of

documented cases of HLH over the last decade [72]. Furthermore, a growing body of work suggests that late-onset HLH is more frequent than originally supposed. While many including the Histiocytic Foundation repeat the statistics offered by Henter et al in 1991 that 70-80 percent of FHL onset occurs within the first year of life, others suggest that onset in adulthood or even old age are more common than the historical literature would suggest [72, 73]. Given all of these factors, it seems impossible at this time to pinpoint an exact figure of incidence for HLH. For the purposes of this discussion it is sufficient enough to say that HLH is a rare disorder.

1.2.2 Clinical Manifestations of HLH

HLH clinical presentations are very heterogeneous, with symptoms potentially manifesting in the respiratory tract, gastrointestinal tract, circulatory system, skin, liver, and/or the central nervous system [74]. When it was first described in 1952, Farquhar and Claireaux described the disease as being rapidly fatal and characterized by erythropenia, fever, jaundice, rash, and enlargement of the liver, spleen, and lymph nodes [75]. Notably the histological analysis from the lymph nodes and spleen showed pronounced phagocytosis of red blood cells by macrophages, prompting Farquhar and Claireaux to name the disease “familial hemophagocytic reticulosis” [75]. The hemophagocytosis of red blood cells often results in cytopenias and anemia in HLH patients [76]. Complications in the central nervous system are also common, with neurological symptoms such as seizures, encephalopathy, hemiplegia, cranial nerve palsies, blindness, demyelization, and convulsions identified in 75% of pediatric cases [61, 77]. The disease is progressive,

eventually leading to multiple organ failure, severe bleeding, fluid accumulation in the lungs, and ultimately death [70, 76].

In addition to the diverse symptoms mentioned above, hereditary forms of HLH commonly manifest in association with other immune deficiencies such as Chediak-Higashi Syndrome (CHS), Griscelli Syndrome (GS), X-linked lymphoproliferative disorder (XLP), Hermansky-Pudlak syndrome, and fibrous cortical dysplasia [76, 78]. This is in part due to how these disorders are caused by mutations to genes which also can contribute to HLH. For example, both HLH and Chediak Higashi Syndrome can be prompted by mutations to the *LYST* gene [79, 80]. Similarly, both HLH and Griscelli Syndrome II can be prompted by mutations to the *Rab27a* gene [81].

1.2.3 Diagnosing HLH

General symptoms such as malaise, paleness, and lymphadenopathy often contribute to the disease being misdiagnosed for other diseases [70]. The presence of rash—commonly purpuric morbilliform eruptions—often lead doctors to misdiagnose patients as having an infection [76]. While patients often do have an infection which prompted their HLH pathology, it is the immune system's dysregulation and inability to respond to infection which is the root cause of the disease.

One clue that clinicians can look for while assessing a potential HLH patient is the rapid onset of disease. With the exception of neonatal cases, HLH patients can have a sudden onset of a systemic and sustained inflammatory response syndrome (SIRs) and cytokinemias of inflammatory cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, IL-18, and

MCSF [82]. This sudden onset can help differentiate HLH from other diseases, although this rule is by no means absolute [83].

The age of a patient can also be informative when trying to evaluate the possibility of an HLH diagnosis. Primary HLH patients tend to be quite young, with an estimated 70-80 percent of the patients being diagnosed within the first year of life [84]. However there are exceptions to this, particularly in instances of acquired HLH. Secondary HLH has been documented in individuals as old as 66 years old [72], and to date there is no reported maximum age of disease manifestation for either primary or secondary HLH [76].

As noted by attending physicians, perhaps what can be most illuminating for clinicians is what symptoms are *not* present in HLH patients [76]. HLH patients will fall suddenly ill and will have no history of previous hospital admissions or chronic illness. Furthermore, blood and urine samples are frequently sterile, and there will be no improvements in response to treatment with antibiotics. Until the latter stages of disease, both the liver and kidneys will remain functional and no abnormal weight loss will occur. Antinuclear antibody (ANA) tests for lupus will also come back as negative [76].

The first official set of diagnostic guidelines for HLH were presented by the Histiocyte Society in 1991 [84]. These guidelines listed the most common clinical, laboratory, and histopathological findings that define HLH. They are: 1) fever, 2) splenomegaly, 3) cytopenias affecting at least two out of three lineages in the peripheral blood, 4) hypertriglyceridemia and/or hypofibrinogenemia, and 5) hemophagocytosis in the bone marrow, spleen, and/or lymph nodes [84]. Three years later the first prospective international treatment protocol (HLH-94) was introduced, and the diagnostic criteria were updated to include 6) low/absent NK-cell activity, 7) hyperferritinemia, and 8) high levels

of sIL-2r [85]. These guidelines were updated yet again in 2004 to include criteria for (9) the molecular diagnosis of FHL [86], and modified yet again in 2009 to include supportive diagnostic features such as hypofibrinogenemia[1] and hyponatremia [87]. In order to have a confirmed case of HLH, a patient must have a molecular diagnosis and/or five out of eight diagnostic criteria met [86].

Despite considerable effort to create clear diagnostic guidelines, correctly diagnosing HLH remains quite difficult due to the highly variable nature of disease presentation as well as its rarity. In addition, some of the assays such as the NK cell function assay or the soluble CD25 assays take time to complete, which makes them impractical during clinical emergencies [70, 88]. Furthermore, not all hospitals receiving HLH patients have access to resources such as flow cytometers which necessary for some diagnostic assays. Rural communities where consanguineous couples are more common are therefore at a greater risk for having their HLH go untreated [70]. In addition to that, there can still be ambiguity in diagnosing patients with HLH even if five out of the eight diagnostic criteria are met. It is not uncommon for patients to have a subset of criteria met but no molecular diagnosis, leaving physicians unsure of how to treat the patient [89]. Furthermore, because FHL1 has not yet had a specific gene associated with it (although its chromosomal location has been mapped), it is possible that a patient has a disease-inducing

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1 While hypofibrinogenemia and hypertriglyceridemia were included in the HLH 2004 guidelines as the fourth diagnostic criteria for HLH 86. Henter, J.I., et al., *HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis.* Pediatr Blood Cancer, 2007. **48**(2): p. 124-31., the most recent set of diagnostic guidelines has relegated hypertriglyceridemia to being an "other result supportive of HLH" 87. Filipovich, A.H., *Hemophagocytic lymphohistiocytosis (HLH) and related disorders.* Hematology Am Soc Hematol Educ Program, 2009: p. 127-31..

mutation which current HLH molecular diagnostic panels cannot identify. In summary, though great advancements have been made towards providing clear-cut guidelines, HLH diagnosis is often far from definitive.

1.2.4 Primary vs Secondary HLH

1.2.4A Primary HLH (FHL)

After first being described in 1952, the underlying cause of HLH remained largely a mystery. It was not until 1996 that Egeler et al suspected an immunological basis for the disease, particularly with regards to CD8+ T cell and natural killer cell function [90]. The first mapping study attempting to identify a genetic cause for HLH was conducted in 1999 on a Pakistani family. While a locus of disease was identified on chromosome 9q21.3-22, no specific gene for this disease could be identified. Furthermore, aberrancies in this locus could not be identified in an unrelated family of Arab descent [68]. To this day, the exact gene responsible for this form of inherited HLH, termed "familial hemophagocytic lymphohistiocytosis type 1" or "FHL1," has yet to be determined.

Although unsuccessful at pin-pointing a particular gene, the study by Ohadi et al marked the beginning of a rapid succession of gene determination for different sub-types of inheritable HLH. The same year that Ohadi et al published their findings for FHL1, Stepp et al identified *PRF1*, the gene for perforin, as the genetic determinant for FHL2 [91]. Subsequently even more studies elucidated the genetic determinants for FHL types 3, 4 and 5 (see Table 1). Collectively, these disorders are referred to as "primary HLH". If a genetic cause can be identified for a patient, they are said to have "genetic" HLH. If a specific genetic cause cannot be identified for a patient but there is clearly a family

association (e.g. multiple children in a family have the disease), then the patients are said to have “familial HLH” [76].

As with estimating the global prevalence of HLH, estimating the specific frequencies of different familial HLH is difficult and varies between populations. Overall the consensus is that FHL2 is the most common form of primary HLH [92] , comprising an estimated 15-50% of all FHL cases depending on geographic region [71, 76]. In the United States FHL2 is reported to consist of 50% of all HLH cases, while in Japan is accounts for only 40% and in Turkey it only accounts for 30% of all FHL cases [93, 94].

It is important to note that many different mutations within the same gene can give rise to HLH. For example, as of 2017, over 115 mutations to the *PRF1* gene have been reported to contribute to HLH pathology, most of which are missense and nonsense mutations [70]. Importantly, sometimes residual activity remains in a mutated FHL gene, and this can influence the severity of the disease [70, 95].

Table 1 Overview of the sub-categories of primary HLH and associated diseases

Disease Name	**Gene mutated**	**Protein affected**	**Chromosomal location**
FHL1	Unknown	Unknown	9q21.3-q22
FHL2	PRF1	Perforin	10q21-22
FHL3	UNC13D	Munc13-4	17q25
FHL4	STX11	Syntaxin11	6q24
FHL5	STXBP2	Munc18-2	19p13.2-3
CHS-1	LYST	Lyst	1q42.1-q42.2
GS-2	RAB27A	Rb27a	15q21
XLP-1	SH2D1A	SAP	Xq25
XLP-2	BIRC4	XIAP	Xq25

1.2.4B Secondary HLH (sHLH)

In contrast to primary HLH where either a genetic analysis or family assessment suggests a genetic cause of disease, secondary HLH (also referred to as "sHLH" or "acquired HLH") describes a condition in which there is no discernable genetic basis for HLH disease [76]. Rather, the disease is usually induced by an infection. These infections can include 1) fungal pathogens such as Cryptococcus, Candida, Aspergillus, or Histoplasma capsulatum [96]; 2) parasites such as toxoplasma, malaria, strongyloides, leishmania, or babesia; 3) bacteria such as salmonella, chlamydia, tuberculosis or rickettsia; or 4) viruses such as Epstein Barr virus (EBV), cytomegalovirus, rubella, or mumps virus [76, 96, 97]. In addition, malignancies such as lymphomas, myelomas, or inflammation from other disorders such as Kawasaki, juvenile idiopathic arthritis (JIA), polyarteritis nodosa (PAN), or systemic lupus erythematosus (SLE) can also prompt the development of secondary HLH [96]. Other potential causes include immunosuppressive drugs or low numbers of natural killer cells [98]. This has led some authors to further specify acquired HLH as being infection associated (I-HLH), autoimmune-associated (A-HLH), or malignancy associated (M-HLH) [99]. Importantly, different age groups have different frequencies with which they will manifest particular types of secondary HLH [62].

Secondary HLH is reported to have a higher incidence than primary HLH, although at present the evidence for this claim is mostly anecdotal [100, 101] or derived from very small study populations [102]. However, evidence from a nation-wide survey in

Japan suggested that 90% of children with HLH in that country suffer from secondary HLH [103].

Onset of secondary HLH typically happens later in life than in primary HLH [76]. Growing numbers of cases in which onset has occurred in adolescence [104] to old-age [72] have been reported. At present there does not appear to be any age limit for the onset of disease.

1.2.4B1 HLH and COVID-19

At the time of the writing of this dissertation, no single health concern looms larger in the public consciousness than COVID-19, and therefore we would be remiss not to mention how HLH and COVID-19 intersect. As of 5/12/2020, there are 1.4 million confirmed cases of the disease in the USA alone, and over 83,000 deaths as a result of disease, for a mortality rate of 5.9% [105]. A subset of patients with a high mortality risk have been identified by how their presentation is accompanied by a cytokine storm that is akin to secondary HLH disease presentation. Specifically, patients will develop high levels of ferritin, IL-2, IL-6, IL-7, GCSF, IFN-γ, inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1-α, TNF-α [106, 107]. A multi-center retrospective study from Wuhan China found a statistically significant correlation between increased mortality risk and the presence of these hyperinflammation markers [106]. This has led to a call within the medical community to measure ferritin and platelet levels and evaluate erythrocyte sedimentation rate and HScores in patients with severe COVID-19 in order to identify if they have an increased mortality risk as a result of sHLH [108]. Still others warn that the 2004 HLH Diagnostic Guidelines are not designed for the proper

diagnosis of COVID-19 patients. Dr. Ashish Kumar has warned that the current H-Scoring method too frequently leads doctors to a diagnosis of HLH and that this could result in ineffective or even detrimental treatment of patients [109].

Treatment of patients with both COVID-19 and sHLH is evolving quickly as more cases manifest and attempts are made to treat this co-morbidity with proven HLH interventions. Randomized controlled trials of the IL-6 receptor blockade tocilizumab have indicated that this treatment option is safe and efficacious for patients with both COVID-19 and elevated IL-6 levels, and has been approved for use in China [108]. As of April 3, 2020, plans are being made for a phase III clinical trial that will assess the use of JAK1/2 inhibitor Jakruxolitinib (Jakafi) for the treatment of patients with COVID-19 and sHLH [110]. A similar trial was announced on April 27th for testing the efficacy of pacritinib – and inhibitor of JAK2, IRAK-1 and CSF1R—on hospitalized patients with severe COVID-19 to assess whether or not the drug has any efficacy in reducing the need for invasive ventilation, extracorporeal membrane oxygenation, and/or death [111]. While the majority of resources are rightly being directed towards public health efforts to prevent the spread of COVID-19, the authors hope that experts within the fields of HLH disease treatment will speak up and contribute to the efforts to save the lives of patients with both COVID-19 and sHLH.

1.2.5 Treatment of HLH

Phases of Disease Management

The "Initial" phase of HLH therapy treatment consists of the first eight weeks of treatment. In this time, patients are recommended to receive maximum supportive intervention, which includes antibiotics, antivirals, antifungals, gastro-protective agents, and infusions of IVIgG every four weeks. During treatment, patients are closely monitored for their levels of IL-2 receptor, ferritin, triglyceride, hemoglobin, neutrophils, platelets, consciousness, absolute lymphocyte counts (ALC), and hemophagocytosis [112].

In addition, doctors carefully monitor patients for CNS involvement and early signs of irreversible neurological damage. If neuropathology does occur, patients are also administered intrathecal injections containing anti-inflammatory and immune-suppressive drugs [88]. Some patients respond well to this treatment, and lacking a molecular diagnosis and family history of disease, the therapy is eventually discontinued until reactivation occurs [86]. Patients that do not resolve their disease during the initial phase of treatment or any patient with a confirmed molecular diagnosis or family history of disease are recommended to proceed with "continuation therapy" [86]. Continuation therapy may be more or less intense than the initial therapy, depending on the needs of the patient. Reactivations of HLH disease are common and can happen due to a wide variety of triggers including infections and vaccinations. Reactivations are especially liable to happen as a patient is weaned off the aggressive initial therapy and onto a less rigorous treatment regimen. In such cases, therapy is re-established and/or re-intensified [86].

When an HLH patient does not respond to initial therapy, they then are treated with "salvage therapy" [112]. An un-responsiveness to first-line therapies typically can be

determined in the eight weeks used for initial therapy, but this period can also range between two and 70 weeks [112]. The current 2004 HLH protocol makes no specific recommendations for salvage therapy [86]. During this time, patients may receive blood and/or platelet transfusions [112], additional anti-inflammatory drugs, and/or additional antibiotics, antivirals, and/or antifungals [112]. Determining which drugs to use and the appropriate dosage are very case specific [113], and finding relevant information that could inform the attending physician is an often-cited challenge [112].

Drug Treatments

As to the particulars of the drugs used, HLH treatment most commonly consists of immune-suppression. The first major advancements in treating HLH were achieved in using etoposide (VP-16)—a derivative of epipodophyllotoxin, which are a class of anticancer drugs that work by inhibiting the enzyme topoisomerase II, thereby blocking DNA replication in dividing cancer cells [86, 114]. When applied to HLH patients, this drug inhibits the division of activated lymphocytes [115]. Later on, teniposide, another drug with topoisomerase II inhibitory ability, was also shown to be useful in preventing the expansion of inflammatory mediator cells [116]. A little while later, immunosuppressants such as CSA (cyclosporine A) and antithymocyte globulin (ATG) were shown to be effective at helping to resolve neutropenic episodes prompted by viral infection and limiting lymphocyte expansion [117, 118]. Given these early successes, the HLH-94 guidelines recommended combining etoposide and dexamethasone with cyclosporine for HLH patient treatment [85, 86, 119]. As of the writing of this dissertation, the standard of

care treatment for HLH patients still constitutes this combination therapy of dexamethasone and etoposides, with or without the use of cyclosporine [86, 112, 119, 120].

Another method used to treat HLH is the use of neutralizing the high levels of circulating interferon-γ. High levels of IFN-γ are a defining feature of HLH [101], and higher levels are correlated with poor prognosis [121].[2] Mouse studies indicate that these high levels of IFN-γ are primarily the result of increased antigen presentation to CD8 T cells, and secondarily to increased numbers of activated CD8 T cells [101]. IFN-γ levels can be 2-40 times higher in HLH patients than in healthy controls [127]. These high levels of interferon-γ lead to the increased expression of interferon-inducible genes and other inflammatory cytokines, especially from macrophages [128]. In particular, signaling for endocytosis in macrophages dysregulates as a result of high levels of IFN-γ, causing macrophages to phagocytose blood cells and contribute to the development of anemias and cytopenias [128, 129]. In November of 2019 the FDA approved the use of emaplumab, a

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[2] Specifically, high IFN-γ levels are present in the vast majority of HLH cases, but not all. HLH cases have been documented in individuals with either all or partial mutation to IFNγR1 or IFNγR2 122. Staines-Boone, A.T., et al., *Multifocal Recurrent Osteomyelitis and Hemophagocytic Lymphohistiocytosis in a Boy with Partial Dominant IFN-gammaR1 Deficiency: Case Report and Review of the Literature.* Front Pediatr, 2017. **5**: p. 75, 123. Tesi, B., et al., *Hemophagocytic lymphohistiocytosis in 2 patients with underlying IFN-gamma receptor deficiency.* J Allergy Clin Immunol, 2015. **135**(6): p. 1638-41.. Analysis of the PBMCs from children with active HLH have also shown no noticeable increase expression of IFN-γ or interferon-inducible genes 124. Sumegi, J., et al., *Gene expression profiling of peripheral blood mononuclear cells from children with active hemophagocytic lymphohistiocytosis.* Blood, 2011. **117**(15): p. e151-60.. Finally, mouse models of MAS and HLH show that hemophagocytosis and MAS can occur independently of IFN-γ over-expression 125. Canna, S.W., et al., *Interferon-gamma mediates anemia but is dispensable for fulminant toll-like receptor 9-induced macrophage activation syndrome and hemophagocytosis in mice.* Arthritis Rheum, 2013. **65**(7): p. 1764-75, 126.Avau, A., et al., *Systemic juvenile idiopathic arthritis-like syndrome in mice following stimulation of the immune system with Freund's complete adjuvant: regulation by interferon-gamma.* Arthritis Rheumatol, 2014. **66**(5): p. 1340-51.. Therefore while high IFN-γ expression is common in HLH, it is not essential for the disease to manifest.

monoclonal antibody used to bind and neutralize IFN-γ, for the treatment of HLH patients with recurrent, progressive, or non-responsive HLH [130].

Conditioning and Preparing for Transplant

Despite the advances in drug treatment, HSCT remains the only curative measure for HLH patients [82]. Successful HSCT first requires conditioning—the process of using combinations of irradiation and chemotherapy to eliminate the hematopoietic stem cells in order to make space for donated cells to engraft. Conditioning also can help reduce disease intensity (e.g. by reducing the numbers of expanded T cells), and also reduces the risk of developing graft rejection as a result of residual donor immune cells attacking the new graft [131]. HLH patients preparing for transplant receive either the intensive myeloablative conditioning (MAC) consisting of busulfan and cyclophosphamide or a comparatively gentle treatment with reduced intensity conditioning regiment (RIC) consisting of fludarabine and melphalan [132]. The trade-off made between these two regimens is that because bone marrow cells are destroyed by MAC, the chances for graft rejection are lower, but at the cost of greater toxicity. For example, depletion of lymphocytes using drugs like aletuzumab can lead a patient vulnerable to potentially fatal viral infection [112]. Other researchers at Texas Children's Hospital reported that administering cyclosporine in the early phases of treatment as recommended by the HLH-2004 guidelines can have neurotoxic results such as seizures, MRI abnormalities, and intracerebral hemorrhage [133]. Even the use of etoposides carries a risk of the patient suffering from medullar or mucosal toxicity, developing secondary leukemia, and/or exacerbating neutropenia, though most

would acknowledge that the benefits of these drugs far outweigh the risks [134]. In contrast, RIC conditioning protocols are designed to minimize toxic side effects, but at the increased risk of graft rejection. HLH patients can receive either MAC or RIC, but that patients that receive RIC have a three-year survival rate that is double to that of patients that received MAC [135]. While HLH patients that received RIC had over three times the chance of developing donor/recipient chimerism compared to those that received MAC (65% and 18% respectively), most of the patients that developed chimerism could be given additional infusions of donor lymphocytes or stem cells in order to increases the proportion of donor cells and prevent disease relapse [135]. A more recent study confirmed that HLH patients who received RIC are indeed likely to need further interventions and still have a high rate of suboptimal and short-term engraftment [132]. The consensus in the field is that conditioning regimens is an area of HLH treatment that still requires much refinement [132].

Hematopoietic Stem Cell Transplantation

Finding a donor for HSCT can be quite challenging, particularly for patients of underrepresented or mixed racial/ethnic ancestry [136]. One study found that the median wait time for an HLH patient to receive a transplant was 6.1 months [137]. Given that the disease can intensify rapidly, it is imperative that the search for a compatible donor begins as soon as possible [88]. Some treatment centers exhort transplantation within three months of diagnosis, even if a complete donor match is not available [138]. Bone marrow from a sibling, even one that is heterozygotic for FHL, is feasible option [78, 138]. In

patients without a matched donor, partial donor matches, or haploidentical donors, have also been reported to result in successful HSCT [139, 140]. Umbilical cord or placenta transplants, while effective, are generally not recommended unless no other option exists owing the fact that there will be no option for secondary transplantation or donor lymphocyte infusion [141].

Controlling inflammation prior to HSCT greatly increases the chances of a successful transplant. One study found that the five-year survival rate of patients with active disease at the time of transplantation was 58 percent, as opposed to patients without active HLH that had a five year survival rate of 72 percent [137]. Another study found that fifty percent of deaths in HLH patients which received HSCT were directly attributable to active HLH disease, and that disease control was even more important than haplocompatibility in terms of predicting engraftment success [138]. There are numerous ways in which active disease (AD) could reduce the likelihood of successful engraftment. High levels of IFN-γ have been shown to have a negative influence on hematopoiesis [138, 142]. Activated T cells and macrophages could also infiltrate the bone marrow, taking up the niches that HSPCs need in order to engraft and producing cytokines which deter engraftment and hematopoiesis [138]. Given this information, delaying HSCT in order to get the disease under control is often justifiable [78, 82, 137, 138].

Once conditions are right for transplant, hematopoietic stem cells are collected from the donor using either needle aspiration (this can be done with or without treatment with G-CSF), apheresis of the peripheral blood after injection with G-CSF, or the collection

of blood from an umbilical cord or placenta[3] [144]. Depending on the source of these HSCs and protocol being followed, they then might be expanded using additives such as SCF, Flt3L, IL-3 and IL-6 [145-148]. The donor cells are then infused into the patient [145].

Post-Transplant and Long-Term Prognosis

Back in 1983, the long-term survival for FHL patients was 4% [137], with a median survival of less than two months after diagnosis [98]. However, since the publication of the HLH 2004 treatment guidelines, substantial strides have been made in terms of increasing the survival rate [119], and the likelihood that a patient will need additional interventions. Table 2 summarizes the result from some of these most important HLH studies.

Transplant-related mortality (TRM) poses a significant challenge for HLH patients, particularly within the first 100 days after transplant [149]. Veno-occlusive disease (VOD), pneumonia, and a high incidence of primary non-engraftment are leading causes of post-transplant mortality [78]. Survival after HSCT is better if the patient has their disease under control/in remission at the time of transplant [149-153]. Many patients, especially those that received RIC prior to transplantation, do not maintain sufficiently high levels of

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[3] Several studies have investigated using cord blood for HSCT, with mixed results. A Japanese group reported that cord blood works just as well as matched unrelated donor 143. Ohga, S., et al., *Hematopoietic stem cell transplantation for familial hemophagocytic lymphohistiocytosis and Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in Japan.* Pediatr Blood Cancer, 2010. **54**(2): p. 299-306.. A Korean group found the opposite (Yoon et al 2010). Cord blood has the drawback that there is no option for donor lymphocyte infusion when the patient shows dwindling chimerism 78. Seo, J.J., *Hematopoietic cell transplantation for hemophagocytic lymphohistiocytosis: recent advances and controversies.* Blood Res, 2015. **50**(3): p. 131-9..

donor chimerism, and therefore require secondary transplants and/or donor lymphocyte infusions [132].

While theoretically successful engraftment should result in an FHL patient being cured of their disease, in practicality this is not always 100 percent true. Horne et al reported that 3% of patients developed HLH disease after transplant, despite adequate donor engraftment [154], and others have validated this observation [149]. This recurrence of disease typically occurs within 100 days of transplant, but cases have been known to occur as late as 180 days after transplant [149]. In order for successful resolution of the HLH phenotype, levels of chimerism must be maintained at a minimum of 10-20% [138]. If these levels can be maintained, HLH patients may live for longer than 20 years after HSCT [138].

Table 2 Prognosis of clinical HLH studies

Study	Percent of patients that survived long enough to receive transplant	Short Term Result (post-transplant)	Long term result (post-transplant)	Additional intervention (post-transplant)
[155]		One year survival rate: ≈ 95% for patients that received early etoposide treatment; ≈ 55% for patients that received late or no etoposide treatment	4 year survival rate of 78.3%	
[119]	78%	1 year survival rate ≈ 60%	3 year survival rate of 55%	
[138]	72%	92% survival for 4 weeks post-transplant; 10% secondary graft rejection 8-15 months post-transplant	5 year survival rate of 58.5%; Longest surviving patient 20+ years post-transplant	10% of transplanted patients required a secondary transplant
[137]	71%	77% survival rate 100 days post-transplant	5 year survival rate: 54% ± 6%	
[156][4]				18% of patients received a second transplant; survival rate for these patients was 66%
[115]	80%	81% survival for the first 100 days post-transplant; 70% survival in the first year	5 year survival rate of 62%	
[132]	98%	12 month survival rate of 80.4%; 18		35% of patients required a second transplant or donor

[4] Note, the Hartz et al study was a retrospective multi-center study of chimerism. Some of the patients in this study may have been reported on in other studies as well. 156. Hartz, B., et al., *The minimum required level of donor chimerism in hereditary hemophagocytic lymphohistiocytosis.* Blood, 2016. **127**(25): p. 3281-90.

	month survival rate of 66.7%	lymphocyte infusions

Living with HLH

Given the severity of HLH disease manifestation and the poor prognosis, most studies of clinical outcomes of HLH focus entirely on patient survival. However, the reader is encouraged to recognize that ultimately quality of life—not just survival—is the complete measure that HLH patients and their loved ones hope to maximize. Getting treatment for and recovering from HLH is an extremely taxing and uncertain journey. Beyond just fighting for survival, HLH patients and their loved ones must contend with the ambiguity of diagnosis, the difficulties with finding information and treatment for an orphan disease, anxiety over finding a transplant, and the mental, emotional, and financial stresses of extended hospital stays. Furthermore, patients suffer from treatment associated toxicities such as gastro-intestinal distress, difficulty sleeping, mood swings, and irritability. Even worse, patients are vulnerable to CNS sequelae which can have lasting impacts on their cognitive development and capabilities. It is not uncommon for patients to need physical, occupational, and/or speech therapy after receiving HSCT. Anxiety about relapse or flare-ups are ever-present. While we will restrict this text to having a more clinical and scientific tone that is in keeping with the stylistic conventions of dissertation writing, I would like to briefly recognize the hardships and struggles confronting HLH patients and their families. Anyone who is interested in connecting with HLH patients, learning about HLH patient advocacy, or contributing towards HLH research is encouraged to explore the following resources:

- Facebook Group: "HLH / Hemophagocytic Lymphohistiocytosis Family (Survivors & Angels)"

- Liam’s Light House Foundation
- 5K to Fight Histio

1.2.6 Landmarks in HLH Research

- 1939: Scott and Robb-Smith describe "histiocytic medullary reticulosis" as subset of diseases caused by "atypical Hodgkin's lymphoma" [157]
- 1952: Farquhar and Claireaux describe the first two documented cases of HLH, describing the disease as "familial hemophagocytic reticulosis" [75]
    - Similar conditions had been previously described, but given the literature available it is impossible to definitively confirm or deny these cases as HLH.
        - Anderson (1944)
        - Asher (1946)
        - Reese and Levy (1951)
- 1983: Janka reviews 121 published cases of HLH, summarizes diagnostic criteria, and coined the term "familial hemophagocytic lymphohistiocytosis," or "FHL" [98].
- 1986: Fischer et al perform the first HSCT on a patient with HLH [158]
- 1991: The Histiocyte Society first presents five diagnostic criteria for diagnosing HLH [84]
- 1997: The HLH diagnostic guidelines are updated to include three additional criteria [85]
- 1999: Ohadi et al conduct the first mapping approach to investigate the genetic causes of FHL. They identify a 7.8 cM region on chromosome 9q21.3-q22 as being aberrant compared to healthy subjects. To this day, no further specifications have refined our understanding of the FHL1 locus [68].
- 1999: Another genetic locus, this time on chromosome 10q21-22 was identified as another cause of HLH disease [69].
- 1999: Stepp et al identify Perforin as the protein from the gene on locus 10q21-22, and FHL2 is defined as being caused by mutations to the *PRF1* gene [91].
- 2000: Two independent studies confirm mutation to the *Rab27a* is responsible for Griscelli Syndrome Type II [159, 160].
- 2003: FHL-3 is mapped to mutations in the *UNC13D* gene that encodes Munc13-4 protein [161].
- 2004: the first study on gene therapy for Griscelli Syndrome Type II is published [162]. To the author's knowledge, this is the first gene therapy study of HLH/an HLH related disease.
- 2005: FHL-4 is mapped to mutations in the *STX-11* gene, the gene that encodes syntaxin 11 [163].
- 2009: FHL-5 is mapped to mutations in the *STXBP2* gene, the gene that encodes the protein for Munc18-2 [164].
- 2007: The new HLH 2004 treatment protocol was published, and included updated guidelines for HLH diagnosis and treatment [86].
- 2009: HLH Diagnostic guidelines updated to include additional supporting symptoms [87]
- 2015: the first study on gene therapy for HLH—specifically FHL2—is published [92]
- 2016/2017 The first studies on FHL3 gene therapy are published [165, 166]
- 2018: FHL2 patient T cells are successfully transduced using a lentiviral vector [92]
- 2018: XLP patient T cells are successfully transduced using a lentiviral vector [167]
- 2019: The first description of a gamma-retroviral HLH gene therapy is published [168]
- 2020: A third research group publishes on gene therapy for FHL3 [169]

1.3 FHL3

Having completed a general discussion of the HLH, we will focus our attention to the specifics of FHL3 and the basic immunological pathways which are disrupted in patients with this disease. We will begin by examining the *UNC13D* gene and the expression, regulation, and function of Munc13-4 protein. Finally, we will conclude this introductory chapter with a description of the FHL3 (*Jinx*) mouse model, and describe the nascent field of FHL3 disease research.

1.3.1 Expression of the *UNC13D* gene

As noted previously in Table 1, FHL3 is caused by a mutation in the *UNC13D* gene, which encodes for Munc13-4 protein [70]. The *UNC13D* gene is located on chromosome 17q25, and includes 32 exons that range in size from 36 to 235 bp long [161]. The mouse homologue to the human *UNC13D* gene is found on the murine chromosome 11 [170] and has 88.11% sequence identify with human Munc13-4 (NCBI protein BLAST for human vs. mouse Munc13-4, isoform 1).

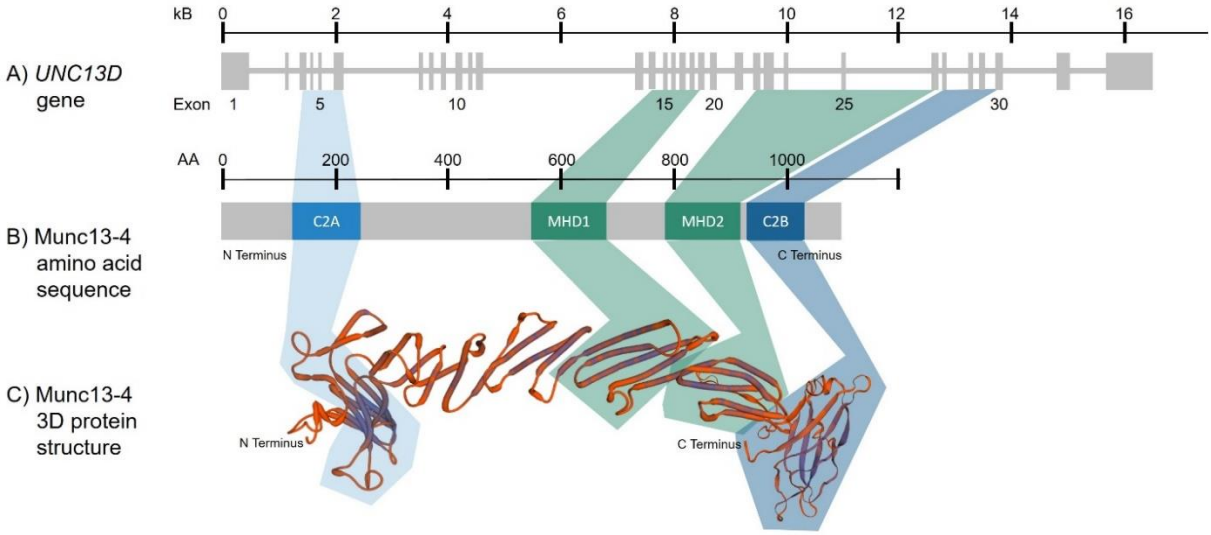
Developing full FHL3 requires receiving a defective copy of the *UNC13D* gene from both parents. While the presence of one working *UNC13D* gene can still mostly compensate [171] patients that are heterozygotic for *UNC13D* mutations are still at risk for other disorders such as autoimmune lymphoproliferative syndrome (ALPS) [172].

The literature has identified a wide variety of missense and nonsense mutations in the *UNC13D* gene which can lead to FHL3 [161, 173-175]. The original study that connected FHL3 to the *UNC13D* gene demonstrated six distinct mutations in ten patients

with FHL3 [161]. Most of these mutations led to truncation of the C-terminus of the Munc13-4 protein, either by introducing a pre-mature stop codon or by inducing a frameshift. One mutation caused an in-frame deletion within a highly conserved region of the MHD1 domain, potentially identifying a functionally critical region of the protein [161]. Since then, authors Santoro et al have suggested that the most common molecular defect in FHL3 patients are mutations that result in alternative mRNA splicing to the *UNC13D* mRNA transcript. Such mutations have been demonstrated in both exons and intronic regions [175].

Figure 1 .

Diagrams for the UNC13D gene, the amino acid sequence it generates, and the subsequently folded mature Munc13-4 protein. A) Human UNC13D (Gene ID 201294) is found on the complementary strand of chromosome 17, and is comprised of 32 exons.5 B) The resulting amino acid chain is 1090 aa long, and consists of a C2A, C2B, and a MUN domain comprised of two MHD domains. C) When folded, the Munc13-4 protein is projected to resemble a classic "dog bone" shape, with the extended MHD1 domains in the middle and the C2 domains forming knobby projections at the N- and C-termini.6



1.3.2 Regulation of the *UNC13D* gene

The *UNC13D* gene is controlled under the promoter ENSR00000098459 [176]. This promoter is active in CD4+ and CD8+ T cells, eosinophils, macrophages, monocytes, naïve B cells, neutrophils, and natural killer cells [176]. The promoter ENSR00000098459 has at least five enhancer regions, some of which are positioned adjacently and other

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[5] Location (NC_000017.11 (75827225… 758444404, complement).

[6] This 3D diagram was generated running a Swiss Model analysis on the amino acid sequence for Munc13-4 protein (NCBI Accession number NP_954712.1), and then combining the templates for Munc13-1 (template 5ue8.1.A) and cytosolic phospholipase A (template 1cjy.1.A). Combined, these two templates cover over 90% of the Munc13-4 protein structure. Nevertheless, it is important to note that in addition to the normal inaccuracies that come with 3D modeling, this one also suffers the limitation of being derived from the summation of two template analyses.

positioned 407.5 kb away. These enhancer regions can be found both down and up-stream of the start signal for the *UNC13D* gene [177]. One enhancer region in particular is located in intron 1 of *UNC13D*, and multiple reports of mutations to this 118-308 bp enhancer region led to the insight that altering these intronic enhancers leads to FHL3 phenotype [178-181].

Bioinformatics data indicates that enhancer regions of *UNC13D* are capable of binding to dozens of different transcription factors such as TRIM28, IRF2, GATA3, and STAT proteins 1, 2, and 3 [177]. More specific studies into the transcriptional regulation of *UNC13D* have highlighted the importance of the transcription factors STAT4, ELF1, and BRG1 [181]. Concisely, a c118-308C>T mutation in intron 1 of *UNC13D* led to the discovery that this region contains critical sites to which ELF1 binds and recruits STAT4 and BRG1 to upstream sites. The involvement of STAT4 is consistent with other research which implicates this transcription factor as a major contributor towards TH1 cell differentiation via the action of tri-methylation of lysine 4 on histone 3 at intergenic and promoter-associated binding sites [182]. Knockdown of STAT4 was shown to reduce Munc13-4 expression by 76% [181]. Therefore, though molecular diagnosis of FHL3 is most often concerned with coding mutations to the *UNC13D* gene, an evaluation of regulatory introns is also warranted. For example, one case study identified an intronic 1 mutation which disrupted NF-κB binding, leading to MAS and juvenile idiopathic arthritis—conditions very similar to HLH [183]. Given the many transcription factors identified as being capable to binding to *UNC13D* enhancer regions, particularly NF-κB and STAT proteins, one might expect that future discoveries in the field of FHL3 research could rapidly be made in this area.

1.3.3 Structure of Munc13-4 Protein

Munc13-4 protein is a member of the larger mammalian uncoordinated 13 (Munc13) family7, of proteins which also includes Munc13-1, Munc13-2, Munc13-3 [187] and Baiap3 [188]. This protein family is known for their role in the exocytic pathways. Munc13-4 is exceptional in that it serves its primary role in the exocytosis of cytotoxic granules from cytotoxic effector cells, and not as regulators of neurological synapses [188]. However, structurally, Munc13-4 is similar to the other members of the Munc13 family in that it contains two C2 domains8 separated by a MUN domain which includes MHD1 and

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7 Note that when Munc13-4 was first described in 2000 by Koch et al, the authors chose to refer to Munc13-4 as being part of a sub-family of Munc13-like proteins 184. Koch, H., K. Hofmann, and N. Brose, *Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform.* Biochem J, 2000. **349**(Pt 1): p. 247-53.. However, subsequent publications have cited Munc13-4 as being part of the Munc13 family proper 161.Feldmann, J., et al., *Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3).* Cell, 2003. **115**(4): p. 461-73, 185.Boswell, K.L., et al., *Munc13-4 reconstitutes calcium-dependent SNARE-mediated membrane fusion.* J Cell Biol, 2012. **197**(2): p. 301-12, 186. Higashio, H., Y. Satoh, and T. Saino, *Mast cell degranulation is negatively regulated by the Munc13-4-binding small-guanosine triphosphatase Rab37.* Sci Rep, 2016. **6**: p. 22539., and I have elected to continue this grouping here. However it is worth noting that while more recent authors have considered Munc13-4 to be a member of the Munc13 family of proteins, it is acknowledged that it is indeed a more distant relation.

[8] Note that some members of the Munc13 family have three, not two, C2 domains. C2 domains are further delineated as C2A, C2B, and C2C in the order that they are present going from the N-terminal to the C terminal of the protein. There is nearly a unanimous consensus within the literature in referring to the two C2 domains of Munc13-4 as "C2A" for the N-terminal C2 domain and "C2B" for the C-terminal C2 domain. The exception to this consensus is the work of van der Sluijs et al, who have elected to refer to the two C2 domains of Munc13-4 as "C2B" and "C2C" for the N and C-terminal C2 domains respectively (189. van der Sluijs, P., M. Zibouche, and P. van Kerkhof, *Late steps in secretory lysosome exocytosis in cytotoxic lymphocytes.* Front Immunol, 2013. **4**: p. 359.). This change in naming convention is probably due to the fact that in Munc13-1 and Munc13-2, the MHD domain is found between the C2B and C2C domains (190. Pei, J., et al., *Remote homology between Munc13 MUN domain and vesicle tethering*

MHD2 domains9 [184]. Other members of the Munc13-like family of proteins have a diacylglycerol-binding C1 domain, an N-terminal extension, and/or a PH domain [161, 192], none of which Munc13-4 has.

As of the writing of this review, three isoforms of Munc13-4 have been described in the literature[193]. The canonical sequence, referred to as Isoform 1, is 1090 amino acids long. By contrast, isoform 2 is only 363 amino acids long, and Isoform 3 is extended to be 1142 amino acids long [193].

C2 domains

The C2 domains exhibit mixed topology, with the first C2 domain (C2A) exhibiting a type I topology and the second C2 domain (C2B) exhibiting a type II topology. This mixed topology means that the N and C termini of the protein are either positioned at

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complexes. J Mol Biol, 2009. **391**(3): p. 509-17.). This would have given van der Sluijs et al justification for saying that Munc13-4 lacks a C2A domain. To the authors' knowledge, this is the single exception to the C2A and C2B convention, but readers of the Munc13-4 literature are exhorted to practice caution when researching these distinct domains.

[9] Note that earlier literature about Munc13-4 refers to this protein has having MHD1 and MHD2 domains, while later works cite only a single MUN domain, for which MHD1 and MHD2 domains are implicit.

MHD1 and MHD2 domains were first identified by Koch et al in 2000 during a search for proteins involved in exocytosis from non-glutamatergic neurons or non-neuronal cells. These two domains correlated with residues 1106-1249 and 1358-1525 of rat Munc13-1 protein, and were found to be present in a wide variety of proteins implicated in secretory pathways (184. Koch, H., K. Hofmann, and N. Brose, *Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform.* Biochem J, 2000. **349**(Pt 1): p. 247-53.). However, subsequent studies found that these regions could not fold properly nor function on their own. Further computational analysis showed that the homology between the C2B and C2C domains of Munc13-1 extended beyond the originally supposed MHD1 and MHD2 domains. Only by including these additional homologous regions could an autonomously folding unit be generated which was capable of rescuing neurotransmitter release in Munc13-1/2 double knockout mice. This larger region, which includes both the MHD1 and MHD2 domains as well as additional amino acid stretches, is today referred to as a "MUN domain" 191. Basu, J., et al., *A minimal domain responsible for Munc13 activity.* Nat Struct Mol Biol, 2005. **12**(11): p. 1017-8..

the very top or bottom of the C2 β sandwich, depending on the circular permutations of the β strands [194, 195]. The two C2 domains of Munc13-4 each have five aspartic acids that give the C2 domains an overall negative charge and form two calcium binding sites referred to as "Ca1" and "Ca2" [161]. These aspartic acids bind only to calcium ions, and not to Mg^{2+}, Ba^{2+} nor Sr^{2+} [185]. Once folded into a three dimensional structure, the locations of these calcium binding sites are positioned in one of three loops formed between beta strands of the C2 domain. Importantly, a large alpha-helix insertion into loop 3 is thought to prevent Ca^{2+} binding within that loop. Although this means that Munc13-4 can only bind two Ca^{2+} ions per C2 domain, this third loop may still be functional in terms of mediating membrane-protein interactions, similar to how inositol polyphosphate binds to Ca^{2+} independent binding sites on synaptotagmin isoforms I, II, and IV [194]. In addition to this potential phospholipid binding site on loop 3, the Ca^{2+} binding sites found on loops 1 and 2 are thought to facilitate binding of Munc13-4 protein to phospholipids [195].

MUN domain

The other functionally important domain in the Munc13-4 protein is the MUN domain, which is shared among other members of the Munc13 protein family. This domain consists of an elongated, arch-shaped architecture comprised of α-helical bundles and a central, highly conserved hydrophobic pocket [196]. The elongated structure spans more than 150 angstroms, and due to its similarity with other proteins like Sec6p, Tip20, and Exo70, has led some researchers to speculate that the MUN domain plays a role in tethering vesicles to presynaptic active zones [196, 197] Such a function was confirmed in stimulated

endothelial cells. In these cells, Munc13-4 is recruited to Weibel-Palade Bodies via its MHD1/MHD2 domains, and tethers the WPBs to the plasma membrane [198].

In addition to potential tethering function, the MUN domain also plays an important role in the priming of vesicles bound for exocytosis. Munc13-1 has a MUN domain which facilitates the transition of syntaxin-1 from a closed to an open conformation [196][10]. This transition is necessary for the maturation of the soluble NSF attachment receptor (SNARE) protein complex [196]. Once mature, the SNARE complex forces the plasma membrane and vesicle membrane into close proximity to allow for membrane fusion to occur and neurotransmitters to be released into the synapses between neurons [196].

The functionality of the MUN domain has been localized to two residues, Asn-1128 and Phe1131 (sometimes referred to as residues "NF"), which are located within the hydrophobic pocket of the MUN domain. It is thought that these residues might bind either syntaxin-1 or other SNAREs, but a definitive substrate has yet to be identified. Also, while the NF residues are critical, other subdomains of the MUN domain enhance SNARE complex assembly, probably by catalyzing the opening of syntaxin-1 [196].

Munc13-4 shares this MUN domain, including the NF residues, with Munc13-1. Therefore, while these processes described above have been studied most extensively in the MUN domains of Munc13-1 proteins and their involvement in neurotransmitter release, it is

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[10] This process is sometimes referred to as transitioning from the Munc18-1-closed syntaxin-1 complex to the SNARE complex 196. Yang, X., et al., *Syntaxin opening by the MUN domain underlies the function of Munc13 in synaptic-vesicle priming.* Ibid.2015. **22**(7): p. 547-54..

reasonable to speculate that the MUN domain of Munc13-4 protein may serve similar roles within the context of exocytosis at immunological synapses.

Post translational modification

Post-translational modifications such as lipidation, glycosylation, or the creation of disulfide bonds can alter the structure, targeting, and subsequent function of a protein. To date, few post translational modifications have been noted for Munc13-4 protein. The best characterized of these is Munc13-4's ability to bind calcium ions with its C2 domains (see previous section) [161] [185]. Other studies have also found that the C2B domain can bind to lipids in liposomes [199]. Bioinformatics analysis indicates the possibility that the serine at position 150 of the Munc13-4 protein can be phosphorylated during the G1/M phases of cell division [200]. The latter was published in a broad bioinformatics analysis of what proteins are phosphorylated in dividing HeLa cells, and further studies are needed to validate the existence and biological significance of this phosphorylation.

1.3.4 Regulation of the Munc13-4 protein

Recruitment to target membranes

Munc13-4 protein associates with a number of membranes including plasma membranes [185, 201-203] lysosomes [192], liposomes [204], secretory granules [199], and transient associations with the membranes of multivesicular bodies (MVBs)[205]. With regards to cytoplasmic distribution of Munc13-4, the currently available literature contains conflicting reports. Munc13-4 has been observed to be dispersed in the cytosol of breast

cancer carcinoma cell lines [205], while others have reported minimal cytoplasmic Munc13-4 expression in mast cell lines [192] and neutrophils [206].

To date, no cohesive understanding of Munc13-4 protein exists, and instead the curious investigator must delve into a body of research that is fragmented according to cell types. Nonetheless, we suspect that the specific mechanism of Munc13-4 recruitment to the specific target membranes is consistent throughout all the different cell types that express it and use it for exocytosis of secretory granules. Specifically, the literature would have us believe that the C2 domains are critical for the recruitment of Munc13-4 to the secretory granule membrane [198] and that association with membranes is dependent on the binding of calcium ions to the C2B domain and subsequent binding to PS [185].

In addition to being calcium ion dependent, Munc13-4 recruitment to exocytic vesicles can be regulated by the type of stimuli the cell receives. For example, resting natural killer cells from healthy patients do not have Munc13-4 recruited to perforin-containing vesicles. Only when a strong stimulus such as activation with PMA/ionomycin, engagement of CD16, or co-engagement of 2B4 or NKG2D is Munc13-4 recruited. Notably weaker signals such as the engagement of LFA-1, 2B4 or NKG2D alone is not a strong enough stimuli to recruit Munc13-4 and therefore NKs that are stimulated in this way fail to degranulate [207]. Similarly, human neutrophils have diffuse levels of Munc13-4 in their cytosols until stimulated with human neutrophil elastase, after which Munc13-4 is recruited to the plasma membrane [206].

Recycling

Another way that Munc13-4 activity can be regulated is by recycling the proteins after fusion of a vesicle with the plasma membrane. Using TIRF microscopy, Woo et al reported that Munc13-4 diffuses into the plasma membrane after fusion of lytic granules with the plasma membrane [208]. Once in the plasma membrane, Munc13-4 associates with lipid rafts enriched in phosphatidylinositol(4,5)-biphosphate (PIP2), after which Munc13-4 is internalized via AP-2/clathrin-dependent internalization [209]. This mechanism is very similar to the mechanism used for synaptic vesicle protein retrieval found in presynaptic neurons [189]. Correlational evidence from PIP5K inhibition studies suggest that a lack of Munc13-4 recycling (as indicated by large accumulations of Munc13-4 at the plasma membranes of cytotoxic cells) is linked to decrease in serial cytotoxicity. While an intriguing hypothesis, definitive causational studies are required to determine the extent to which Munc13-4 recycling is essential and/or rate-limiting in the context of serial cell killing.

1.3.5 Functions of Munc13-4 Protein

1.3.5A Expression of Munc13-4 in different cell types

The other members of the Munc13 family are primarily expressed in the tissues of the CNS where they regulate the release of neurotransmitters into the synapses between neurons [187]. Bioinformatics analysis shows that in contrast, Munc13-4 is expressed broadly in the spleen, thymus, peripheral leukocytes, the goblet cells of the lung, and the placenta, but not in the small intestine, prostate, ovary, colon, brain, heart, kidneys, or skeletal musculature [161, 184]. Further investigation into specific cell types that express

Munc13-4 protein show that CD19+ B lymphocytes, CD4 and CD8 positive T lymphocytes, NK cells, and mononuclear cells, whether resting or active, express high levels of Munc13-4 protein [161]. Beyond just bioinformatics, dedicated studies have also described Munc13-4 expression in natural killer cells [210], T cells [161, 181, 203, 211, 212], platelets [185, 213-215], epithelial cells [216], endothelial cells [198], mast cells [185, 186], and neutrophils [204, 206, 217-220]. Broadly, Munc13-4 is used in the trafficking of endocytic vesicles and the priming of granules for exocytosis. The following is a summary of the current understanding for the role of Munc13-4 with regards to both of these processes, organized by cell type.

1.3.5B Cytolytic cells

Munc13-4 protein plays an indispensable role in the release of cytotoxic granules (also referred to as "lytic granules,", "LGs," secretory lysosomes," or "lysosome-related organelles" [203] from CD8+ effector T cells and natural killer (NK) cells. These granules are comprised of an acidic proteoglycan core, soluble lysosomal enzymes, and lysosome-associated membrane proteins such as Lamp1 (CD107a), Lamp2 (CD107b), and Lamp3 (CD63) [201, 203]. Once a naïve CD8+ T cell encounters an antigen, it takes an average of 5-8 days in the secondary lymphoid tissues for that cell to differentiate into a CTL (during which perforins and granzymes are synthesized and packaged into cytolytic vesicles), proliferate, and migrate back into the tissues [221]. In contrast, natural killer cells are constitutively primed for targeted cell killing [202]. Upon encountering a target cell while patrolling through the peripheral tissue, a CTL will take 20-30 minutes to degranulate and kill the offending cell [202].

Cytotoxic degranulation can be broken into five distinct phases: 1) recognition of the target cell by the cytotoxic cell, 2) adhesion and formation of a synapse between the cytotoxic cell and the target cell, 3) rapid reorientation of the Golgi complex and the microtubule organizing center (MTOC), 4) polarization of lytic granules towards the immunological synapse, and 5) vesicle release [222]. For the purposes of the introduction, we will confine our discussion to the very end stages of this process and the role that Munc13-plays in it. For a more a more generalized discussion of this process, please consult de Saint Basile's excellent 2010 review [202].

The final step in cytotoxic granule release can be further broken down into the processes of 1) vesicle docking to the plasma membrane, 2) priming, and 3) fusion of the vesicle and target membranes. Docking of mature cytotoxic granules occurs when Rab27a protein interacts with membrane-anchored synaptotagmin-like protein 1 or 2 (SLP1 or SLP2) [202]. This interaction is facilitated by the interactions of Munc18-2 and syntaxin 11 in the closed formation [221].

Once docked, the second step of in cytotoxic granule release is priming. For priming to occur, binding of Rab27a at a region of Munc13-4 between the C2A and the MUN domains is essential [189, 192, 203], as is Ca^{2+} binding to both of Munc13-4's C2 domains [185, 208]. Specifically, Ca^{2+} binding to the C2A domain facilitates but is not necessary to the binding of Munc13-4 protein to the H3 domain of SNAREs/SNARE motifs of plasma membrane associated syntaxins 1, 2, 4, and syntaxin 11. As noted previously, Munc13-4's syntaxin binding site is likely within the hydrophobic pocket of its MUN domain [196]. Furthermore, binding of Ca^{2+} to the C2B domain strongly facilitates Munc13-4 interactions with the plasma membrane components phosphatidylcholine and

phosphatidylserine. While the C2A and C2B domains can act independently of each other, in order for exocytosis of lytic granules to occur, both must be functional in their binding to Ca^{2+} ions [185]. The result of these calcium-dependent interactions is the formation of 70 and 110 kD SNARE complexes at the plasma membrane [185]. Without the priming step, lytic vesicles accumulate at the plasma membrane but are unable to fuse [161].

The final step in cytolytic vesicle release is fusion of the plasma and lytic vesicle membranes. This is mediated by interactions between the t-SNARE, v-SNARE[11], and SNAP proteins of the vesicle and target membranes occur. A four-helix bundle forms, with three alpha-helices coming from t-SNAREs (typically two from SNAP-23, and one from syntaxin 11) and one v-SNARE (typically VAMP7) [224].[12,13] Finally, complexes of Munc18 proteins and SEC1 proteins clasp across the SNARE complex bundle[14] and causes

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[11] The terms "t-SNARE" and "v-SNARE" were originally coined to describe SNAREs respectively present on either the target or vesicle membrane. This terminology has fallen somewhat out of favor in exchange for more descriptive terms like "R-SNARE" (an arginine containing SNARE) or Q-SNARE (a glutamine containing SNARE). 223. Pattu, V., et al., *SNARE protein expression and localization in human cytotoxic T lymphocytes.* Eur J Immunol, 2012. **42**(2): p. 470-5.

[12] This four-helix bundle structure is also found at the fusion sites of other exocytic granules. The specific proteins that form this structure can vary amongst cell types, but it typically contains three Q-SNAREs and one R-SNARE, though exceptions to this rule have been described 223. Ibid.. For example, in mast cells, VAMP7, VAMP8, syntaxin4, and SNAP23 create a complex 225. Liu, S., et al., *Zoledronate modulates intracellular vesicle trafficking in mast cells via disturbing the interaction of myosinVa/Rab3a and sytaxin4/VAMP7.* Biochem Pharmacol, 2018. **151**: p. 18-25, 226. Sander, L.E., et al., *Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells.* Eur J Immunol, 2008. **38**(3): p. 855-63., but in eosinophils and neutrophils VAMP8 has not been implicated in this process 224. Chitirala, P., et al., *Cytotoxic Granule Exocytosis From Human Cytotoxic T Lymphocytes Is Mediated by VAMP7.* Front Immunol, 2019. **10**: p. 1855..

[13] It is also important to note that the specific cell machinery is not the same in humans and mice. In mice, VAMP2, not VAMP7, is the primary mediator of LG degranulation 227. Matti, U., et al., *Synaptobrevin2 is the v-SNARE required for cytotoxic T-lymphocyte lytic granule fusion.* Nat Commun, 2013. **4**: p. 1439..

[14] The human genome contains 26 tSNAREs and 9 vSNAREs, but only a subset of these SNAREs are known to be expressed in CTLs 223. Pattu, V., et al., *SNARE protein expression and localization in*

fusion of those membranes [221]. After granule release, LAMP-1 associated membranes are recycled back into the cell [228].

In some studies, Munc13-4 has been demonstrated as being the rate-limiting step during vesicle fusion. Boswell et al found that the rate of lipid mixing was dependent on the concentration of Munc13-4 protein in the presence of Ca^{2+} [185]. In another study of cultured natural killer cells, the recycling of Munc13-4 from the plasma membrane after lytic granule fusion was the rate-limiting step for subsequent serial cytotoxic granule release [209].

In addition to its role in the final step of fusing an LG with the plasma membrane, Munc13-4 has also been suggested to be important for the maturation of those LGs. Whilst perforin and granzyme are transported in LGs to the plasma membrane, Munc13-4 and Rab27a have been shown to be co-transported to the plasma membrane on endosomes [201]. These endosomes were also shown to sometimes fuse with Rab11+ recycling endosomes, similar to the phenomena observed in neutrophils [201]. Fusion of these separately trafficked endosomes with the LG membrane occur only when a CTL conjugates with a target cell, and only after this fusion are the LGs capable of secretion. Until that point, the lytic granules behave as lysosomes [201]. Whether or not Munc13-4 plays an

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human cytotoxic T lymphocytes. Eur J Immunol, 2012. **42**(2): p. 470-5, 224. Chitirala, P., et al., *Cytotoxic Granule Exocytosis From Human Cytotoxic T Lymphocytes Is Mediated by VAMP7*. Front Immunol, 2019. **10**: p. 1855.. A comprehensive list of all the proteins involved in the process of LG fusion with the plasma membrane at an immunological synapse would be very useful. At the time of this writing, over 23 different SNARES have been shown to be expressed in stimulated T cells and associate with lytic vesicles 223. Pattu, V., et al., *SNARE protein expression and localization in human cytotoxic T lymphocytes.* Eur J Immunol, 2012. **42**(2): p. 470-5..

active role in LG maturation or if it is “just along for the ride” has yet to be experimentally demonstrated.

An important point worth noting is that cytotoxic cells that cannot release cytotoxic granules due to defective or absent Munc13-4 proteins might still be able to kill target cells via Fas/FasL interactions. Indeed, patients with HLH have been documented has having higher levels of soluble FasL in their serum [229]. Investigations into single perforin $^{-/-}$ and DC Fas $^{-/-}$ as well as double knock-out mice have shown that while Fas : FasL interactions can indeed lead to killing of over-expanded inflammatory cells, the combination of perforin and FasL killing have a synergistic effect [187]. Furthermore, FHL3 patients are unable to compensate for their condition by over-expressing the Fas-mediated apoptotic pathway. Therefore while lymphoproliferation may be able to be somewhat abated by the Fas-mediated apoptotic pathway, this is not to say that perforin/granzyme killing is redundant or replaceable by this pathway.

1.3.5C Goblet cells

As noted previously, cells of the respiratory tract express high levels of Munc13-4 protein [161, 184]. Munc13-4 is expressed at particularly high levels in “goblet cells,” which are a type of secretory cell that are interspersed throughout the epithelial cells of the lung. These goblet cells secrete mucins such as MUC5AC which in turn act to hydrate and lubricate the lung epithelia and aid in the clearance of pathogens. The process of MUC5AC secretion involves calcium-dependent exocytosis, similar to the process of lytic granule release in cytotoxic cells. Notably, it is VAMP8 and not VAMP7 that is the v-SNARE that interacts with SNAP23 in goblet cells. ([206]. While basal levels of MUC5AC are

primarily regulated by Munc13-2, Munc13-4 has been shown to be essential to the hyper-secretion of MUC5AC which is prompted by stimulation of the cells with human neutrophil elastase [206].

Given this information, one might expect that FHL3 patients might have chronic respiratory problems, perhaps associated with an inability to secrete adequate mucins from goblet cells. In fact, a retrospective analysis of HLH patients found that even though lung involvement in HLH receives little attention, over half of patients present with lung-related pathology—most commonly dyspnea and coughing. Mortality rates were higher in HLH patients with lung involvement, and lung infections were a significant predictor of death. Still, just under half of patients did not present with lung pathology, suggesting the workings of a compensatory mechanism for goblet cell secretion [161], potentially from the contributions of Munc13-2 in constitutive mucin secretion [206].

1.3.5D Platelets

Munc13-4 protein has also been implicated in the secretion of ADP and serotonin containing dense granules from platelets [230]. Secretion of these dense granules is important for hemostasis and thrombosis formation. Specifically, Munc13-4 co-precipitates with GTP-Rab27a and -Rab27b, and has been shown to be able to compensate for inhibited dense granule degranulation as a result of unprenylated Rab27a (which cannot associate with target membranes) [213]. The mechanistic value of Munc13-4 as a tethering agent was further underscored by experiments in which Munc13-4 was observed to bind to liposomes which had increased levels of phosphatidylserine as a result of calcium ion

stimulation. This binding requires both C2 domains, although the functionality of the C2B domain could be localized to just the calcium-ion binding regions [231].

The importance of Munc13-4 in platelet granule exocytosis has also been studied in Munc13-4 deficient mice. *Jinx* platelet counts and average size are no different from those of WT mice [230], but these platelets are reportedly unable to release dense granules, and have inhibited release of α-granules and lysosomes [214, 215, 231]. This contributes to increased tail-bleeding times as a result of impaired platelet aggregation. In fact, Munc13-4 has been suggested to be the rate-limiting factor in the process of platelet granule secretion [214]. These results were corroborated in other studies that used either global KO or conditional platelet Munc13-4 knockout mice to show that Munc13-4 deficiency increased venous bleeding time, increased time to arterial thrombosis, was the rate limiting step in the formation of thrombi, and finally caused a decrease in allergic airway inflammation [230, 232]. Much of this decreased platelet functionality can be attributed to the lack of ADP release from dense granules of platelets, which under normal circumstances would act as a as a major positive feedback loop for thrombus growth and stability [230].

Given the mechanistic importance of Munc13-4 in platelets, the lack of clinical evidence indicating FHL3 pathology related to impaired platelets is surprising. Reports of bleeding disorders, bleeding diathesis, and brain edema are most commonly reported in patients with FHL5, Chediak-Higashi Syndrome, and Hermansky-Pudlak syndrome type II, or secondary HLH [233-236]. Broadly, HLH patients have been treated with platelet infusions, recombinant human thrombopoietin (rhTPO), and/or recombinant factor VIIa (rFVIIa) in order to reduce gastrointestinal bleeding, urinary tract bleeding, and pulmonary bleeding [236, 237]. However, with regards FHL3 patients, the literature on associated

bleeding disorders is strikingly sparse. A single case report of two FHL3 patients observed that these patients had normal platelet counts, normal platelet aggregation, and had no major bleeding episodes, but that these platelets had severely inhibited release of dense granules and upregulation of CD107a and cell adhesion molecules at the plasma membrane [238]. The extent to which impaired platelet function contributes to FHL3 very much remains an open question. It would be very interesting to analyze retrospective clinical data and see if perhaps FHL3 patients required extra care during surgery or were prone to bleeding events.

1.3.5E Endothelial Cells

Compounding on the idea that FHL3 patients should theoretically be prone to bleeding disorders is the fact that Munc13-4 is also necessary for the exocytosis of Weibel-Palade bodies (WPBs) from endothelial cells (the cells that line the blood vessels and the heart). Normally, WPBs are released from endothelial cells as a result of tissue damage or inflammation. The contents of those WPBs include von Willebrand factor that binds to factor VIII to promote platelet adhesion to wound sites and p-selectin which is used to recruit leukocytes. Knock-down studies have shown that Munc13-4 is necessary for the histamine-induced release of WPBs [198]. At rest, Munc13-4 is present on WPBs as well as in the cytoplasm and on the plasma membrane. Upon stimulation, Munc13-4 concentrates on WPBs and at the plasma membrane, presumably at sites of active membrane fusion [198]. Mutation studies demonstrated that this re-distribution is dependent on both C2 domains, but association with the WPBs is dependent on the MHD1/2 domains. Furthermore, the recruitment of Munc13-4 to WPBs as well as

Munc13-4 mediated WPB exocytosis are independent of Rab27a. Rather, at least in endothelial cells, it was shown that histamine stimulation induces plasma membrane recruitment of annexin A2, and that this protein facilitates the recruitment of Munc13-4 through direct interactions between the C2 domains of Munc13-4 and the S100A10 subunit of annexin A2 [198].

One might therefore expect that FHL3 patients would be particularly prone to bleeding disorders, but as discussed in the previous section, they are not. It is possible that another mechanism for WPB exocytosis, such as cAMP-dependent WPB exocytosis, can compensate for the lack of Munc13-4 protein in FHL3 patients [198].

1.3.5F Mast Cells

Another cell-type well known for its secretory functions is the mast cell. Mast cells are myeloid cells that reside in connective and mucosal tissues and which release histamine, heparin, proteases, and chemokines/cytokines as a result of IgE binding to their FcεRI receptors and subsequent calcium ion influx [239]. The release of these factors induces a typical allergic inflammation response, characterized by vasoconstriction, reddening, tissue remodeling via the destruction of extracellular matrices, and the recruitment of other immune cells. Munc13-4 has been shown to be essential for mast cell degranulation, specifically calcium-ion induced degranulation [240-242].

As with other cell types, part of the functionality of Munc13-4 is attributable to the calcium-ion sensing abilities of C2 domains [240]. Mutation of the C2 domain regions

impairs mast cell degranulation both in terms of the number of fusion events, the rate at which individual fusion events happen, and the size of individual fusion pores [240].

Given the above information, one might therefore suspect that FHL3 patients would be particularly susceptible to parasitic infections, have diminished allergy responses, and have impaired anaphylaxis. Indeed, numerous case reports indicate that HLH patients are prone to parasitic infections, especially malaria and leishmaniasis [72, 243-245]. Furthermore, mastocytosis, or the accumulation of mast cells either systemically or in the organs, has been documented in HLH patients [246]. In contrast to these dramatic examples of mast cell involvement, the effect of impaired mast cell function on allergies would be predictably subtle, and at the time of this writing no studies have documented any attenuation of allergic responses in HLH patients. However, in the grand scheme of things, this question is a minor curiosity compared to the concerns of treating HLH patients and understanding their disease pathology. From an immunological standpoint however, much has yet to be understood about mast cell involvement in FHL3.

1.3.5G Neutrophils

A final and very important cell type that express Munc13-4 and which could contribute to FHL3 pathology are neutrophils. Neutrophils a short-lived leukocyte that act as first-responders to inflammation and bacterial infections. They are capable of releasing granules laden with proteases and reactive oxygen species, releasing their chromatin and antimicrobial peptides to form neutrophil extracellular traps (NETs), phagocytosing pathogens for intracellular killing, and secreting cytokines to create an inflammatory environment. Many of these functions require fusion of membrane-bound organelles, and

Munc13-4 plays a critical role in this process. In fact, compared to all other cell types, neutrophil biology has contributed the most towards the understanding of Munc13-4 protein. While specific mechanistic details may be restricted to just neutrophils, many insights from this field of research can be extrapolated to thinking about the role of Munc13-4 in other cell types and overall its role in FHL3 disease.

It will likely not surprise the reader the Munc13-4 plays an important role in the release of inflammatory granules from neutrophils. Neutrophils can secrete granules in response to stimulation and subsequent calcium ion influx. These granules contain proteases, and cytotoxic agents such as matrix metalloproteinases (MMPs and myeloperoxidases (MPOs) [199]. Neutrophils can also secrete reactive oxygen species (ROSs) into the extracellular environment at sites of infection via $p22^{phox}$ expressing secretory granules [219, 247]. In resting neutrophils, Munc13-4 distribution is mostly cytosolic, but upon stimulation (e.g. with fMLF) Munc13-4 is rapidly recruited to membranes in a calcium ion-dependent way and associates with those membranes using calcium-ion independent binding with its C2 domains [204]. As in the case of endothelial cells, this process of redistribution is independent of Rab27a binding. At least in neutrophil differentiated PLB-985 cells (a myeloid cell line) which were stimulated with fMLF, Munc13-4 is necessary for the exocytosis of matrix metalloproteinase-9 (MMP-9) containing tertiary granules and CD63 positive primary granules [204]. Similarly, extracellular release of ROSs is also dependent on Munc13-4 mediated exocytosis [219].

It was recently discovered that Munc13-4 mediated exocytosis is complexly regulated by two proteins—STK24 and CCM3. STK24 can suppress Munc13-4 mediated exocytosis by interacting with the C2B domain of Munc13-4 and competing for its lipid

binding sites, thereby not allowing Munc13-4 to associate with the target membranes [199]. This degranulation inhibition can be reversed with CCM3, which interacts with the C2A domain of Munc13-4 and recruits STK24 away from Munc13-4's C2B domain [199].

In addition to its role in exocytosis, Munc13-4 also plays an important role in the maturation of late endosomes. Late endosomes are dynamic organelles with heterogeneous composition according to their stage of maturation [248]. The complex maturation process that transforms an early endosome to a late endosome includes homotypic fusion of multiple endosomes to form larger, maturing vesicles, conversion of RAB5 to RAB7 [249], the formation of intraluminal vesicles [250], heterotypic partial fusion (aka "kiss-and-run") of the late endosome with lysosomes [248], accumulation of lysosomal-associated membrane proteins (LAMPs), and modification to lipids such as the conversion of phosphatidylinositol 3-phosphate to phosphatidylinositol 3,5-biphosphate) [251]. Munc13-4 plays an indispensable role in this process by aiding in the many fusion events that are required to enlarge, acidify, and ultimately mature the LE. Upon calcium ion signaling, Munc13-4 associates on late endosomes in a calcium-dependent way with syntaxin 7 and in a calcium-independent way with VAMP8 [248]. This triplex aids in both heterotypic and homotypic fusion events that help the LE mature [248].

Mature LEs in neutrophils can further develop into phagosomes. Similar to the fusion events that are necessary for the maturation of an LE, phagosomal maturation requires the fusion of primary/azurophilic granules and multivesicular bodies [219]. Absence of Munc13-4 impairs this fusion, meaning that phagosomes never receive the myeloperoxidase and serine proteases and therefore cannot generate HClO [219]. This

contributes overall impaired intracellular bacterial killing from Munc13-4 deficient neutrophils [219].

Aside from mediating the maturation of phagosomes, Munc13-4 also contributes to neutrophil intracellular bacteria killing by regulating intracellular ROS production. In neutrophils, ROSs are produced from NADPH oxidase—a multi-subunit enzymatic complex consisting of cytosolic factors $p67^{phox}$, $p40^{phox}$, membrane associated flavocytochrome b_{558}, and the accessory proteins Rac2(13) and Rap1a [219]. Taken together, this complex can reduce oxygen to produce superoxide anions (O_2^-)[219]. In Munc13-4 null mouse neutrophils, it was observed that $p22^{phox}$ (one of the two subunits that make up the flavocytochrome b558) can traffic to the site of NADPH oxidase assembly on intracellular vesicle membranes, but that fusion of those intracellular vesicles never occurs. As such, both intracellular and extracellular ROS production is prevented, contributing to still further impairment of neutrophilic function. Experimental evidence suggests that neutrophils attempt to compensate for this deficiency by upregulating the release of NETs (neutrophil extracellular traps), but even this is not enough to recover their overall cell killing abilities [219].

Maturing endosomes need not just be degradative in nature, but can also have cell signaling properties, such as TLR9 signaling to activate neutrophils in response to CpG DNA [248]. Innate immune cells express TLR9 on their endoplasmic reticulum until CpG DNA (a type of DNA that is generally associated with bacteria) is endocytosed and transported into a tubular lysosomal compartment. TLR9 and its downstream effector proteins (e.g. MyD88) then also traffic to this compartment and execute their signaling functions in order to activate the immune cell [252]. In neutrophils, these many fusion

events are mediated by Munc13-4, and an absence of Munc13-4 leads to impaired TLR9 signaling [248]. Thus FHL3 neutrophil dysfunction is not just a property of inhibited cell killing, but also a function of impaired immune activation.

Interestingly, Munc13-4 has been shown to be important in endosomal recycling. Endosomal recycling is the process which occurs after proteins presented on the cell membrane (e.g. receptors, ion channels, ion pumps, and/or adhesion molecules) are internalized via endocytosis. Some of these proteins will be targeted for degradation, but other will need to be targeted for recycling back up to the plasma membrane. This complex process allows a cell reuse valuable proteins and help it maintain homeostasis [253]. At least in neutrophils, the final step in endosomal trafficking, the fusion of recycled endosomes with the plasma membrane, has been shown to be mediated by Munc13-4 protein. Specifically, Munc13-4 binds to Rab11+ recycled endosomes, helping facilitate their fusion with the plasma membrane. This binding between Munc13-4 and Rab11 is mediated by calcium signaling. Munc13-4 deficient neutrophils have normal endocytosis, but impaired trafficking and retention of Rab11 vesicles to the plasma membrane. Endosomal recycling is used in many cell types beyond just neutrophils, and if these cells also rely on Munc13-4 mediated endosome recycling the resulting dysregulation could affect many cell types beyond the myeloid and lymphoid lineages. Admittedly, this phenotype would likely be subtle as cells would likely compensate for lack of endosome recycling by instead upregulating more protein expression. The question as to the role of endosome recycling in FHL3 disease and its effects on patients before and after HSCT is a fascinating immunological question that will warrant addressing as the field of FHL3 disease management evolves.

Another, more recently discovered function of Munc13-4 in neutrophils is its ability to associate with STX7 and regulate macroautophagy[15]—the process of degrading and digesting entire organelles and bulky cytoplasmic components by sequestering them into a large autophagosome and subsequently fusing it with a lysosome [254]. This process is regulated by nutrient availability and cell stress. While Munc13-4 is necessary for endosomal trafficking/late endosome maturation[16], it is not necessary for the fusion of autophagosomes with lysosomes. This is presumed not to happen because Munc13-4 does not bind to STX17—a SNARE that localizes on the outer membranes of autophagosomes and which helps regulate autophagosome/lysosome fusion) [254]. Munc13-4 deficient neutrophils have both impaired endosomal maturation/late endosome trafficking and increased macroautophagy, degradation of long-lived proteins, and numbers of autophagosomes [254].

Originally it was proposed that Munc13-4's involvement in macroautophagy was related to its ability to bind to RAB11 (which has been shown to aid in the fusion of recycling endosome membranes [255] and VAMP8 (which has been shown to recruit STX17 and mediate the fusion of autophagosomes and lysosomes [256]. However, Munc13-4 deficient cells still retain high levels of RAB11 and VAMP8 positive vesicle fusion with autophagosomes, suggesting that another mechanism for Munc13-4 involvement in autophagy is at work. To date, one of the best hypothesis is that the

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[15] "Macroautophagy" is sometimes just referred to as "autophagy."

[16] Again, this process results from Munc13-4 binding to STX7—an SNARE which regulates endo-lysosomal fusion

increase in macroautophagy observed in Munc13-4 deficient neutrophils happens as a result of compensatory action of the transcription factors TFEB and TFE3, which upregulate autophagy related genes such as *Atg9b* and *Ulk1 [254]*. A lack of autophagy regulation has been implicated as a mechanism for HLH disease pathology [257], but at present it is mostly a matter of speculation. Still others have observed ATP-induced autophagy is associated with impaired degradation of phagocytosed bacteria, which could theoretically contribute to increased susceptibility to bacterial infections.

FHL3 patients are particularly susceptible neutropenias, as well as bacterial and fungal infections [72, 118, 245, 258], potentially indicating neutrophil dysfunction as another mediator of disease. In FHL2 mouse models, neutrophil infiltration correlates with increased tissue damage and increased inflammation. Furthermore, treating LCMV-infected perforin null mice with neutrophil depleting antibodies exerts a protective effect on these mice [259, 260]. Given all of the above, it seems safe to say that Munc13-4 deficient neutrophils play an important role in mediating FHL3 disease.

1.3.5H Munc13-4 and homologous DNA repair

A single study from researchers at the Chinese Academy of Medical Sciences in Tianjin China reported that siRNA knock-down of Munc13-4 protein expression caused a reduction in homologous recombination repair of double-stranded DNA breaks [261]. Based on these results, the authors suggest that FHL3 disease is in part mediated by impaired ability of cells to repair double stranded DNA break. Unfortunately for Western readers, this article was not translated into English, and at present not even a copy of the manuscript is available to the public for scrutiny of the figures. Without further

evidence, it seems dubious that Munc13-4 plays any role in homologous recombination repair because of how FHL3 patients have no documented hypersensitivity to radiation. Furthermore, correction of FHL3 can be achieved through HSCT, which would not affect other Munc13-4 expressing cells such as endothelial cells. If Munc13-4 was necessary for successful repair of double-stranded DNA breaks, one would anticipate that accumulating dsDNA breaks would rapidly lead to severe disease even after HSCT. Therefore, unless further evidence arises, it is doubtful that Munc13-4 plays a role in homologous recombination repair.

1.3.6 The FHL3 Mouse Model

Given the need to better understand FHL3 disease and the basics of Munc13-4 related biology, the modern researcher can be considered fortunate to live in a time where an FHL3 mouse model exists. The creation of a Munc13-4 deficient mouse model arose out of research pertaining to genes that confer susceptibility to MCMV infection. In their 2006 publication *Analysis of MCMV Resistome by ENU Mutagenesis*, authors Crozat et al described eight mutations generated from random mutagenesis that created MCMV susceptibility in C57BL/6 mice. One of these mutations was published under the name "*Jinx*[17] codominant," and it was observed that mice bred to homozygosity for this mutation suffered from splenic MCMV viral loads four to five times higher than WT C57BL/6 mice

[17] The name "Jinx" is a reference to Halle Berry's character "Giancinta 'Jinx' Johnson" in the 2002 James Bond movie *Die Another Day*. Mouse strains produced from the Scripps University's ENU mutagenesis studies were named for other female characters from James Bond movies, including "Domino," "Solitaire," "Moneypenny" and "Goodnight" 262. Crozat, K., et al., *Analysis of the MCMV resistome by ENU mutagenesis.* Mamm Genome, 2006. **17**(5): p. 398-406.(Personal correspondence with Crozat, 2020).

and had low cytotoxic activity of their NK cells [262]. Shortly after this initial finding, Crozat et al followed up their work with a more detailed analysis of the *Jinx* phenotype, which was further fleshed out to include how these mice had normal pigmentation, behavior, and primary and secondary lymphoid organs prior to infection, but that after infection they suffered from exaggerated induction of IL-12, IFN-γ, and type I interferons. Furthermore, the NKs and CTLs of these mice demonstrated a profound defect in their ability to degranulate, as demonstrated by a lack of CD107a on the surfaces of these stimulated cells [170, 262].

Intrigued, the researchers first mapped the *Jinx* mutation to a 4.60 million base pair region on mouse chromosome 11, and subsequently zeroed in on the *UNC13D* gene as the cause of the *Jinx* phenotype. Analysis of cDNA from *Jinx* splenocytes revealed that the mutations to the *UNC13D* were due to a single C→A transversion within intron 26. This single nucleotide mutation leads to an alternative splice site that is preferred to the exclusion of the normal splice site. The abnormal splicing has two consequences. First, the altered splice site results in the inclusion of 53 intronic nucleotides, which in turn leads to the addition of 20 aberrant amino acids into the translated Munc13-4 protein. Second, translation of the mutated Munc13-4 polypeptide is prematurely terminated due to an in-frame TGA stop codon after amino acid 859. This pre-mature termination causes the exclusion of the second C2 domain and part of the second MHD domain (See Figure 8). The result is a non-functional Munc13-4 protein [170][18].

[18] Note that the original publication from Crozat et al described the critical point mutation as being a change from G → T, and that it was located within the 26th exon. This error arose from analyzing the minus strand

Upon connecting the *Jinx* phenotype with the mutated *UNC13D* gene, authors Crozat et al sought to develop a reproducible mouse model for human FHL3. Their research first centered on using MCMV infection. *Jinx* mice that received a low dose of MCMV (2 x 10^4 PFU) were able to resolve the infection and any phenotypic resemblance to HLH disease within 12 days, thereby making it a less than ideal mouse model for rigorous FHL3 study. When they increased the dose to 10^5 PFU/mouse, the infected mice died within a week. This led the researchers to want to try another type of infection, and because the FHL2 mouse model had recently been established using LCMV infection of perforin knock-out mice [263], LCMV was selected as the infectious trigger for disease [170]. The resulting disease pathology induced by LCMV Armstrong infection of *Jinx* mice included classical hallmarks of HLH: splenomegaly, elevated IFN-γ, severe anemia, thrombocytopenia, neutrophilia, upregulation of activation markers CD69 on CD8 T cells and CD80 on APCs, massive expansion of cytotoxic cells, and hemophagocytosis. The mice were unable to resolve their infections, and by twelve days after LCMV challenge they had viral tiers within their liver over a thousand times greater than similarly challenged WT mice [170].

Most researchers infect their *Jinx* mice with the Armstrong strain of LCMV [170]. This strain is so named after Charles Armstrong, the man who serendipitously isolated this first strain of LCMV from monkey brain tissue while studying epidemic encephalitis in St.

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of DNA instead of the plus strand. A correction to the article was published in 2007, and can be accessed here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2275393/

Louis in 1933 [264]. LCMV is a natural infection in mice which infects this natural reservoir using both horizontal and vertical transmission [265]. In the case of LCMV Armstrong, this infection is acute, and within a week, WT mice are able to clear the virus. This particular strain of virus is neurotropic [266].[19] This has made LCMV Armstrong one of the longest standing and best characterized tools for immunologists[20] and the best characterized infectious trigger in *Jinx* mice sources [169, 170, 267, 268]. Other research groups have also infected *Jinx* mice with LCMV WE strain. The WE strain was originally isolated from a man with meningitis [265, 269]. This strain induces particularly acute disease in infected mice, in part due to this strain's tropism for viscerotropism—particularly for the kidneys and lungs [265]. To date, more than 30 strains of LCMV have been isolated from mice, hamsters, and humans [265]. Further discussions about the use of different LCMV strains will follow in Chapters 2 and 3 of this dissertation.

Since its initial characterization, the *Jinx* mouse model has further helped to elucidate the role of Munc13-4 in many different cell types—particularly those of the

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[19] "Neurotropic" can be a bit of a misnomer given that most strains of LCMV can infect the CNS tissue. However, other strains of LCMV also infect the viscera, inducing T cell exhaustion and therefore not leading to observable meningitis and encephalitis that can occur as a result of "neurotropic" LCMV strain infection 266. Welsh, R.M. and M.O. Seedhom, *Lymphocytic choriomeningitis virus (LCMV): propagation, quantitation, and storage.* Curr Protoc Microbiol, 2008. **Chapter 15**: p. Unit 15A 1..

[20] LCMV Armstrong infected mice are often contrasted with mice which have been infected with LCMV Clone 13—another strain of LCMV which induces chronic infection. The striking differences between these two strains is astonishing given that Clone 13 was derived from LCMV Armstrong and that the two strains differ by only five out of 10,600 nucleotides. Where Armstrong has a K at position 1079, Clone 13 has a Q, and at position 260 Armstrong has an F while Clone 13 has an L. These two amino acid switches lead to Clone 13 being less immunogenic than Armstrong. Consequently, Clone 13 can persist indefinitely in a mouse, while Armstrong is quickly eliminated by a CTL response. Because the goal in creating an FHL3 mouse model is to create an acute inflammatory reaction that mimics sudden FHL3 disease onset, Clone 13 has not been used in creating an FHL3 mouse model.

myeloid lineage [214, 230] [270]. The lab that originally developed the *Jinx* mouse model has continued to study these mice within the context of NK cell biology—particularly with regards to NK cell maturation [271]. Other NK researchers have used *Jinx* mice to parse out critical events in the cytotoxic pathways [272], and activation of NK cells [273]. With regards to HLH, LCMV infected *Jinx* mice have been used to elucidate the critical role that MyD88 plays in generating the FHL3 phenotype [267], and the non-critical role that Munc13-4 plays in prompting hepatic neutrophil recruitment [217]. Given the variety of cells that express Munc13-4 protein and its function in a wide array of cellular activities, it is reasonable to expect that the *Jinx* mouse model will continue to be a useful tool in multiple fields of research.

Although the vast majority of studies on the *UNC13D* gene and Munc13-4 have been conducted using *Jinx* mice, other Munc13-4 mouse models also exist. Specifically, researchers from the University of Texas MD Anderson Cancer Center developed both global and conditional *UNC13D* KO mice in order to study the role of Munc13-4 deficiency in platelets and mast cells [232, 242]. This mouse model was first described in 2018, and since then only two studies have utilized it. While Munc13-4 deficiency is well characterized in *Jinx* mice, it would be very interesting if repeating them same studies in a true knock-out mouse would yield similar results. Furthermore, the ability to turn the *UNC13D* gene off in targeted cell populations will in the future allow for studies that examine individual cell-type contributions towards FHL3 disease pathology.

Given the current understandings of the genetic basis of FHL3, as well as an ever-deepening understanding the basic biology of the Munc13-4 protein, applying gene therapy

to FHL3 seems like a potential avenue to curing this disease. The following is a discussion of potential applications of gene therapy towards curing FHL3.

1.3.7 Gene therapies with regards to FHL3

To date, three different research groups have published results from studies on gene therapy for FHL3. The first to enter into this field was a group based out of the Paris Descartes University, which in 2016, published two abstracts for the annual conference of the American Society of Gene and Cell Therapy (ASGCT). The first abstract, which six months later was expanded on in a letter to *Blood*, described how the authors created VSV-G and H/F pseudotyped lentiviral vectors which were used to successfully transduce CD8+ T cells. These cells subsequently demonstrated reconstitution of the degranulation phenotype, as assessed by *in vitro* degranulation assays. Transplant of these transduced CD8+ T cells into SCID mice afflicted with B-LCL lymphoma led to tumor regression and persistence of transduced T memory stem cells (TSCM cells) in the peripheral blood one week after transduction [274] [165].

The second 2016 ASGCT abstract published by the Paris Descartes University group was also followed by a paper publication, this time in 2017 in *Blood Advances.* Both of these works described using a *UNC13D-GFP* lentiviral vector[21] to transduce HSCs from *Jinx* mice. Transplantation of these transduced HSCs into recipient *Jinx* mice was reported

[21] Neither the 2016 ASGCT abstract nor the 2017 Blood Advances paper specified which envelope protein was used to pseudotype the lentiviral vector, however a reader might assume that the H/F pseudotype might have been preferred given the superiority in transducing T cells that was demonstrated in their previous work.

to lead to engraftment of gene modified cells and the reconstitution of the immune system with cells derived from the modified HSCs. Impressively, their transplanted mice had an average copy number of about four copies per cell in the splenocytes population, although it is worth noting that increased copy number was not associated with increased production of Munc13-4 mRNA. In addition, the splenic CD8 T cells of the gene therapy modified mice experienced a 10% boost in their ability to degranulate in response to CD3 antibody. Finally, challenging gene therapy modified mice with the WE strain of LCMV demonstrated improved immune response to the virus compared to non-transplanted or sham-transplanted *Jinx* mice as assessed by measurements of serum levels of IFN-γ, body temperature and weight, liver damage, and viral load within the liver [166, 275].

The second research group to publish on gene therapy for FHL3 was a team from the University of Freiburg, which in 2018 published in *Human Gene Therapy* on their work to develop a retroviral based T-cell gene therapy for FHL3. The authors demonstrated that their gamma-retroviral UNC13D-ΔCD271 construct could successfully transduce T cells and that this transduced cell population could be enriched via selection for surface-expressed ΔCD271. Furthermore, these transduced cells exhibited an enhanced cytotoxic ability and could increase CD107a and IFN-γ expression levels upon stimulation.[22] Interestingly, the gene modified cells also expressed dramatically higher levels of granzyme B upon stimulation compared to un-transduced or sham-transduced cells, indicating that perhaps something about the expression of particular transgene affects the cell signaling pathways connected to granzyme B expression.

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[22] This group used PHA, not CD3 antibody, to stimulate their cells

The third and final research group to publish on FHL3 gene therapy is our own group from Emory University. In 2020 we published our work which described transduction and improvements to cytotoxic cell functionality and FHL3 related disease outcomes in human T cells and murine HSCPs in *Human Gene Therapy* [169]. That work, as well as many additional studies which have influenced the project, are described here.

In summary, three research groups are currently developing gene therapies for FHL3. One group has described the use of a gamma-retroviral vector to correct FHL3 patient T cells, and the other two (our group included) have used lentiviral vectors to correct FHL3 patient T cells as well as the hematopoietic stem cells in FHL3 gene therapy mice. Our hope is that this thesis will better inform future lentiviral gene therapy projects, as well as provide the most comprehensive body of literature within the field of FHL3 disease research.

Chapter 2

Construct, Protocol, and Mechanistic Validation for FHL3 Gene Therapy

Some of this work was presented as part of the 2020 American Society of Cell and Gene Therapy 2020 Conference

Sarah Takushi, Reginald Tran, Andrew Fedanov, Christopher B. Doering, Shanmuganathan Chandrakasan, and H. Trent Spencer

Optimizing Munc13-4 Expression from Lentiviral Vector Transduction

Andrew Fedanov produced and characterized the lentiviral vectors. Reginald Tran provided and tested the microfluidics devices. Shanmuganathan Chandrakasan assisted in the observation of LCMV infected mice. Sarah Takushi performed the rest of the experiments depicted here. Sarah Takushi, Chris Doering, Shanmuganathan Chandrakasan, and H. Trent Spencer, conceived the experiments and made editing contributions.

2.1 ABSTRACT

Familial hemophagocytic lymphohistiocytosis type III (FHL3) is a primary immunodeficiency caused by a mutation to the *UNC13D* gene and a subsequent lack of Munc13-4 protein. This disorder primarily affects cytotoxic lymphocytes which are rendered incapable of releasing their cytotoxic granules in response to an immunological challenge. There are virtually no published studies pertaining to gene therapy for FHL3, therefore requiring us to take a ground-up approach to developing one. This required a series of preliminary studies to determine the feasibility of moving the project forward. The feasibility studies described in this chapter provide the foundation on which to aggressively test a preclinical model of HLH. This preliminary work involves designing our lentiviral vectors, validating them *in vitro*¸ optimizing protocols for LV production and purification, isolation of target cells, transduction, and assays for testing gene correction. We also performed definitive mechanistic studies which vindicated Munc13-4 of involvement in dynamin dependent endocytosis and inflammatory granule release in neutrophils. The following chapter describes all of this work and discusses its applications towards developing an FHL3 gene therapy as well as broader applications to LV-based gene therapy research.

2.2 INTRODUCTION

As previously discussed, FHL3 is a primary immuno-deficiency disease that could theoretically benefit from gene therapy intervention. In 2017 our research group began investigating the feasibility of developing such a treatment. At the time, no research

studies on gene therapy for FHL3 were published, and so our approach needed to begin from the ground up by addressing a number of different issues that are relevant to the research and development of all lentiviral vector based gene therapies.

Different gene therapy protocols vary considerably in how they calculate the number of transduced cells used for treatment (Table 3), but without exception, producing this quantity of gene-modified cells remains one of the major hurdles for LV based gene therapy research.[23] Manufacturing and quality control of sufficiently large quantities of LVs in a facility certified as having current good manufacturing practice (cGMP) have been identified as some of the main drivers of cost for LV based gene therapies [278, 279]. Therefore it is essential to characterize the minimal requirements for therapeutic benefit in future gene therapy products. Furthermore, any methodologies that could bolster LV transduction efficiency or could reduce the amount of virus needed for transduction would greatly benefit the field of LV gene therapy.

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[23] For example, a clinical trial seeking to correct X-linked chronic granulomatous disease (CGD) specifies that each patient be infused with at least 3 x 10^6 CD34+ cells per kg of body weight transduced *ex vivo* with 1 x 10^8 IG/mL of lentiviral vector to achieve > 0.3 integrated copies per cell (NCT02234934). Even the youngest patients eligible for this trial (23 months of age) would have an average body weight of 19 kg for boys 276. CDC, *Birth to 24 months: Boys Head circumference-for-age and Weight-for-length percentiles.* 2009. and 11.5 kg for girls 277. CDC, *Birth to 24 months: Girls Length-for-age and Weight-for-age percentiles.* 2009., meaning that these patients would respectively require a minimum of 1.71 *10^7 and 1.035 *10^7 transduced CD34+ cells.

Table 3 Different clinical trials use different methods for calculating the quantity of lentiviral vector modified cells which will constitute a single dose or treatment

ClinicalTrials.gov Identifier	**Disease Target**	**Recruitment status as of March 2020**	**Method for calculating number of transduced cells needed**	**Link**
NCT03351829	Beta Thalassemia	Not yet recruiting	One infusion for $5x10^6$~$1x10^7$ gene-modified cells; or more infusions depending on the circumstances	https://clinicaltrials.gov/ct2/show/NCT03351829
NCT03601286	X-linked Severe Combined Immunodeficiency	Recruiting	A minimum of 2.5 x 10^6/kg CD34+ cells after transduction with a minimum transduction efficiency of 0.7 copies/cell is required for infusion into the patient.	https://clinicaltrials.gov/ct2/show/NCT03601286
NCT02234934	X-linked Chronic Granulomatous Disease	Active, not recruiting	At least 3 x 10^6 cells per kg of body weight transduced *ex vivo* with 1 x 10^8 IG/mL of lentiviral vector to achieve > 0.3 integrated copies per cell.	https://clinicaltrials.gov/ct2/show/NCT02234934
NCT03645486	CGD	Recruiting	Infusion of lentiviral TYF-modified autologous stem cells at 1~$10x10^6$ gene-modified cells per kg body weight	https://clinicaltrials.gov/ct2/show/NCT03645486
NCT03645460	ADA-SCID	Recruiting	Infusion of TYF-ADA-modified autologous stem cells at 1~$10x10^6$ gene-modified cells per kg body weight; or more infusions depending on the circumstances	https://clinicaltrials.gov/ct2/show/NCT03645460
NCT03157804	Fanconi Anemia Patients Subtype A (FANCOLEN-1)	Active, not recruiting	Patients will be infused with between $3x10^5$ and $4x10^6$ CD34 + cells / kg of patient body weight.	https://clinicaltrials.gov/ct2/show/NCT03157804

The first phase in the process of *ex vivo* LV-based gene therapy is the production of LVs. The most standard method for producing third-generation, self-inactivating LVs is

to transfect HEK 293T cells with four separate plasmids: one for Rev, one for Gag/Pol, one for the envelope protein, and one that encodes for the transgene. Eighteen hours after transfection, the transfection media is replaced with fresh media, into which LVs can bud from the HEK 293T cells over the course of the next one to two days [280]. LVs are then purified from the supernatant using ultracentrifugation [280], sucrose cushion/gradient purification [280], chromatography, and/or commercially available purification columns [281, 282]. Modifications to the producer HEK 293T cell line such as transforming them to become suspension cells instead of adherent [283, 284], knocking down the expression of receptors for envelope proteins on producer cells [285], and transfection using flow electroporation [286] have also been shown to yield higher titers of virus.

The second step in the process of *ex vivo* gene therapy modification is the isolation of target cells. For hematopoietic stem cell based gene therapy, the richest source for HSCs comes from the bone marrow, where there is an estimated one stem cell per 10^4 bone marrow cells [287]. However, peripheral blood is often used as an alternative source for HSCs because of how stem cells can be mobilized from all bones, instead of just being harvest from the femur. While the numbers of HSCs in the peripheral blood are comparatively low, these numbers can be bolstered by treating a patient with granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and/or stem cell factor in order to mobilize bone-marrow derived progenitor cells into the peripheral blood [287]. HSCs have also been obtained from human umbilical cord blood [141] and human fetal livers [288-290], though limited availability makes these tissues less promising HSC sources for gene therapy.

A third step which can influence the final infusion-ready LV-based gene therapy product is the transduction protocol. Different protocols have optimized the use of different LV MOIs [291], incubation times of the target cells in the transduction media [291, 292], and the type of transduction media used [293, 294]. The major challenge seems to be in maximizing the contact of LVs with target cells. To enhance this process further, scientists have experimented with using vessels that hold only extremely small volumes (i.e. microfluidics devices) [295, 296]. Researchers have also compared different envelope proteins and their ability to bind to and facilitate fusion of LVs with target cell membranes [165, 297, 298]. Finally, a number of different transduction enhancers including polybrene [299], protamine sulfate (PS) [300], retronectin [301], amphiphilic poloxamer synperonic F108 (aka Lentiboost) [302], Cyclosporine A [303], and Cyclosporine H [304] have all been suggested as extra additives which can supplement transduction media for the purposes of increasing transduction efficiency (see Table 4).

In this chapter, we address each of these steps in order to create an optimized protocol for the production and administration of an FHL3 gene therapy. We begin by comparing several different LVs and examining their ability to transduce HSPCs that were isolated using different protocols. We next describe modification of the construct for the purpose of transducing primary human T cells, and describe protocols for the generation of those LVs and the enrichment of the transduced cell population. We then investigate some of the mechanistic functions of Munc13-4 with regards to how it plays a role in the endocytic events required for transduction with VSV-G pseudotyped LVs. Finally, we optimize reporter assays that can be used to determine the extent to which gene therapy modification corrects disease in the FHL3 mouse model.

Table 4 Transduction enhancers and their mechanism of action

Transduction enhancer	Mechanism of action
Polybrene (Hexadimethrine bromide)	Polybrene neutralizes charge repulsion between virions/LVs and sialic acid residues on target cells [305, 306]. Polybrene is widely used in LV transduction protocols, but there is some evidence that this transduction enhancer can disrupt the transmembrane potential of sensitive cell types [307].
Protamine disulfate	Like polybrene, protamine sulfate is a polycation which neutralizes charge repulsion forces between virions/LVs and target cells. Comparisons between polybrene and protamine sulfate have indicated that it is more effective as a transduction enhancer than polybrene [300].
Fibronectin	Fibronectin is an extracellular matrix molecule. Its carboxy-terminal 30/35-kD fragment has multiple binding domains and has been shown to enhance transduction by binding viral particles/LVs and target cells, bringing them in close enough proximity for transduction to occur [308, 309].
Retronectin	Retronectin, a recombinant form of human fibronectin, acts by binding LVs with the heparin-binding domain, and integrins VLA-4 and/or VLA-5 on target cells with its C-domain and CS-1 sites [310]. Consequently, LV particles and transduction target cells are brought into close proximity with each other, facilitating higher transduction efficiency [301].
LentiBOOST (poloxamer synperonic F108)	Poloxamers interact with lipid membranes and decrease microviscosity, thereby enhancing lipid exchange [311]. This enhancement of lipid exchange helps viral/LV membranes fuse with target cell membranes [299, 302].
Vectofusion-1 (LAH4-L1)	This short, cationic, amphipathic peptide sequence enhances the adhesion and fusion steps of viral/LV entry to a target cell [312-314].
PGE2 (Prostaglandin E2, dinoprostone)	PGE2 is an inflammatory mediator [315] that has been shown to increase transduction efficiency of HSPCs [316, 317]. To date, the specifics about this mechanism of action are unknown [316, 317].
Rapa (rapamycin)	Rapamycin increases LV transduction of human and murine HSPCs by enhancing cytoplasmic entry [317, 318]. Further specifics of this mechanism have yet to be elucidated.
Stauro (staurosporine)	Staurosporine is a small molecule serine/threonine kinase inhibitor which has been shown to increase transduction efficiency of HSCPs with LVs [319]. Stauro is hypothesized to act by enhancing phosphorylation of the protein cofilin [319], a protein which when activated helps mediate the actin dynamics that are essential for lentiviral entry and nuclear migration within a target cell [320].
Cyclosporine A	Some cells such as murine pluripotent stem cells express cyclophilin A (CypA) [303], which has been shown to bind to the capsid protein of HIV [321, 322] and inhibit un-coating, initiation of reverse transcription [323, 324], nuclear import, and integration of the viral genome into the host's DNA [325, 326]. Cyclosporine A binds to CypA [303] and inhibits cyclophilin A restriction [303, 327-331].
Cyclosporine H	In response to interferon-γ signaling, some cell types such as human HSPCs upregulate the viral restriction factor IFITM3. IFITM3 normally restricts the fusion of viral/LV and endosome membranes [332]. One study found that Cyclosporine H can degrade IFITM3 [304], resulting in enhancement of transduction [304, 333].

2.3 MATERIALS AND METHODS

Cloning

All cloning was done into an expression vector which has been FDA approved for a hemophilia A gene therapy products and which was supplied to us by Expression Therapeutics. In humans, *UNC13D* is comprised of 32 of exons [161], therefore in constructing our *UNC13D* lentiviral vector we used the sequence from the *Homo sapiens* unc-13homolog D (*UNC13D*) mRNA (NCBI reference Sequence: NM_199242.2). When translated, this nucleotide sequence yields isoform 1 of the Munc13-4 protein (protein Sequence NP_954712.1, UniProt identifier Q70J99-1). The sequence for the tCD271 and streptavidin-binding protein came from Matheson et al [334]. Codon optimization was performed by GenScript according to the algorithm previously described [335]. All clones were screened using restriction digests and sequenced by Genewiz.

Lentiviral Vector Packaging and Titering

Third generation, self-inactivating, VSV-G pseudotyped lentiviral vectors were constructed using a standard HEK 293T transfection protocol [336]. Specifically, HEK 293T/17 cells were transfected with a four plasmid system consisting of the cloned, transgene-expressing plasmid and packaging plasmids provided by Dr. Denning at Expression Therapeutics for VSVG, REV, and GAG-POL proteins. The molar ratios of these plasmids was 3:1:1:1, unless otherwise specified. The following day the media was replaced with DMEM containing 4.5 g/L glucose, L-glutamine & sodium pyruvate, supplemented with 10% FBS and 1% penicillin/streptomycin. Supernatant from these

transfected cells were collected on days two and three post-transfection. Viral vector was pelleted out of the supernatant by overnight ultracentrifugation at 10,000 g, 4°C. Where indicated, this was done with the supernatant layered over a 10% sucrose cushion. Pelleted viral vector was subsequently filtered through a 0.22 micron filter and re-suspended in either StemPro or TC media.

All lentiviral vector preparations were titered by applying polybrene (aka "hexadimethrene bromide," 8 μg/mL, Sigma) and 3, 9, or 27 microliters of the new lentiviral vector onto HEK 293T/17 cells, incubating overnight, and then culturing in fresh media for five days. DNA was subsequently isolated from these cells using a Qiagen DNA Micro kit (Qiagen; 56304). Quantitative PCR was performed using Power SYBR Green Master Mix (ThermoFisher; 4367659) with RRE primers (Forward primer: TGG AGT GGG ACA GAG AAA TTA ACA, Reverse primer: GCT GGT TTT GCG ATT CTT CAA) to determine the average number of copies of viral vector DNA that were integrated per cell.

Some experiments used a GFP LV that was similarly prepared and titered by Lentigen.

Viral Copy Number (VCN) Analysis

The same RRE primers described above, SYBR Green Power PCR Master Mix, and PCR protocol that were used for the titering of new lentiviral vectors were also used in the qPCR-based copy number analysis of transduced cells.

Transduction

Cell line transductions: Cell lines used in *in vitro* experiments were transduced once with a single overnight application of lentiviral vector using the specified MOIs. Where specified, transduction media was supplemented with polybrene (8 μg/mL, Sigma).

Murine HSPC transductions: Isolated Sca-1 cells were cultured for thirty-six hours in stimulation media consisting of StemPro nutrient-supplemented media (Gibco; 10640-019), recombinant mouse IL-3 (20 ng/mL; R & D; 403-ML), recombinant human IL-11 (100 ng/mL; R & D; 218-IL), recombinant human Flt3/Fc (100 ng/mL; R & D 368-ST), mouse mSCF (100 ng/mL; R&D 455 MC), L-glutamine (HyClone; SH30034.01), and penicillin and streptomycin (Lonza; 09-757F). These cells were subsequently transduced overnight with the specified lentiviral gene therapy vector in polybrene containing media (8 μg/mL, Sigma).

Conventional T-cell transduction: Primary human T cells were thawed, washed in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin, and stimulated overnight using CD3/CD28 beads (Thermo Fisher; 111.31D) in T-cell media (X-VIVO 15 media (Lonza; 04-418Q) supplemented with 10% FBS, 1% penicillin/streptomycin, IL-2 (50 ng/mL, PeproTech; 200-02) and IL-7 (5 ng/mL, PeproTech; 200-07)). The following day the beads were magnetically removed, the cells were washed, and then 200,000 cells were plated in 100 microliters of T-cell media in a 96-well plate. The cells were transduced for 12 hours, after which they were washed yet again and cultured at 37 degrees in 5% CO_2 for the specified duration of time.

Microfluidics based transductions: Microfluidics devices were generously provided by the Lam laboratory in Georgia Institute of Technology and were used according to the previously described protocol [296]. Cells were re-suspended at a

concentration of 20 million cells/mL in 50% T-cell media (see above section on T-cell transduction) and 50% LV prep (which was also re-suspended in T-cell media). Re-suspended T cells were subsequently loaded into each device, and incubated for eight hours. Cells were then flushed from the devices, washed, and incubated in T-cell media at 37 degrees in 5% CO2 for the specified duration of time.

Western Blot

A standard Western blot protocol [337] was performed using cell lysates prepared using Cell Lysis Buffer (Cell Signaling Technology; 9803). Blotting was performed on a PVDF membrane (BioRad; 162-0261) and staining was done for Munc13-4 (Abcam; Clone ERP4914, ab109113), and Beta-actin (GenScript; Clone 2D1D10, A00702). Secondary antibodies (IRDye 800 CW and IRDye 680, Licor; respectively catalogue numbers 926-32213 and 026-68072) were used to clearly distinguish between the Munc13-4 and beta-actin bands. The blots were imaged using a Li-Cor Odyssey CLx imaging device, and subsequent image analysis was don using either Image Studio Version 4.0 or ImageJ software.

CFU Assays

One mL of Methocult Culture media (StemCell Technologies; 03434) containing one thousand cells/mL was plated and incubated for 7-10 days. Colonies were subsequently counted, assessed using a viability stain, and the number of successfully transduced CFUs was determined either by copy number assay or flow cytometry on the pooled cells.

Magnetic sorting

T cells were suspended in cold separation buffer (PBS supplemented 0.5% BSA and 2 mM EDTA) and 1 μL of CD271 antibody (Biolegend; 345122) per million cells for 15 minutes on ice. Cells were then centrifuged, re-suspended in separation buffer and streptavidin microbeads (Miltenyi Biotec; 130-048-101) and incubated for another 15 minutes on ice. Additional separation buffer was added and the cells were washed through an MS column (Miltenyi Biotec; 130-042-201), collecting the magnetically labeled fraction in a tube as the final CD271+ cell population.

T-Cell Degranulation Assay

For splenic tissue sources, homogenized splenic tissue was passed through a 70 micron filter and washed with PBS containing 5% FBS. For assays on peripheral blood T cells, 150 μL of blood was collected via fascial bleed, centrifuged to remove the plasma, and treated with RBC lysis buffer (Thermo Fisher; 00-4333). All cells were plated at 300,000 cells/FACS tube in RPMI containing 10% FBS and 1% penicillin/streptomycin and supplemented with mouse IL-2 (10 ng/mL, Biolegend; 575402). Cells were then incubated for 30 minutes in either the presence or absence of 3 μg/mL of CD3 (BioLegend; Clone 145-2C11, 100301) and 1 μg/mL of CD28 antibody (BioLegend; Clone 37.51, 102101) to produce "stimulated" and "unstimulated" sample groups respectively. Where specified, cells were instead stimulated with PHA-L ("Leucoagglutinin", Sigma; L2769) or PMA/ionomycin (Fisher; 00-4970-93). Thirty minutes after initial stimulation, CD107a antibody (BV421, BD; 121617) and monensin (Biolegend; 420701) were added to all of the cultures, and the cells were incubated for the specified number of hours. Staining for

analysis consisted of a live/dead cell stain (ThermoFisher; 65-0865-14), CD8 (PE, BioLegend; 108739), CD44 (UV379, BD; 740215), CD69 (FITC, BD; 557392).

Neutrophil degranulation assay

Isolation: Bone marrow was flushed from the tibias and femurs of WT and *Jinx* mice and isolated using a mouse neutrophil isolation kit (Miltenyi Biotec; 130-097-658).

Transmigration: Transmigration was performed by members of the Tirouvanziam lab based on their previously published protocol [338]. Briefly, three weeks prior to neutrophil isolation, H441 human Club cells ([338, 339]) were cultured in an air/liquid interphase created using an Alvetex (Reinnervate) 200 μm-thick inert 3D scaffold with >90% porosity. On the day of transmigration, these H441 cultures were placed with the apical compartment exposed to RPMI supplemented with leukotriene B4 (LTB4, 100 nM). One million neutrophils were loaded onto the 200 μm-thick basal compartment of the Alvetex scaffold and allowed to migrate at 37°C at 5% CO_2 through the collagen and epithelial layers into the chemo-attractant containing apical compartment.

Stimulation: neutrophils were re-suspended in neutrophil stimulation buffer (HBSS supplemented with Cytochalasin B (Abcam; 4930-96-2), 10% BSA, and (where specified) 160 μM CaCl2 and placed on ice for 15 minutes. They were then stimulated for ten minutes (unless otherwise specified) at room temperature with either fMLF (10-5 μM, Sigma Aldrich; Cat # F3506-5MG), PMA/ionomycin (Fisher; 00-4970-93), or E.coli lysates (prepared by boiling overnight cultures of live E. coli bacteria). Stimulation was stopped by putting the cells on ice.

Mice

Jinx (Unc13d^{Jinx}, unc-13 homolog D) mice were obtained from Jackson Laboratories (MGI ID 3628822) and heterozygotes were bred to produce either homozygotic *Jinx* mice or homozygotic WT (C57BL/6J) mice. Genotyping was performed using a custom-made Taqman probe based PCR assay to identify the point mutation responsible for creating the *Jinx* mouse.

LCMV Infection

Jinx mice were infected intraperitoneally with 2 x 10^5 PFU of LCMV Armstrong, which was generously provided to us by the Ahmed lab.

Primary Cell and Cell Lines

Healthy human donor T cells were isolated by negative selection from donor PBMC sourced from leukoreduction filters, as previously described [340, 341].

The following cell lines were used in this study: NIH 3T3 cells (ATCC number CRL-1658), Jurkat clone E6-1 cells (ATCC number TIB-152), and H441 human Club cells ([338, 339].

Mouse HSPCs

Bone marrow was flushed from the tibias and femurs or WT or *Jinx* mice, washed with PBS, and subjected to the specified protocols for either the MACS Sca-1 isolation kit

(Miltenyi Biotec; 130-106-641), the StemCell Sca-1 isolation kit (StemCell Technologies; 18756) or the StemCell Lineage depletion kit (StemCell Technologies; 19856).

Cell Culture

The following reagents were used for cell culture: DMEM (Corning; 10-017-CV), RPMI (Corning; 10-040-CV), StemPro (Gibco; 10640-019), FBS (Atlanta Biologicals; S11150H), penicillin/streptomycin (Lonza; 09-757F), X-VIVO 15 media (Lonza; 04-418Q), human IL-2 (PeproTech; 200-02), human IL-7 (PeproTech; 200-07), mouse IL-2 (Biolegend; 575402), recombinant mouse IL-3 (R & D; 403-ML), recombinant human IL-11 (R & D; 218-IL), recombinant human Flt3/Fc (R & D 368-ST), mouse mSCF (R&D 455 MC), L-glutamine (HyClone; SH30034.01), Bexin-1 (Glixx Labs), cyclosporine H, and recombinant human IFN-gamma (Peprotech; 300-02).

Flow Cytometry

The following reagents were used throughout the various flow cytometry based experiments: eFluor 780 viability staining (ThermoFisher; 65-0865-14), PE mCD8 (BioLegend; 108739), UV379 mCD44 (BD; 740215), FITC mCD69 antibody (BD; 557392), BV421 mCD107a (BD; 121617), APC mLy6G (Biolegend; 127613), UV395 mCD11b (BD; 563553), PE mCD63 (Biolegend; 143903), BV421 mCD66a (Biolegend; 134531), PE hCD3 (BD; 552127), CD271 (Biolegend; 345122) and streptavidin-BV421 (Biolegend; 405226). APC-LCMV GP33 and GP276 tetramers were generously supplied to us from the Ahmed lab.

Statistical Analysis

Statistical analysis was done using both paired and un-paired T tests, Kruskal-Wallis H test, and one-way ANOVA tests. All statistical tests were performed using GraphPad Prism Version 8.

2.4 RESULTS

2.4.01 Optimization of the lentiviral construct

We began our gene therapy studies for the correction of Munc13-4 deficiency by designing several alternative lentiviral vectors (Figure 2A). We began by cloning the cDNA for the full *Homo sapien* Munc13-4 protein into a standard lentiviral backbone. Subsequently, we added the sequence for green fluorescent protein to act as a marker for transduced cells. Next, we replaced the Ef1α promoter with the synthetic MND promoter[24]. After that, we included a Kozak sequence in some of our constructs to enhance translation of transgene mRNA [343]. Finally, we created a codon optimized version of the canonical *UNC13D* transgene using the optimization strategy we previously developed in our lab [344]. All cloned plasmids were checked for their accuracy using restriction digests and DNA sequencing.

We were reliably able to produce these self-inactivating lentiviral vectors at similar titers using our previously described HEK 293T transfection protocol [345] (Figure 2B). We demonstrated that these vectors could be used to transduce NIH 3T3 cells (a mouse embryonic fibroblast cell line that does not express Munc13-4) and robustly express Munc13-4 protein. We achieved twice as much protein expression from cells that were transduced with the Ef1α_Kozak_Codon-Optimized-*UNC13D* ("EKCOU") construct

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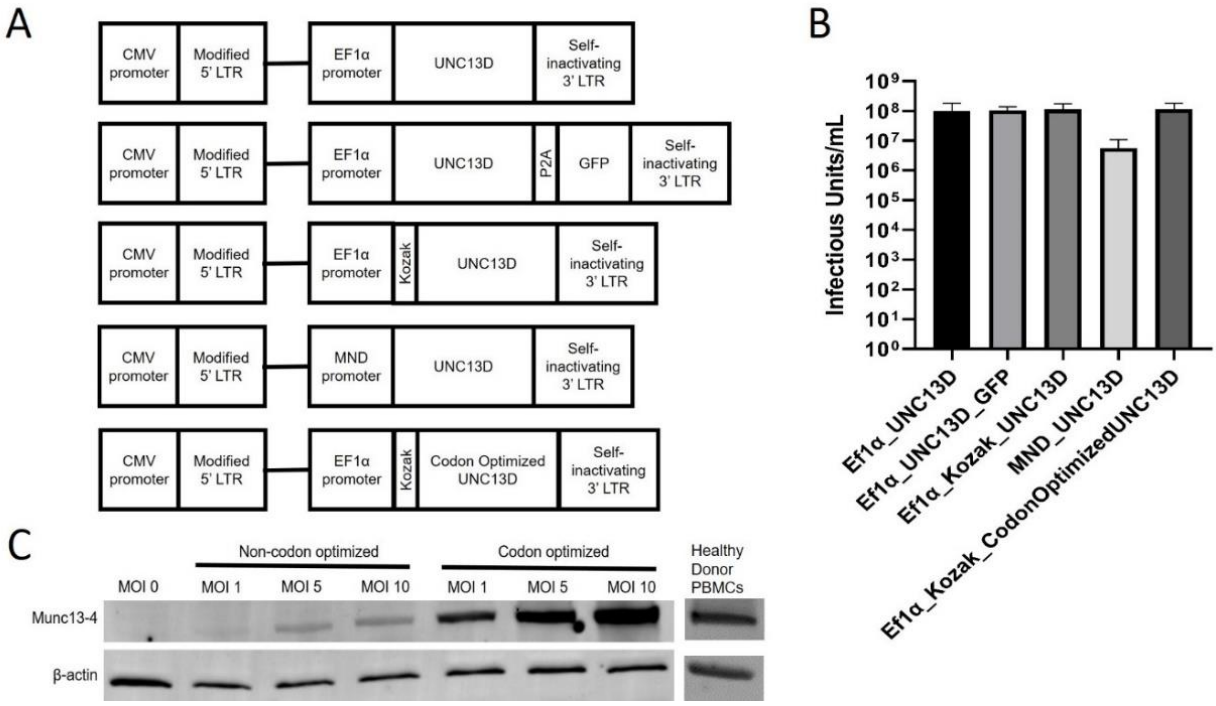
[24] The MND promoter is a synthetic promoter which was adapted from Moloney murine leukemia virus and myeloproliferative sarcoma virus elements 342. Challita, P.M., et al., *Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells.* J Virol, 1995. **69**(2): p. 748-55..

compared to the non-codon-optimized construct at an MOI of 5, and three times more protein expression at an MOI of 10 (Figure 2C), and therefore we chose to focus the majority of our subsequent transduction experiments on this particular lentiviral construct.

Figure 2

A) Diagram of the cloned lentiviral vector constructs. B) Modification to the original lentiviral construct did not produce any change in viral titer as assessed by transduction of HEK 293T cells and subsequent VCN analysis (Kruskal-Wallis H test, $p = 0.48$) C) Versions of the lentiviral construct that were under the control of the Ef1α promoter and included a Kozak sequence demonstrated that the codon-optimized version of the UNC13D gene is expressed more highly in NIH 3T3 cells than the non-codon-optimized version of the gene. At MOIs of 5 and 10, the level of Munc13-4 expression exceeded physiological expression levels of Munc13-4 in PBMCs from a healthy donor. VCNs for the non-codon optimized and codon optimized versions of the construct were respectively 0.22 and 0.18 at an MOI of 10.



2.4.02 Optimization of HSPC isolation

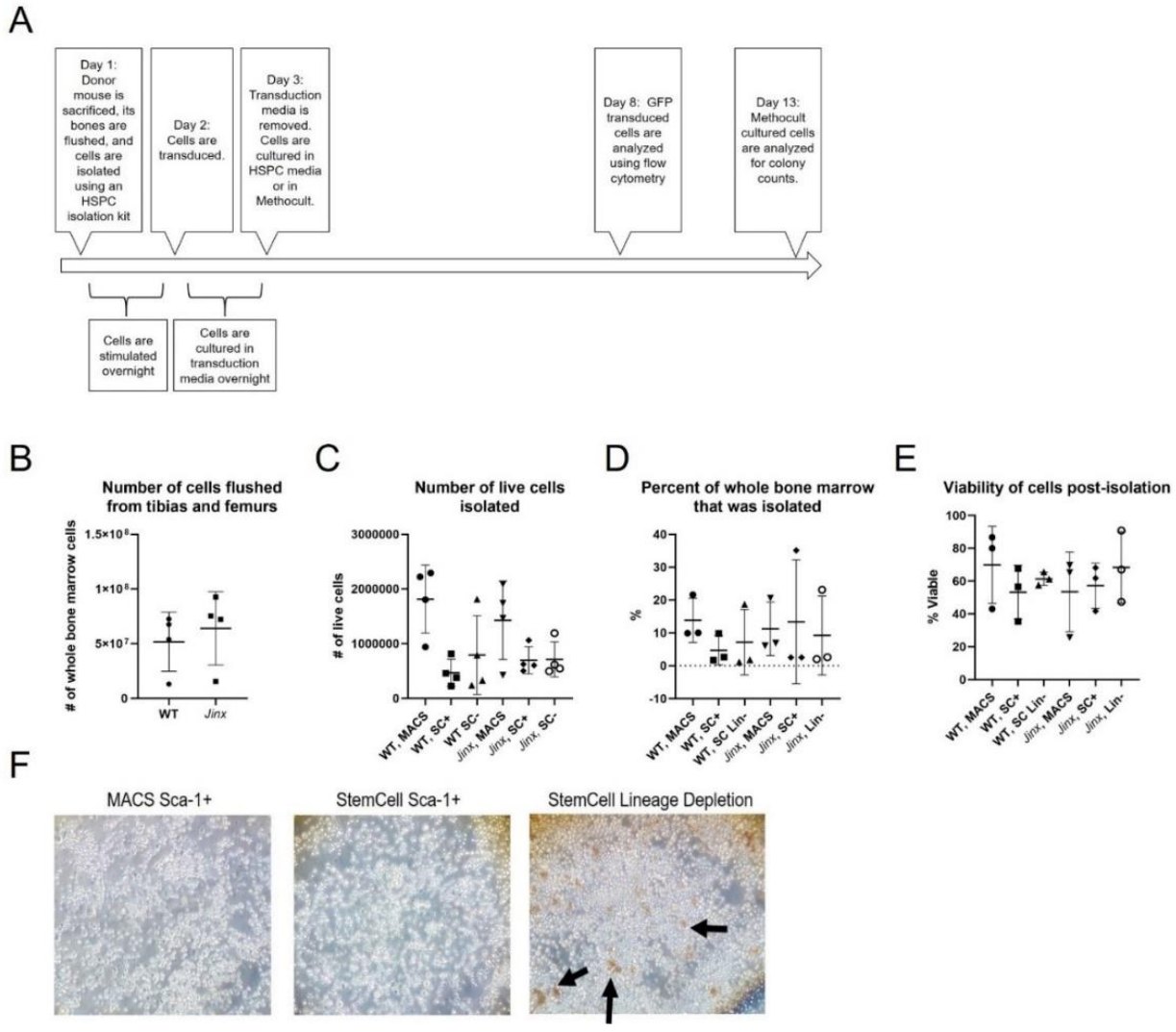
In order to pave the way towards developing an HSPC based gene therapy for Munc13-4 deficiency, we next wanted to optimize our protocols for the isolation of murine hematopoietic stem and progenitor cells. No literature existed on the quality of *Jinx* bone

marrow cells after isolation, so we performed a series of experiments to compare the viability, transduce-ability, and ability to differentiate and expand post transduction. To begin, we compared WT and *Jinx* mouse HSPCs isolated using mouse Sca-1 cell isolation kits from MACS and StemCell Technology, as well as a Lineage Depletion kit from StemCell Technology. After isolation, cells were transduced with either our in-house made EKCOU lentiviral vector, or Lentigen's GFP LV in order to determine if different isolation methods exerted an effect on transduction efficiency. End point analysis was done using either flow cytometry or MethoCult colony counts. Figure 3A diagrams this work flow.

Importantly, WT and *Jinx* mice had no significant difference in the numbers of bone marrow cells flushed from their femurs and tibias (Figure 3B). The number of HSPCs isolated from WT and *Jinx* whole bone marrow using different isolation methods showed significant variation between different treatment groups, with the largest number of cells available coming from WT bone marrow which was subjected to the MACS Sca-1 isolation it (Figure 3C). However, when this large cell number was interpreted as a percent of the whole bone marrow starting material, no significant variation between any of the treatment groups was observed (Figure 3D). Viability of these isolated cells did not differ significantly between treatment groups (Figure 3F).

Figure 3

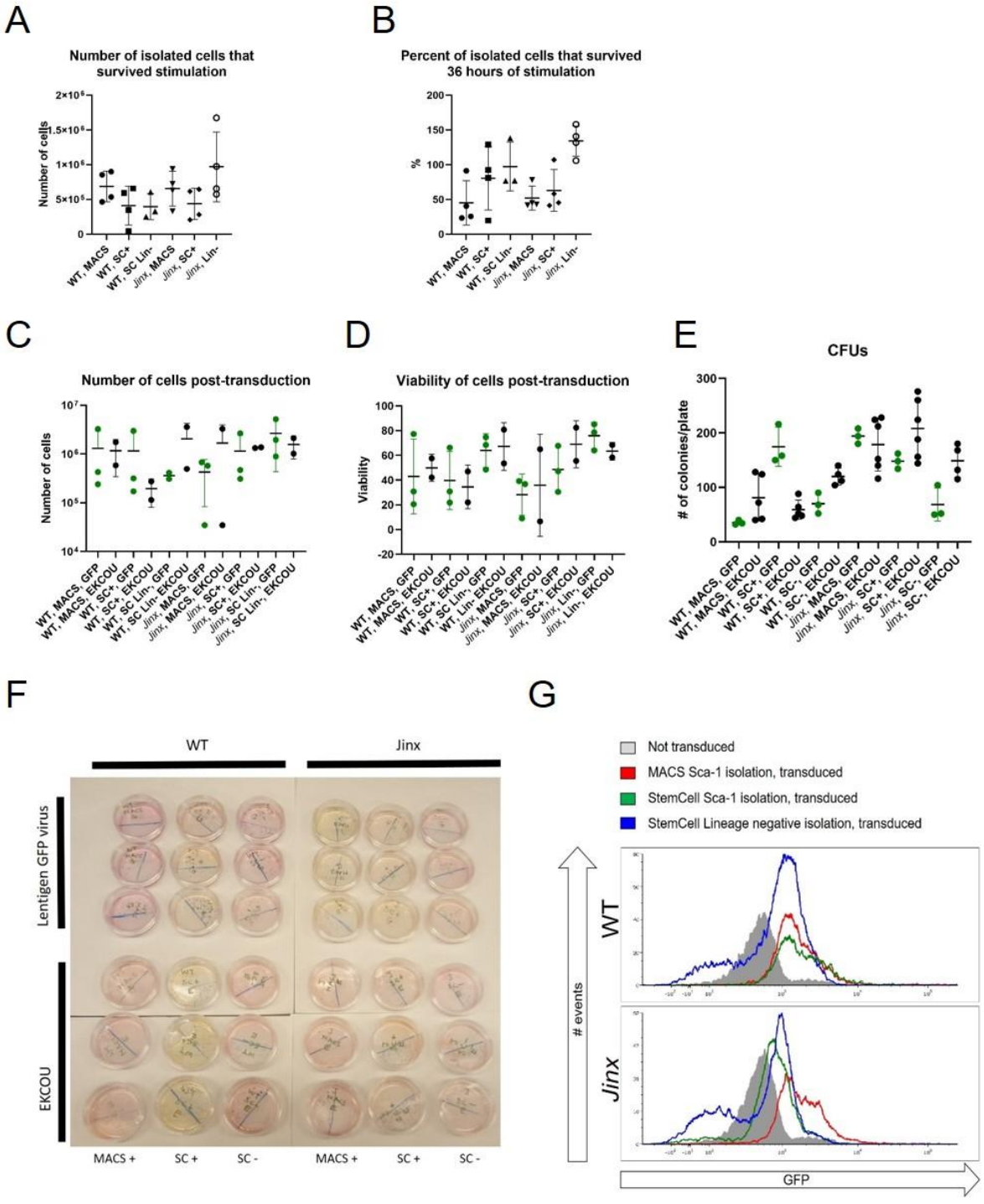
A) Diagram of the workflow for the optimization of isolation, transduction, and endpoint analysis for murine HSCPs. B) WT and *Jinx* mice had no significant difference in the number of whole bone marrow cells flushed from their tibias and femurs (Mann Whitney U test, p = 0.3429). C) The number of HSPCs isolated from WT and *Jinx* whole bone marrow using different isolation kits showed significant variation between different treatment groups (ordinary one-way ANOVA, p = 0.01). D) The percent of the whole bone marrow that remained in the final isolated HSPC product did not show any significant variation between different treatment groups (ordinary one-way ANOVA, p = 0.89) E) The viability of the HSPCs post-isolation did not show any significant variation between treatment groups (ordinary one-way ANOVA, p = 0.80). F) 100X observation of freshly isolated cells shows that the Sca-1 isolation product is consistent between MACS and StemCell isolates, but that the StemCell Lineage negative HSPC isolation appears to leave magnetic beads or magnetically conjugated debris (black arrows) in the final cell product.



Isolated HSPCs were stimulated for thirty-six hours. Interestingly, while the total numbers of cells post-stimulation did not vary from group to group, cells that were isolated using the StemCell lineage depletion kit on average expanded more than cells isolated using other methods (Figure 4A and B). All HSPCs were subsequently transduced overnight with either EKCOU or GFP constructs in polybrene supplemented media. This transduction exerted no significant change on the total cell counts or viability of HSPCs from one treatment group compared to the others. This indicates that our in-house made EKCOU LV construct is no more cytotoxic than the commercial product from Lentigen (Figure 4C and D). After transduction, some cells were reserved for CFU assays, while others were maintained in HSPC media (sans polybrene) for five days before flow analysis to determine GFP expression. CFU assay results showed that for both GFP and EKCOU constructs, *Jinx* HSPCs that were isolated with the MACS Sca-1 kit produced the largest number of colonies and appeared to be the most metabolically active (Figures 4E and 4F). VCN analysis showed no significant differences between the copy numbers for the different groups (data not shown, average = 2.32, standard deviation of 0.34 copies/cell). Furthermore, *Jinx* HSPCs isolated with the MACS Sca-1 kit also and had the greatest GFP expression compared to other isolation methods (Figure 4G).

Figure 4

A) No significant difference was observed in the number of cells that survived 36 hours of stimulation (ordinary one-way ANOVA, $p = 0.11$). B) When compared to the initial number of post-isolation HSPCs, *Jinx* cells which were isolated using StemCell's lineage negative selection kit showed much greater expansion after stimulation (ordinary one-way ANOVA, $p = 0.01$). C) After transduction, no significant difference was observed for the number of cells available (ordinary one-way ANOVA, $p = 0.81$) or D) viability (ordinary one-way ANOVA, $p = 0.22$). E) After transduction, HSPCs were plated for CFU assays and cultured for ten days, after which significant variations in the number of colonies (E, ordinary one-way ANOVA, $p < 0.0001$) and metabolic activity of the plated colonies (F) were observed. G) Flow cytometry analysis showed that GFP-LV transduced HSPCs from both WT and *Jinx* mice expressed GFP, regardless of the method of their isolation.

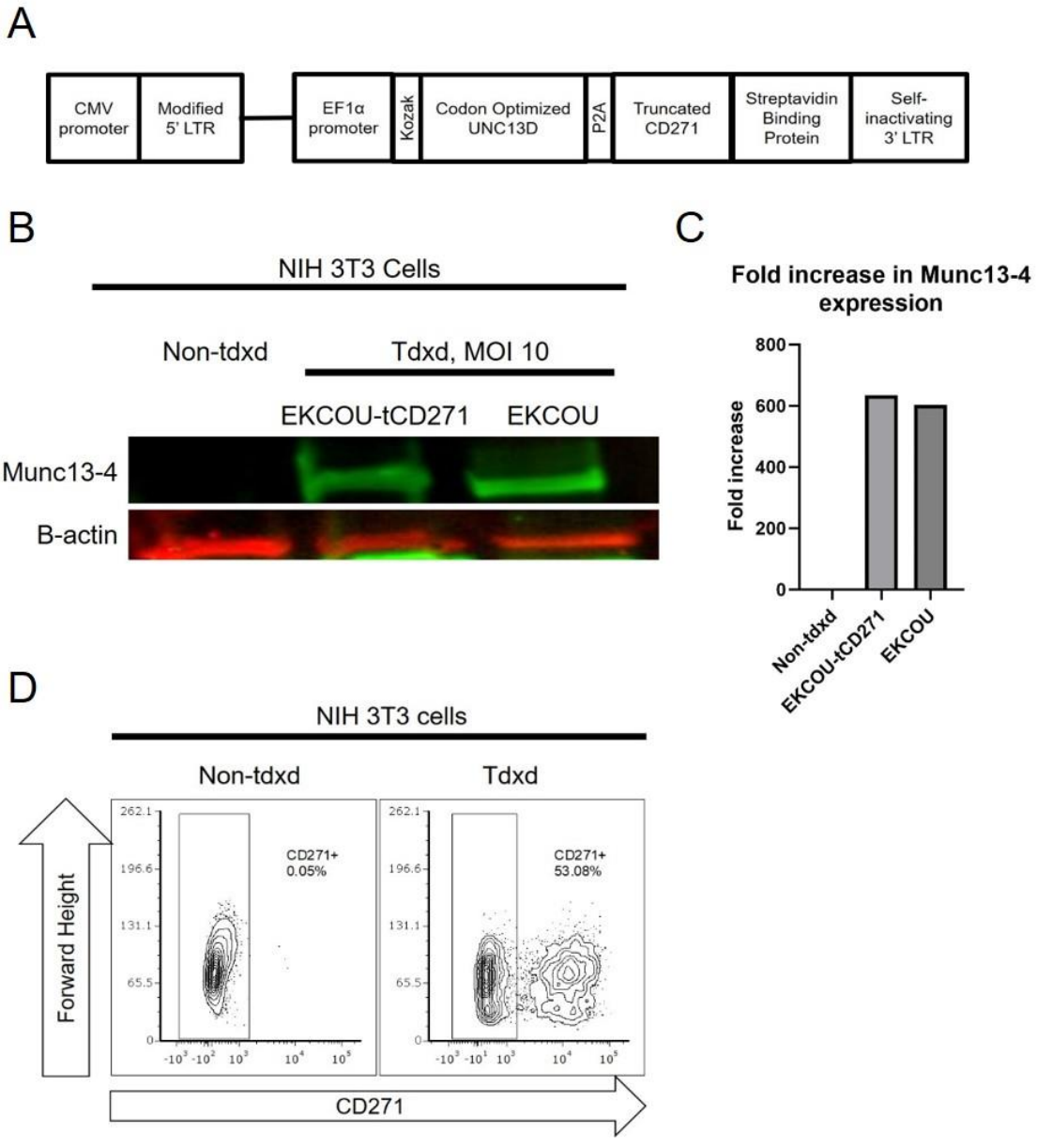

A
Number of isolated cells that survived stimulation
Number of cells
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
WT, MACS
WT, SC+
WT, SC Lin-
Jinx, MACS
Jinx, SC+
Jinx, Lin-
B
Percent of isolated cells that survived 36 hours of stimulation
%
200
150
100
50
0
C
Number of cells post-transduction
Number of cells
10⁷
10⁶
10⁵
10⁴
WT, MACS, GFP
WT, MACS, EKCOU
WT, SC+, GFP
WT, SC+, EKCOU
WT, SC Lin-, GFP
WT, SC Lin-, EKCOU
Jinx, MACS, GFP
Jinx, MACS, EKCOU
Jinx, SC+, GFP
Jinx, SC+, EKCOU
Jinx, SC Lin-, GFP
Jinx, SC Lin-, EKCOU
D
Viability of cells post-transduction
Viability
100
80
60
40
20
0
-20
WT, Lin-, GFP
WT, Lin-, EKCOU
Jinx, MACS, GFP
Jinx, MACS, EKCOU
Jinx, SC+, GFP
Jinx, SC+, EKCOU
Jinx, Lin-, GFP
Jinx, Lin-, EKCOU
E
CFUs
# of colonies/plate
300
200
100
0
WT, SC-, GFP
WT, SC-, EKCOU
Jinx, SC-, GFP
Jinx, SC-, EKCOU
F
WT
Jinx
Lentigen GFP virus
EKCOU
MACS +
SC +
SC -
G
Not transduced
MACS Sca-1 isolation, transduced
StemCell Sca-1 isolation, transduced
StemCell Lineage negative isolation, transduced
events
WT
Jinx
GFP

2.4.03 Design and validation of the codon-optimized-tCD271 lentiviral construct

In order to have an extracellular marker which could be used to identify and positively select for successfully transduced cells using an *in vitro* cell sorting process, , the cDNA for a truncated CD271 ("tCD271," indicating the removal of the cytoplasmic domain of this protein) was cloned into the existing EKCOU construct (Figure 5A). This new construct, herein referred to as the EKCOU-tCD271 construct, was first validated in NIH 3T3 cells, which are negative for Munc13-4 expression. Western blot confirmed that transduced NIH 3T3 cells expressed transgenic Munc13-4 protein (Figures 5B and C), and flow cytometry validated the expression of tCD271 at the plasma membrane (Figures 5D).

Figure 5

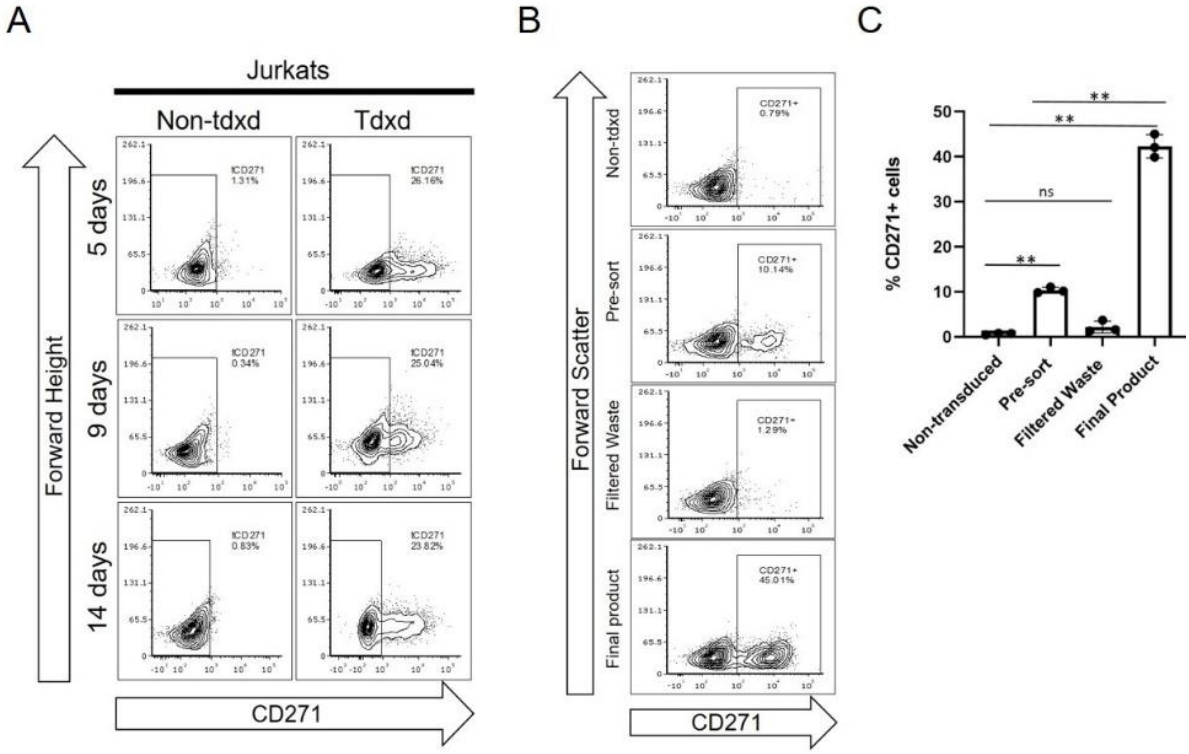
A) Diagram of the EKCOU-tCD271 construct. B) Western depicting Munc13-4 expression in lysates from NIH 3T3 cells that were transduced with an MOI of 10 of either the EKCOU-tCD271 or EKCOU lentiviral vectors. Lysates were collected five days post transduction. C) Fold increase in the amount of Munc13-4 expression from transduced NIH 3T3 cells compared to non-transduced NIH 3T3 cells. Fold increase was calculated as the ratio of Munc13-4 to Beta actin for transduced cells divided by the Munc13-4 to Beta actin ratio for non-transduced cells. D) Surface expression of the extracellular domain of CD271 in NIH 3T3 cells that were transduced with the EKCOU-tCD271 lentiviral vector (MOI 10).

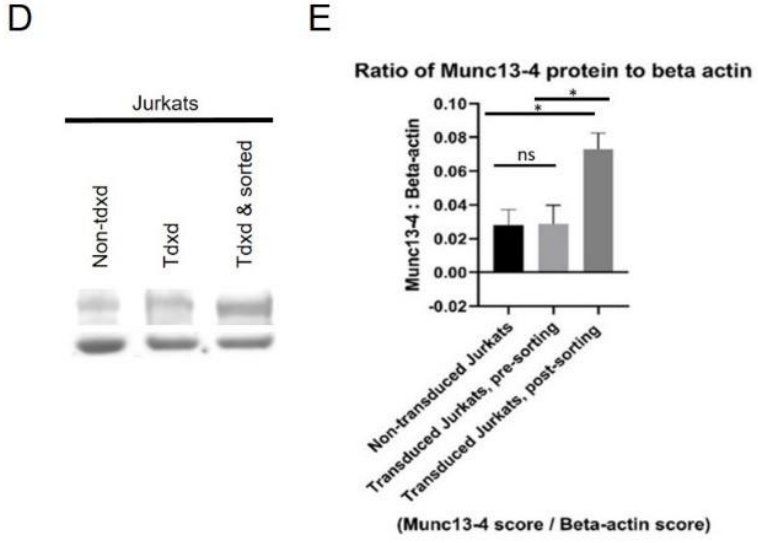


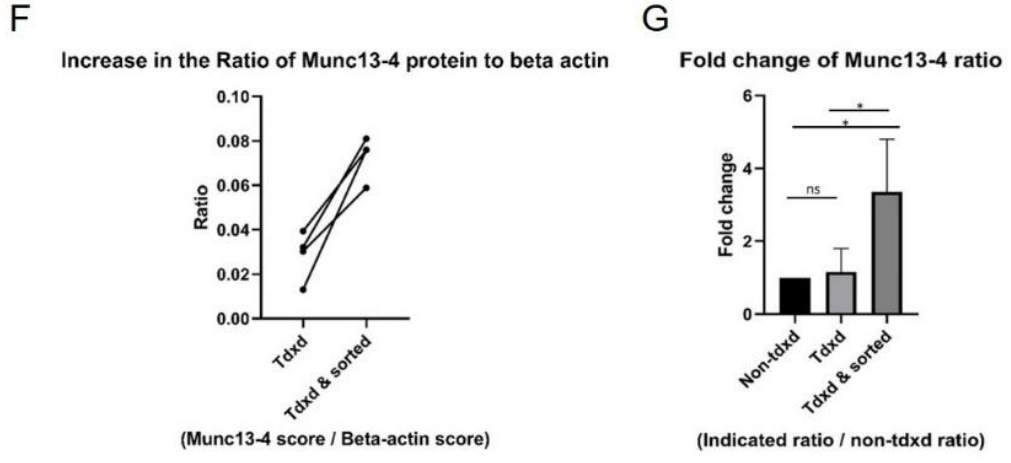
Having validated the EKCOU-tCD271 construct in NIH 3T3 cells, we repeated this validation process in Jurkat cells, which are a T-cell line and therefore more similar to the FHL3 T cells which we ultimately hope to transduce. Jurkats were transduced, and observed to express transgene proteins stably over time (Figure 6A). Next, we selected for transduced cells using biotinylated anti-CD271 antibody and magnetic beads conjugated to streptavidin. We grew the sorted cell product for an additional two days post-magnetic sorting to allow for the freeing of CD271 epitopes, and compared CD271 expression in the pre-sorted cell population, the filtered waste, and the final sorted product. We found that this procedure could significantly increase the percent of CD271+ cells (Figures 6B and C). Western blot confirmed that while Jurkats express endogenous Munc13-4, transduced and magnetically enriched Jurkats express three to four-fold more Munc13-4 (Figures 6D-G).

Figure 6

A) Jurkats experience stable expression levels of CD271 after transduction with the EKCOU-tCD271 construct, as evidenced by flow cytometry performed 5, 9, and 14 days post transduction. B) Magnetic selection for CD271+ Jurkats yielded an enriched transduced cell population, as assessed by flow cytometry. C) EKCOU-tCD271 transduced Jurkats that underwent magnetic selection showed statistically higher CD271 expression than transduced and un-sorted cells or non-transduced cells ($n = 3$, paired t-tests, ** indicates $p < 0.0025$) D) Jurkats express endogenous Munc13-4, and therefore Western blot assessment of EKCOU-tCD271 transduced Jurkats must examine the up-regulation of this protein compared to non-transduced cells E) EKCOU-tCD271 transduced Jurkats that underwent magnetic selection showed statistically higher Mun13-4 expression than transduced and un-sorted cells or non-transduced cells ($n = 4$, paired t-tests, * indicates $p < 0.05$) The ratio (F) and the fold change (G) of Munc13-4 to beta-actin increased from EKCOU-tCD271 transduced Jurkats to EKCOU-tCD271 transduced + magnetically enriched Jurkats.


A
Jurkats
Non-tdxd
Tdxd
5 days
9 days
14 days
Forward Height
CD271
CD271 1.31%
CD271 26.16%
CD271 0.34%
CD271 25.04%
CD271 0.83%
CD271 23.82%
B
Forward Scatter
CD271
Non-tdxd
Pre-sort
Filtered Waste
Final product
CD271+ 0.79%
CD271+ 10.14%
CD271+ 1.29%
CD271+ 45.01%
C
% CD271+ cells
Non-transduced
Pre-sort
Filtered Waste
Final Product
ns
**


D
Jurkats
Non-tdxd
Tdxd
Tdxd & sorted
E
Ratio of Munc13-4 protein to beta actin
Munc13-4 : Beta-actin
ns
Non-transduced Jurkats
Transduced Jurkats, pre-sorting
Transduced Jurkats, post-sorting
(Munc13-4 score / Beta-actin score)

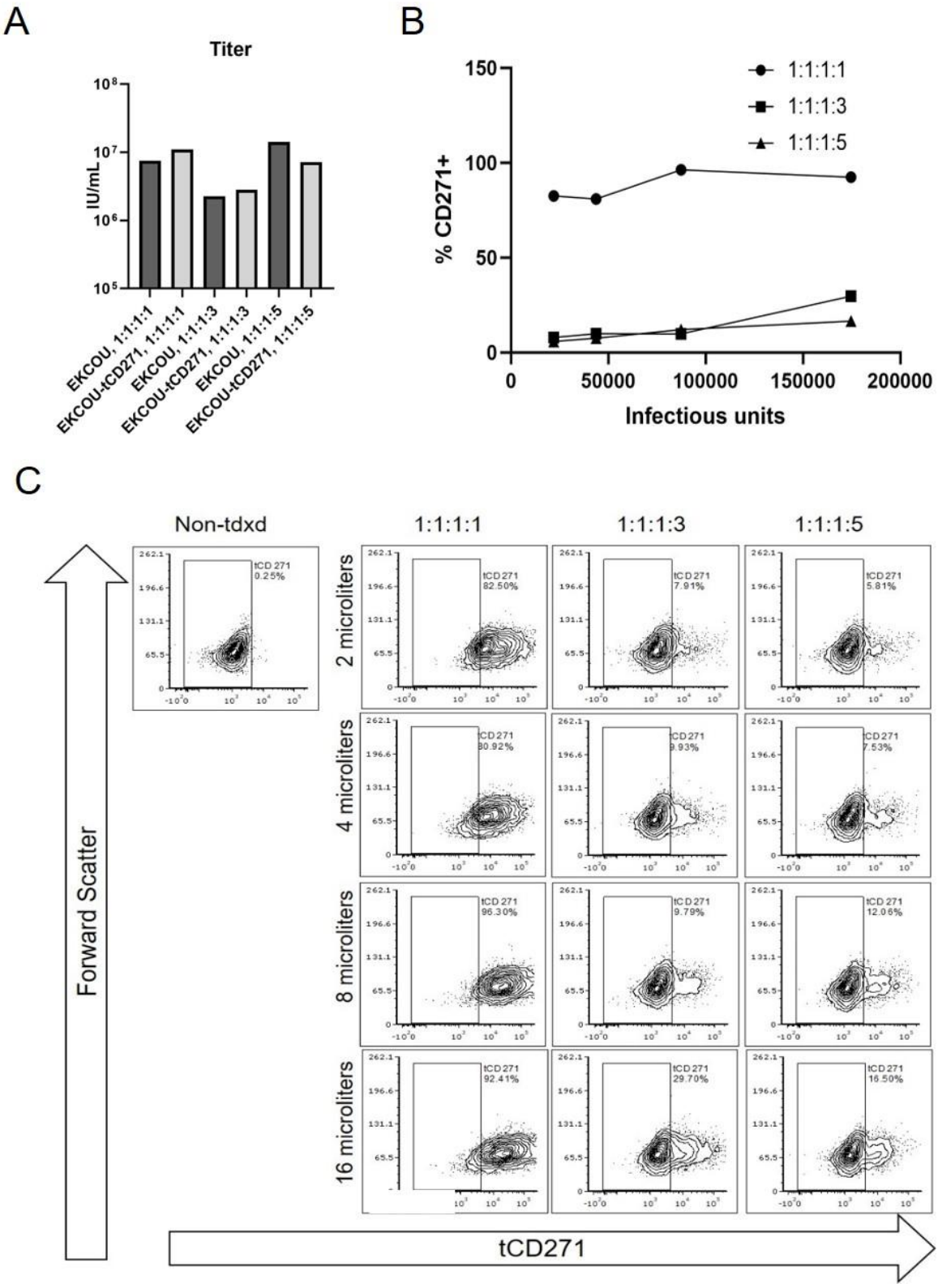

F
Increase in the Ratio of Munc13-4 protein to beta actin
Ratio
Tdxd
Tdxd & sorted
(Munc13-4 score / Beta-actin score)
G
Fold change of Munc13-4 ratio
Fold change
ns
Non-tdxd
Tdxd
Tdxd & sorted
(Indicated ratio / non-tdxd ratio)

2.4.04 The effect of transfection plasmid ratios on LV producer cells and transduced target cells

To produce lentiviral vectors, our lab has used a four plasmid transfection system consisting of separate plasmids for Gag/Pol, Rev, VSV-G, and a plasmid containing the transgene of interest. HEK 293T cells are transfected with these plasmids using a molar ratio of 1:1:1:3 respectively. We wanted to see if we could obtain higher titers of virus using different ratios of these plasmids during transfection. We compared the health of the transfected HEK 293T cells, the titers of the produced EKCOU and EKCOU-tCD271 lentiviral preparations (Figure 7A), and ability of those lentiviral vector preparations to transduce Jurkats (Figures 7B and C). Statistical analysis showed no significant difference as a result of varying plasmid ratios on viability (data not shown, ordinary one-way ANOVA, $p = 0.21$) or on titer (Figure 7A), but the LVs made under the 1:1:1:1 conditions showed significantly higher tCD271 expression in transduced Jurkats (Figures 7B and C). We explore why this might be in the corresponding discussion section, but briefly we suppose that this might be due to differences in how Jurkats respond to residual producer cell inflammation or different levels of VSV-G expression compared to HEK 293T cells in which the LVs were originally titered.

Figure 7

A) No variation in titers were observed for EKCOU and EKCOU-tCD271 LV constructs prepared using different ratios of plasmid (n = 3, paired t-test, p = 0.78). Transduction of Jurkats yielded higher CD271 expression in cells that were transduced with the LVs produced with 1:1:1:1 molar ratios, even when adjusted for titer (B) or volume (C) (one-way paired ANOVA, p = 0.0002).

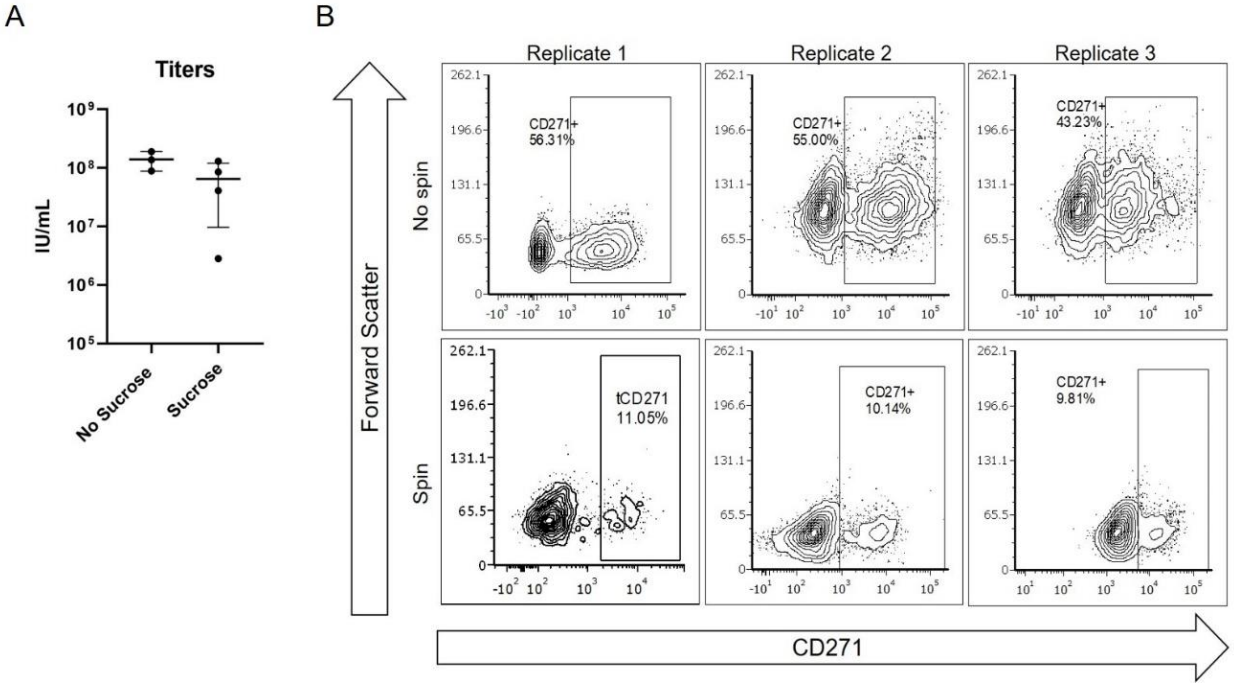

A
Titer
IU/mL
EKCOU, 1:1:1:1
EKCOU-tCD271, 1:1:1:1
EKCOU, 1:1:1:3
EKCOU-tCD271, 1:1:1:3
EKCOU, 1:1:1:5
EKCOU-tCD271, 1:1:1:5
B
% CD271+
Infectious units
1:1:1:1
1:1:1:3
1:1:1:5
C
Non-tdxd
1:1:1:1
1:1:1:3
1:1:1:5
2 microliters
4 microliters
8 microliters
16 microliters
Forward Scatter
tCD271
tCD 271 0.25%
tCD 271 82.50%
tCD 271 7.91%
tCD 271 5.81%
tCD 271 80.92%
tCD 271 9.93%
tCD 271 7.53%
tCD 271 96.30%
tCD 271 9.79%
tCD 271 12.06%
tCD 271 92.41%
tCD 271 29.70%
tCD 271 16.50%

2.4.05 A comparison of different lentiviral vector purification methods

Lentiviral vector preparations that were ultracentrifugation purified with and without a 10% sucrose cushion did not show any difference in final titer (Figure 8A). However, spinning the LV product at 7,000 g for five minutes immediately prior to transduction produced a notably diminished transduction efficiency compared LV preps that were not spun. Originally, this extra five minute spin was suggested as a method to help increase the viability of the cells, but no noticeable increase in viability was observed (data not shown).

Figure 8

A) LVs prepared with and without sucrose cushion purification produce no significant difference in titer (un-paired t-test, $p = 0.12$). B) Spinning lentiviral vector preps at 7,000 g for five minutes immediately prior to transduction significantly reduces transduction efficiency in Jurakts and subsequent tCD271 expression ($n = 3$, paired t-test, $p = 0.009$) despite producing no significant changes in cell viability (data not shown, $n = 3$, paired t-test, $p = 0.56$).

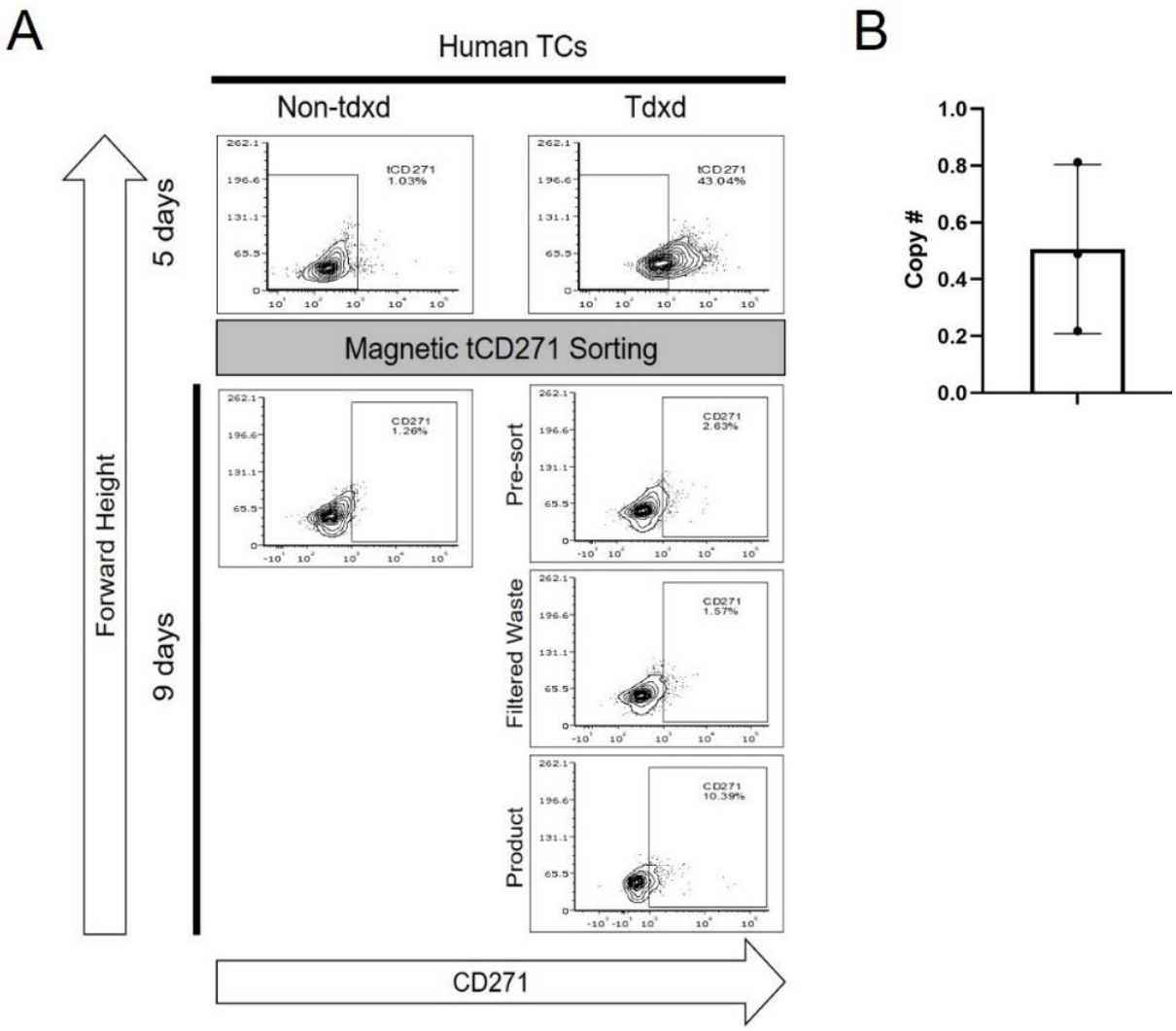


2.4.06 Primary human T cells do not maintain their CD271 expression over time

The same CD271 selection process previously described as efficacious in Jurkat cells was performed on transduced primary human T cells. Given that primary human T cells do not divide as quickly as Jurkat cells, the sorted primary T cells were washed and incubated for an additional four days after sorting in order to for magnetic beads to disassociate from the CD271 epitopes. Without exception, CD271 expression was greatly reduced in T cells which had been incubated for nine days compared to T cells which had been incubated for only five days. This was true for even the T cells which had been magnetically labeled but were not subjected to the magnetic sorting process (the "pre-sort" population) (Figure 9A). This was surprising given that at five days post-transduction, all samples that were used in the sorting process had a copy number of greater than zero (Figure 9B). This phenomena can be explained by pseudo-transduction, or when the RNA LV genome enters the cell, is reverse-transcribed into DNA, but fails to integrate into the host genome. Such DNA can still express the transgene proteins and will have a reported VCN > 0, but because the transgene is not integrated both VCN and transgene protein expression will decrease over time. This topic is covered more thoroughly in the discussion.

Figure 9

A) (Top panels) Primary human T cells showed signs of EKCOU-tCD271 transduction five days after transduction, as evidenced by CD271 expression. However, after nine days, T cells lost CD271 expression in magnetically sorted and un-sorted T cell populations. B) Copy number analysis showed that integration of the LV transgene had occurred within these T cells (n =3).



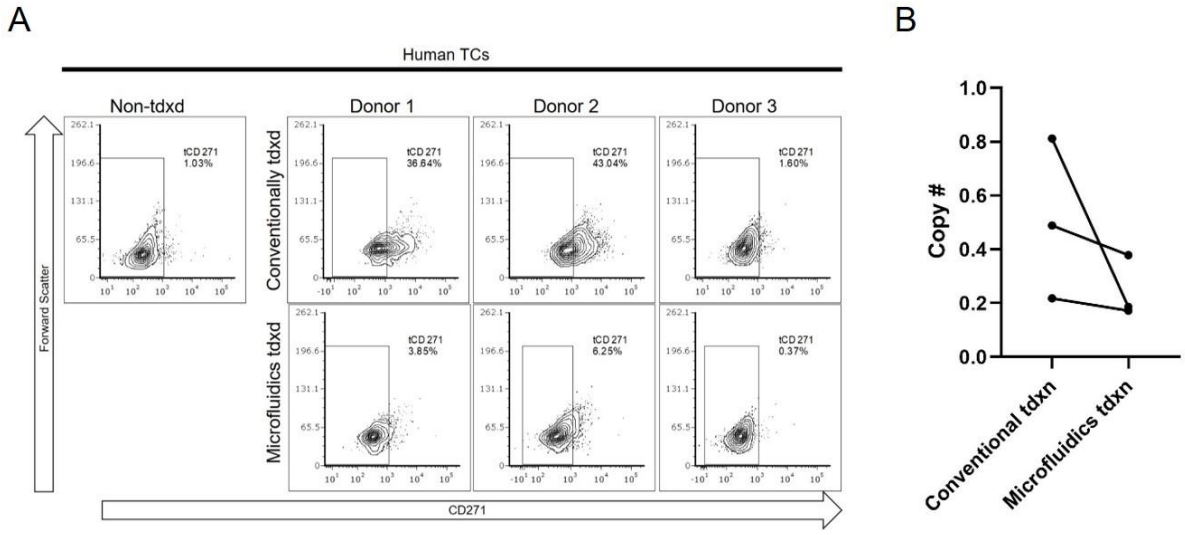
2.4.07 Microfluidics based transduction

Lack of contact between LVs and target cells is one of the main factors which limit transduction [296]. Therefore, some researchers have experimented with shrinking the distance between LVs and target cells by conducting transduction in minimal volumes of media. This approach to transduction is called “microfluidics,” and requires the use of

vessels capable of holding cells and virus in volumes around the order of 10 µLs. We obtained several of these microfluidics devices from collaborators at Georgia Institute of Technology, and conducted a head-to-head comparison of microfluidics transduced vs. conventionally transduced human T cells using the EKCOU-tCD271 LV. We observed no statistically significant difference between conventionally and microfluidics transduced cells (Figure 10A), but it is worth noting that this may be due to donor variation in transduction capacity. We suspect that increasing the number of donors sampled will elucidate a significant difference between these two methods. Copy numbers in conventionally transduced primary human T cells trended higher than those in microfluidics transduced cells, but this difference was not statistically significant (Figure 10B).

Figure 10

A) Primary human T cells that were transduced with the EKCOU-tCD271 construct expressed similar levels of CD271 in cells that were transduced conventionally and those that were transduced using a microfluidics device (n =3, paired t-test, p = 0.17). The two different methods showed no statistically significant variation in the viability of the cells on the day of flow analysis (data not shown, n = 3, paired t-test p = 0.17). B) Copy numbers in conventionally vs. microfluidics transduced human T cells showed no statistically significant difference (n = 3, paired t-test, p = 0.29).

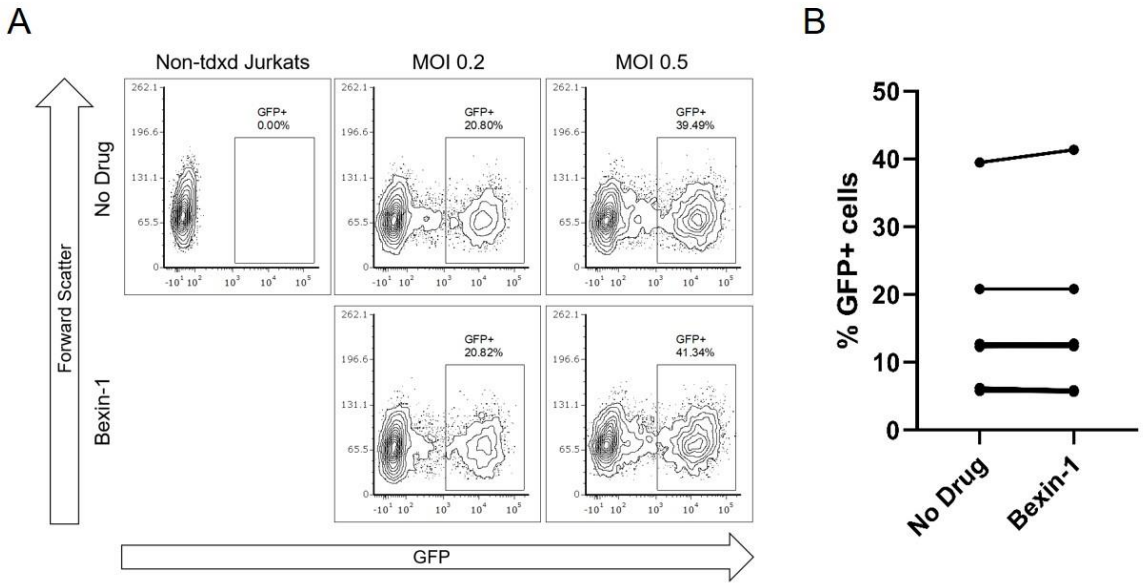


2.4.08 The impact of Munc13-4 expression on transduction efficiency

Munc13-4 is a widely described mediator of exocytosis [161, 192, 230], but some evidence also suggests that it could play a role in endocytosis [208, 248]. VSV-G pseudotyped LVs are internalized with the target cell membranes and fuse with target cell membranes in the acidified endosomes [346-348]. We hypothesized that Munc13-4 plays a role in endocytosis, and therefore a lack of functional Munc13-4 would inhibit endocytosis and subsequent transduction of target cells with VSV-G pseudotyped LVs. To test this, Jurkats, which express Munc13-4 endogenously, were transduced with a GFP construct in the presence or absence of the Munc13-4 small molecule inhibitor Bexin-1. Five days after transduction these cells were analyzed by flow cytometry to determine the percent of GFP+ cells. No statistically significant difference in GFP expression was observed between Jurkats that had been transduced with Bexin-1 as opposed to without Bexin-1 (Figures 11A and B).

Figure 11

A) Jurkats were transduced with a GFP vector in the presence or absence of the Munc13-4 small-molecule inhibitor, Bexin-1 and subsequently analyzed by flow cytometry. B) No statistically significant difference was observed in the GFP expression of cells that had been transduced in media supplemented with Bexin-1 compared to un-supplemented media (n = 6, paired t-test, p = 0.46).



2.4.09 A comparison of different transduction enhancers

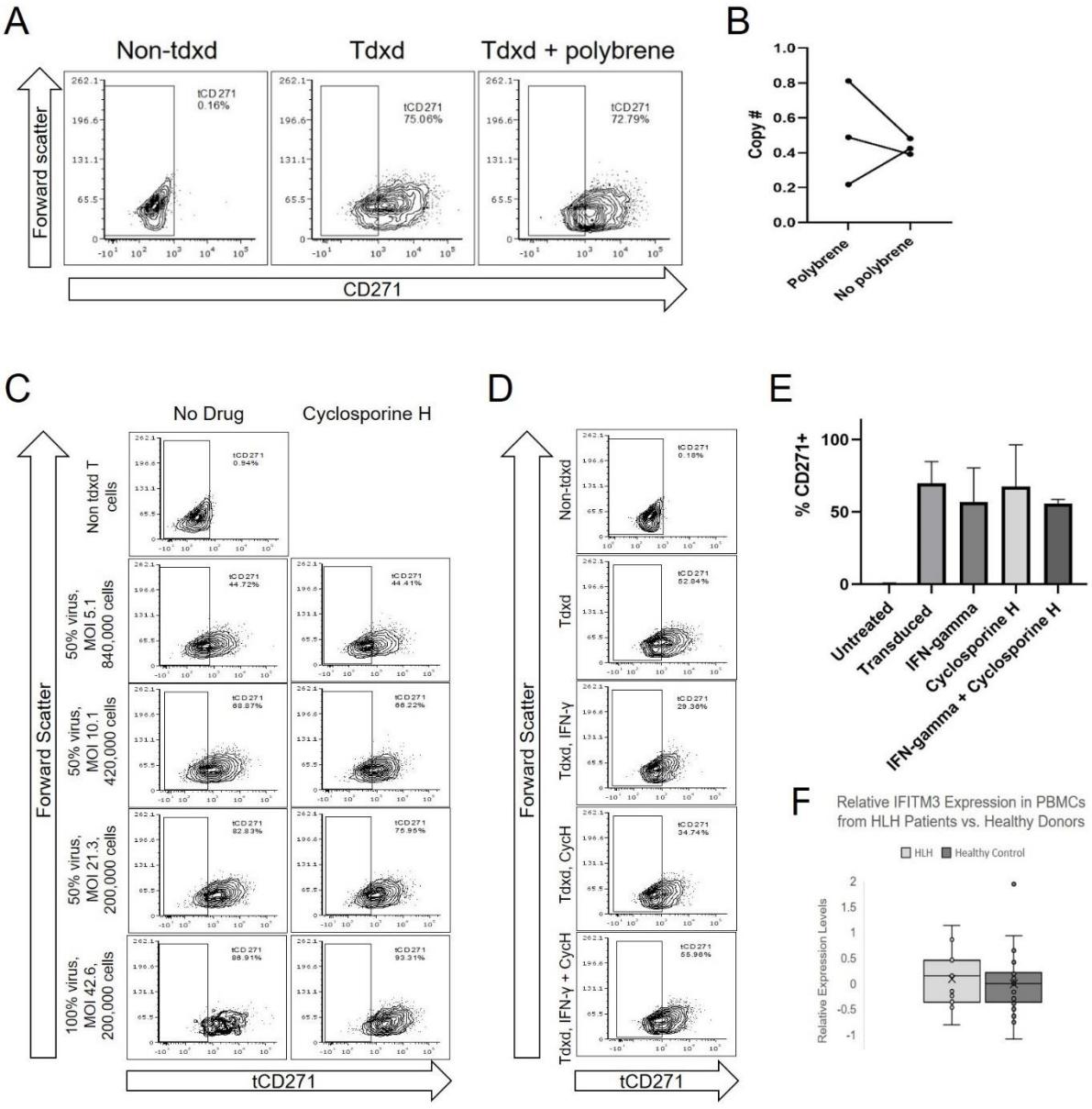
Primary human T cells were transduced with EKCOU-tCD271 LVs in the presence or absence of polybrene (8 μg/mL), incubated for five days, and analyzed for CD271 expression. No statistically significant difference in the viability (data not shown), amount of tCD271 expression (Figure 12A), nor copy number (Figure 12B) was observed.

Primary human T cells were transduced with EKCOU-tCD271 LVs in the presence or absence of 10^{-6} M of cyclosporine H, which is non-toxic to cells (Supplemental Figure 1) and which has been described as the effective dose for treating T cells [304]. Five days post-transduction, no difference was observed in the CD271 expression of treated

vs. un-treated cells (Figure 12C). We hypothesized that IFITM3 was not up-regulated on the surface of these cells on the day of transduction, so we treated T cells with IFN-γ during the overnight stimulation period prior to transduction as a way to increase the expression of IFITM3. However, IFN-γ treatment did not reduce CD271 expression compared to non-treated cells. Furthermore, cells treated with IFN-γ and cyclosporine H did not increase CD271 expression compared to cells that were just treated with IFN-γ. Overall, no additive or combination of additives was observed to increase transduction efficiency over the conventional protocol (Figures 12C, D, and E). Finally RNAseq analysis of HLH patient vs. healthy control PBMCs showed no significant difference in IFITM-3 expression (Figure 12F)[124], indicating the cyclosporine H would not dramatically enhance transduction in HLH patient cells.

Figure 12

Primary human TCs transduced with EKCOU-tCD271 in the presence or absence of polybrene did not show any difference in viability (data not shown), CD271 expression (A), nor copy number (B) (n = 3, paired t-tests, respectively p = 0.44, 0.68, and 0.39). C) Cyclosporine H does not exert an effect on CD271 expression in transduced primary human T cells, even with varying numbers of target cells and volumes of virus (paired t-test, n = 4, p = 0.90). D and E) Primary human T cells that were pre-treated with IFN-γ showed no statistically significant change in CD271 expression compared to non-treated transduced controls (n = 3, paired t-test, p = 0.13). Cells that were treated with IFN-γ and cyclosporine H did not show any difference in CD271 expression compared to cells that were treated only with IFN-γ (n = 3, paired t-test, p = 0.95). In summary, no statistically significant variation between transduced treatments groups was observed (n = 3, One-way ANOVA, p = 0.41). F) RNAseq analysis shows that IFITM3 expression does not differ between HLH patients and healthy controls (11 HLH patients, 33 healthy controls, un-paired t-test, p = 0.62).



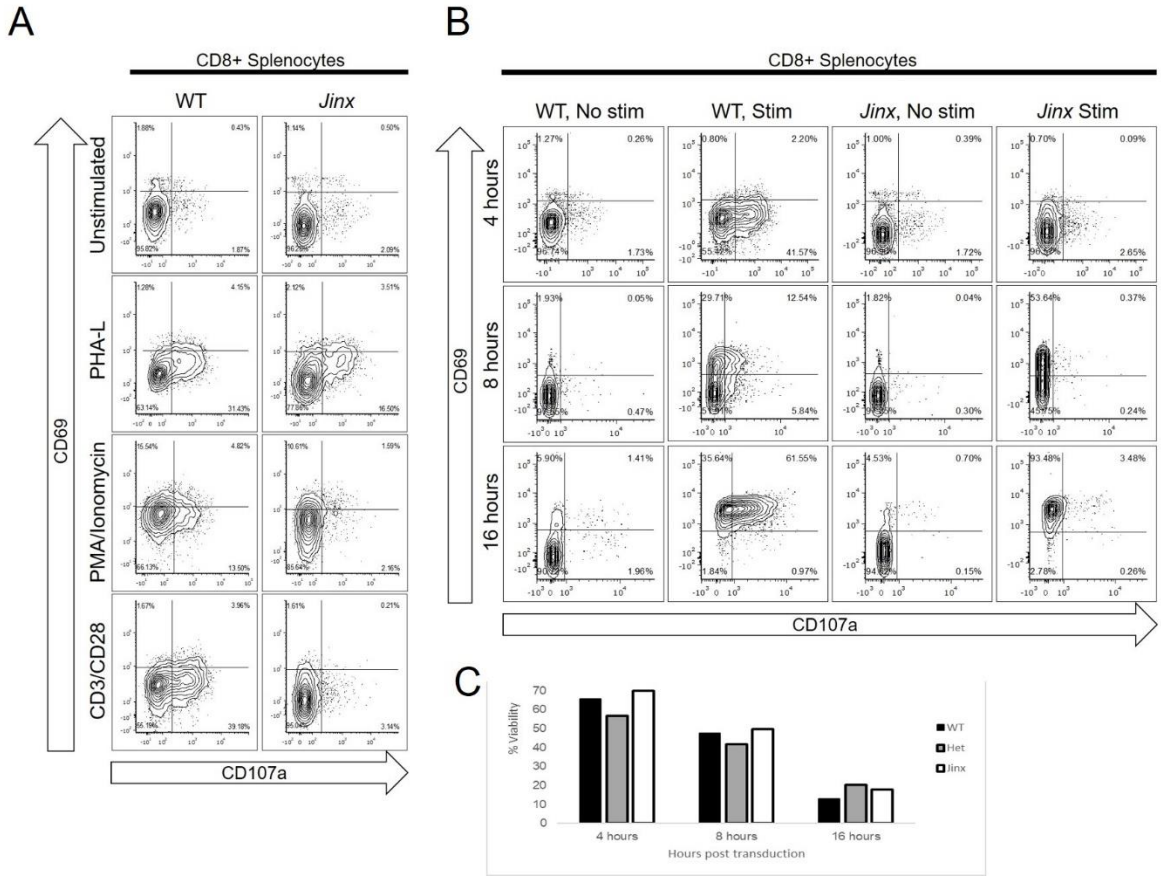
2.4.10 Optimization of the T-cell degranulation assay

Splenocytes from WT and *Jinx* mice were collected, washed, and stimulated with one of three commonly used T-cell stimulants. After four hours, CD8+ splenocytes were analyzed by flow cytometry for their upregulation in CD69 (an activation marker) and CD107a (a degranulation marker) (Figure 13A). Based on these results, we elected to focus our efforts to optimize the T-cell degranulation assay using CD3 and CD28 antibodies. This meant that we analyzed CD8+ splenocytes rather than CD3+ CD8+ splenocytes because stimulation with CD3 and CD28 antibody induces internalization of CD3 (Supplemental Figure 1).

We hypothesized that the time spent in stimulation media might affect the robustness of the activation and degranulation of the splenic T cells. Therefore, we ran a time-course experiment to compare four, eight, and sixteen hours or stimulation. We found that a robust result could be obtained with as little as four hours of incubation with minimal detriment to the viability of the cells, but that a more robust CD107a result could be obtained for longer incubation times (Figure 13B and C).

Figure 13

Stimulation with CD3/CD28 yields the more robust and distinct differences in activation (CD69) and degranulation (CD107a) of CD8+ splenocytes from WT and *Jinx* mice compared to PHA-L and PMA/ionomycin stimulation. B) Activation and degranulation responses in splenocytes from WT, heterozygous, and *Jinx* mice which were cultured for four, eight, and sixteen hours in the presence of CD3/CD28 antibody. While degranulation was most robust at the sixteen hour mark, the cells at this time point also had greatly reduced viability (C).

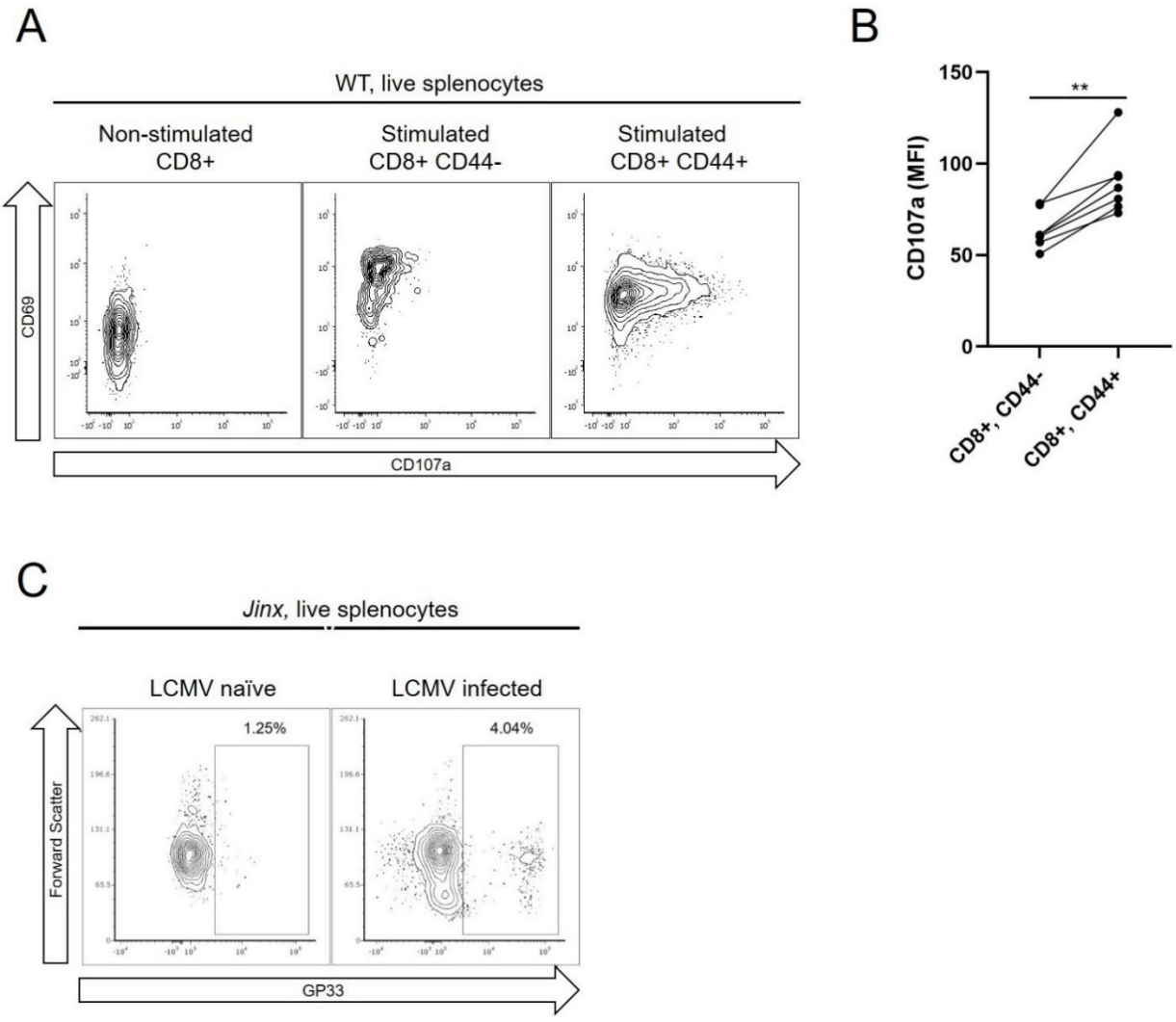


The FHL3 mouse model consists of LCMV infected *Jinx* mice [170]. We were therefore interested in using the T-cell degranulation assay to examine LCMV-specific T cells for evidence of gene therapy modification and functional correction. We obtained LCMV tetramers for the LCMV-specific peptides GP33 and GP276, and were able to observe a small population of LCMV-specific cells in LCMV Armstrong infected mice ten days after infection (Figure 14C). However, this population was not always detectable, and

therefore we elected to focus subsequent experiments onto CD44+ CD8+ splenocytes, which have a memory phenotype and exhibit a more robust degranulation response than CD8+ CD44- splenocytes (Figure 14A and B).

Figure 14

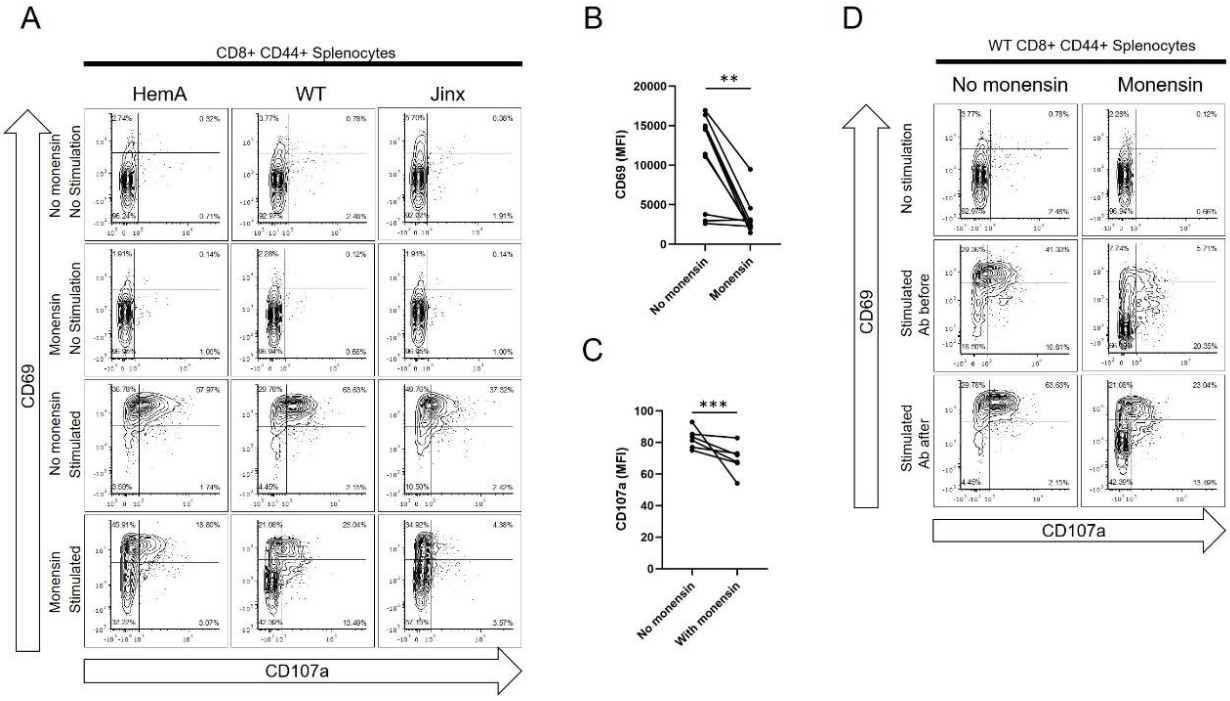
Jinx mice were intraperitoneally infected with 2 x 10^5 PFU of LCMV Armstrong, and ten days after infection their splenocytes were subjected to the CD3/CD28 degranulation assay. A and B) CD8+ CD44+ splenocytes showed more robust upregulation of CD107a upon stimulation compared to CD8+ CD44- splenocytes (n = 7, paired t-test, p = 0.001). C) Staining for LCMV specific T cells was done using GP33 and GP276 tetramers. A small but distinct population of such cells could be observed in some but not all mice.



Monensin, also referred to sometimes as GolgiStop, limits intracellular trafficking and is included in some degranulation assay protocols (see Table 5). We found that excluding monensin caused a significant upregulation in CD69 and CD107a expression in CD44+ CD8+ splenocytes (Figure 15A, B, and C), but that this was most noticeable if the antibodies were added after the stimulation period (Figure 15D).

Figure 15

A) CD44+ CD8+ splenocytes from HemA and WT mice (positive controls) and *Jinx* mice (negative control) with or without CD3/CD28 antibody stimulation in the presence or absence of monensin. B) Monensin significantly down-regulated CD69 expression (n = 10, paired t-test, p = 0.0014) and C) CD107a expression (n = 11, paired t-test, p = 0.0006). Adding monensin did not significantly alter the viability of these cells (data not shown, n = 12, paired t-test, p = 0.0626). D) Adding the antibody for CD69 before or after stimulation alters the final CD69 expression profile.



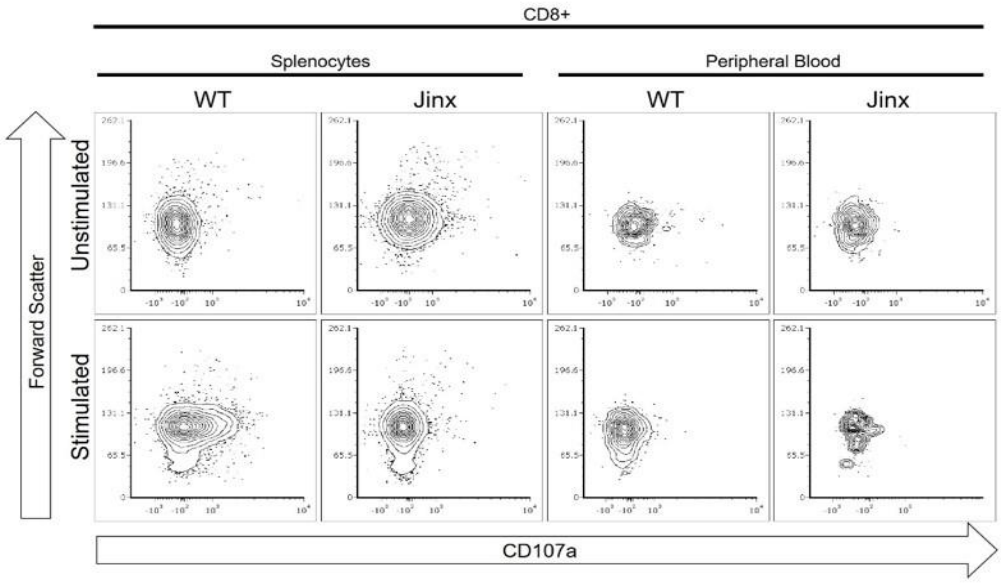
Degranulation assays are most commonly performed on splenocytes (Table 5), but we wanted to see if the assay could also be optimized using T cells from the peripheral mouse blood. The maximum volume of peripheral blood (150 microliters) was facially bled from WT and *Jinx* mice. After red blood cell lysis, cells were stimulated with CD3

and CD28 antibody for eight hours. Peripheral blood CD8 T cells did not activate in either the WT or *Jinx* mice (Figure 16A). Furthermore, not enough CD44+ CD8+ cells could be obtained from facial bleeds for meaningful flow analysis on degranulation and activation (Figures 16B and C).

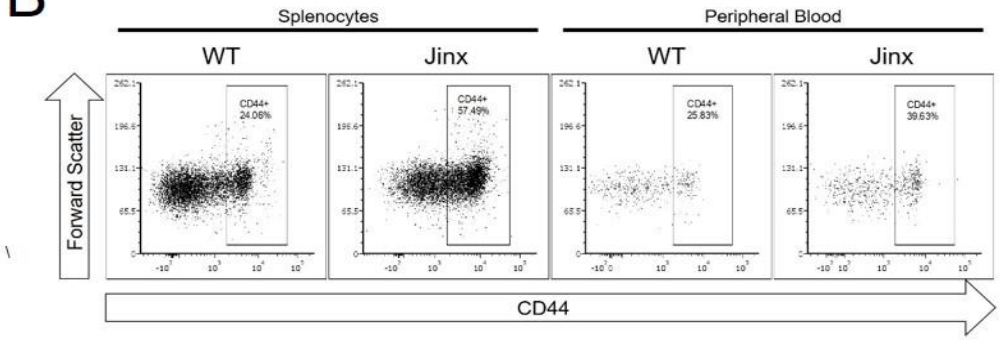
Figure 16

A) WT peripheral blood CD8+ cells do not respond as robustly to the degranulation assay conditions as WT splenic CD8+ cells. B) Fewer CD44+ CD8+ cells are observed in the peripheral blood compared to the quantities which can be harvested from the spleen. C) As with bulk CD8+ cells, CD44+ CD8+ cells from the peripheral blood do not respond as robustly as CD44+ CD8+ splenocytes, and their low abundance makes meaningful analysis difficult.

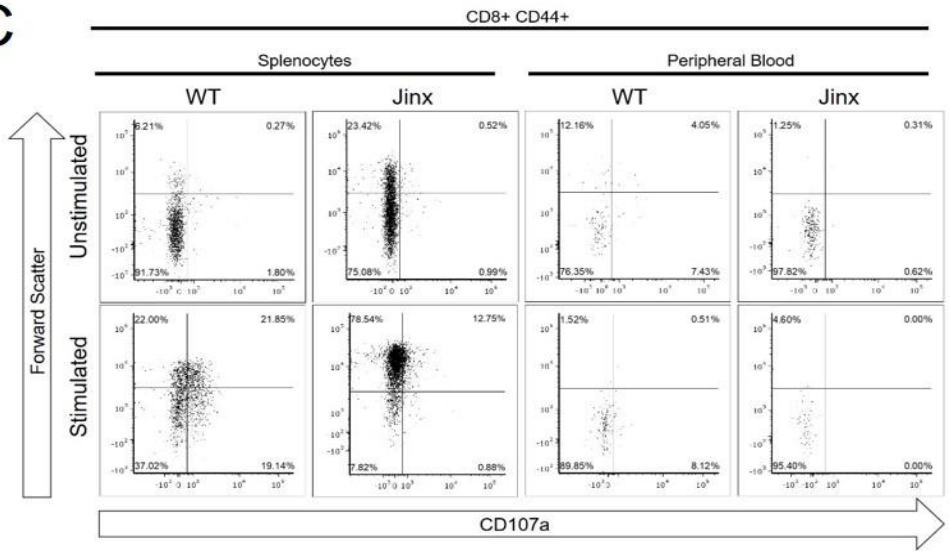
A


CD8+
Splenocytes
Peripheral Blood
WT
Jinx
WT
Jinx
Unstimulated
Stimulated
Forward Scatter
CD107a

B


Splenocytes
Peripheral Blood
WT
Jinx
WT
Jinx
CD44+
24.06%
CD44+
57.49%
CD44+
25.83%
CD44+
39.63%
Forward Scatter
CD44

C

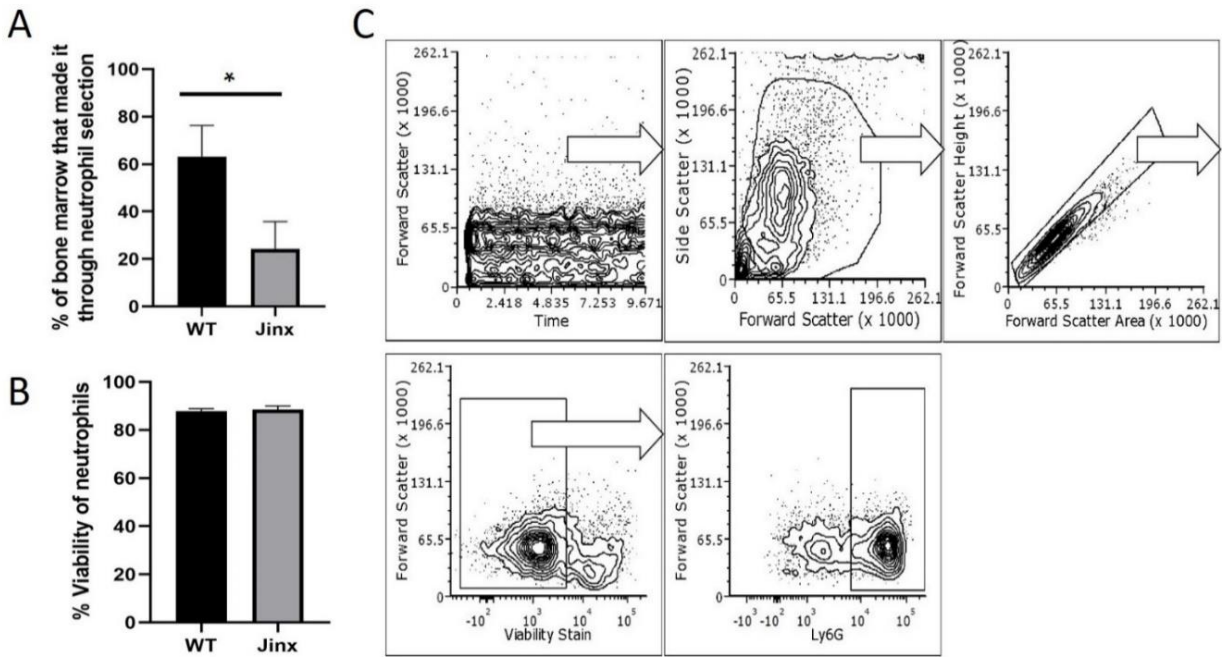

CD8+ CD44+
Splenocytes
Peripheral Blood
WT
Jinx
WT
Jinx
6.21%
0.27%
91.73%
1.80%
23.42%
0.52%
75.08%
0.99%
12.16%
4.05%
76.35%
7.43%
1.25%
0.31%
97.82%
0.62%
22.00%
21.85%
37.02%
19.14%
78.54%
12.75%
7.82%
0.88%
1.52%
0.51%
89.85%
8.12%
4.60%
0.00%
95.40%
0.00%
Unstimulated
Stimulated
Forward Scatter
CD107a

2.4.11 Neutrophil degranulation assay

Though understanding of FHL3 disease pathology centers around T cell and natural killer cell dysfunction, myeloid cells are also affected by Munc13-4 deficiency. In particular neutrophils normally express Munc13-4 protein [204, 219], and clinical evidence suggests that FHL3 patients are particularly susceptible to bacterial and fungal infections [72, 118, 245, 258] which might normally be combatted by neutrophils. We therefore wanted to optimize an assay that could assess functional correction in neutrophils as a way to assess functional correction in these cells. Neutrophils from WT and *Jinx* mice were isolated using a murine PMN isolation kit (Figures 16A and B). Subsequent flow analysis was performed on the Ly6G+ cell populations (Figure 2C).

Figure 17

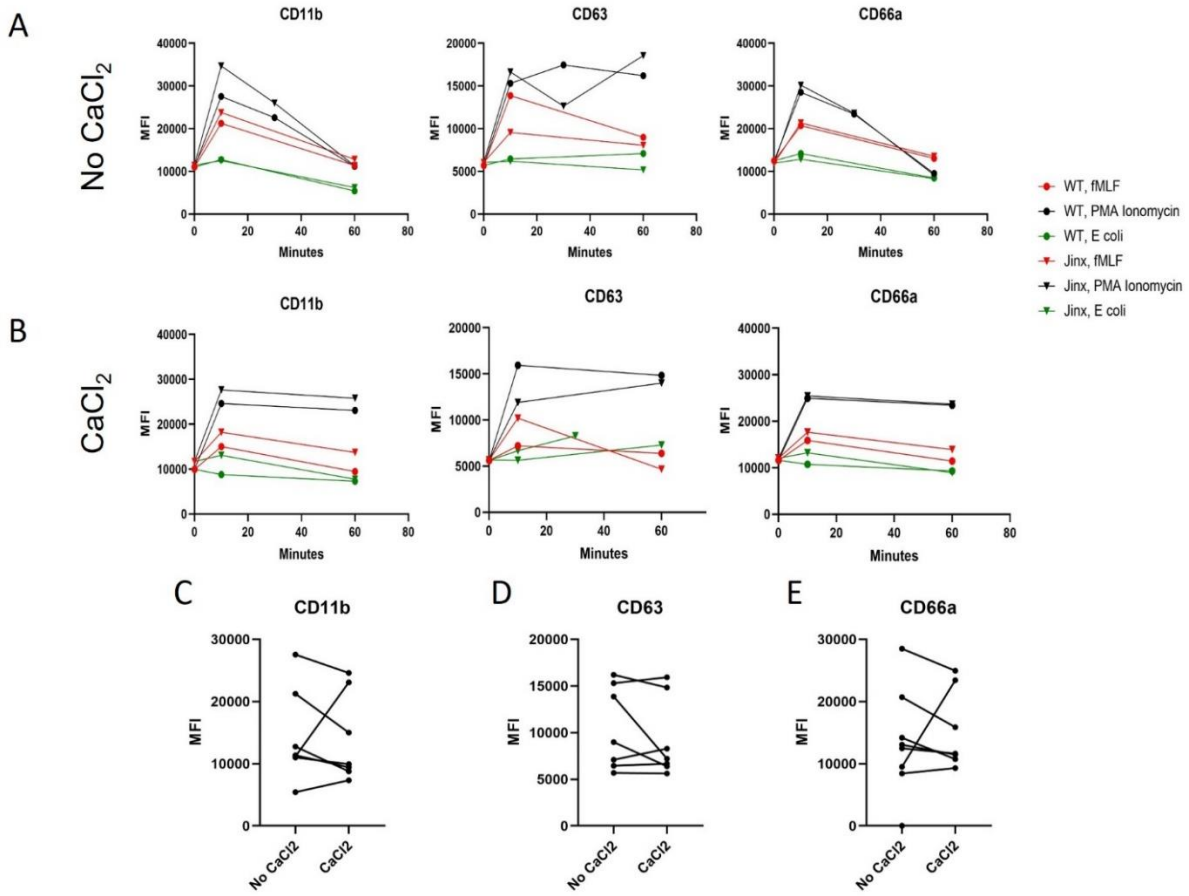
A) Selection of neutrophils yields a smaller percent of the bulk bone marrow in *Jinx* mice compared to WT mice (n = 3, un-paired t-test, p = 0.018). B) Neutrophils from WT and *Jinx* mice show similar viability (n = 3, un-paired t-test, p = 0.574). C) Flow gating strategy for the selection of mouse neutrophils.



WT and *Jinx* neutrophils were stimulated with either fMLF, PMA/ionomycin, or lysates from *E. coli* bacteria, and their degranulation response was assessed via flow analysis for CD11b, CD63, and CD66a markers. While both fMLF and PMA/ionomycin stimulation caused an increase in WT neutrophil degranulation markers, this up-regulation was also observed in *Jinx* neutrophils (Figure 18A). The experiment was subsequently performed again, this time with supplementation of the stimulation media with 160 μM $CaCl_2$. $CaCl_2$ addition did not yield any significant difference in neutrophil degranulation compared to un-supplemented media (Figure 18B and C).

Figure 18

Time course experiments showing the effect of different neutrophil stimulants on WT and *Jinx* neutrophils over time without A) and with B) 160 μM $CaCl_2$. C-E) No significant difference was observed in the CD11b, CD63, and CD66a expression of WT neutrophils treated with or without $CaCl_2$ (paired t-tests, n = 7, p = 0.89, 0.27, and 0.98 respectively.

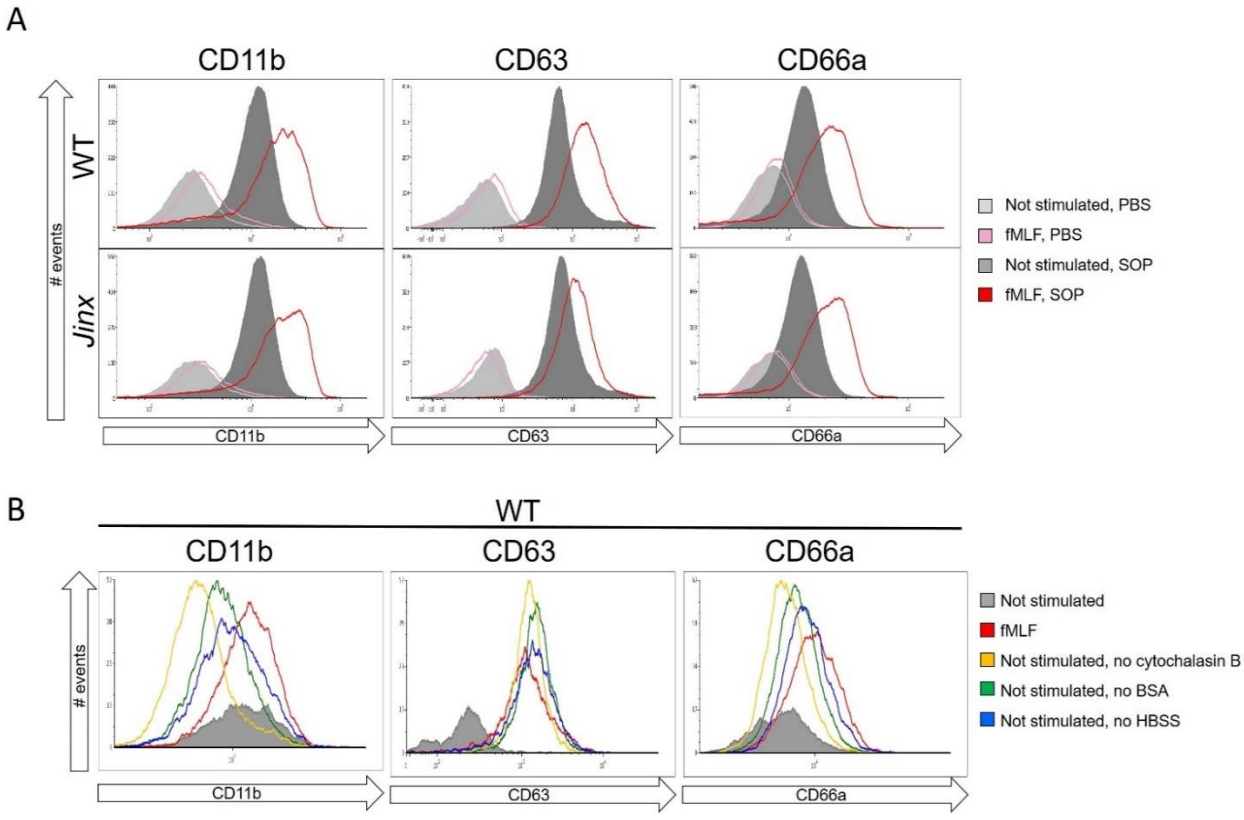


In an effort to discover if some part of the neutrophil degranulation assay was unintentionally activating neutrophils and causing degranulation in both the unstimulated and stimulated controls, flow analysis was performed on neutrophils that were kept in cold PBS immediately after isolation and neutrophils that were subjected to the full neutrophil degranulation assay protocol. All stimulated cells were stimulated with 10^{-5} molar fMLF. Neutrophils which were incubated in cold PBS could not degranulate in response to fMLF,

while even non-stimulated neutrophils incubated in media showed up-regulation of degranulation markers (Figure 19A). Wondering if a particular component of the neutrophil media was responsible for this up-regulation in degranulation markers, we systematically excluded each additive from the media. We observed no significant decrease in degranulation marker expression between unstimulated neutrophils incubated in deficient media vs. complete media (Figure 19).

Figure 19

A) Neutrophils cannot degranulate in response to fMLF stimulation if incubated in PBS alone. Furthermore, neutrophils cultured in media upregulate all degranulation markers compared to neutrophils kept in cold PBS. B) Excluding different components of neutrophil media does not reduce degranulation marker expression in non-stimulated neutrophils.



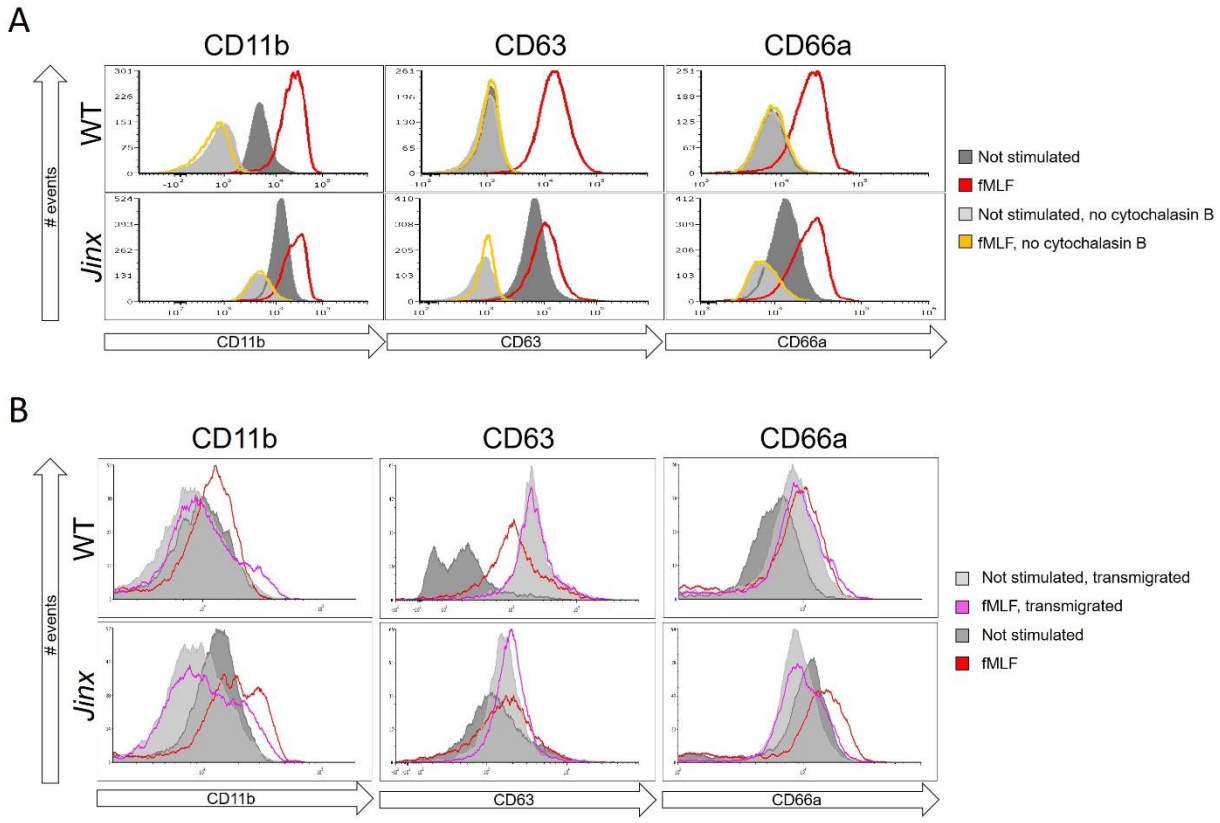
Cytochalasin B is an inhibitor of actin polymerization, and while it is called for in conventional neutrophil degranulation assay protocols (Table 6), we wondered if excluding

it would enhance upregulation of neutrophil degranulation markers. However, no difference was observed between the unstimulated and stimulated neutrophils incubated in cytochalasin B deficient media in either WT or *Jinx* mice (Figure 20A).

Neutrophils were isolated and subjected to *in vitro* transmigration in order to increase their responsiveness to stimuli and their affinity for degranulation. However, this transmigration process did not noticeably increase the degranulation response in WT neutrophils which were stimulated vs. those that were not (Figure 20B).

Figure 20

A) Excluding cytochalasin B from the media does not augment degranulation in response to fMLF stimulation. B) Transmigration does not have affect expression of neutrophil degranulation markers.



2.5 DISCUSSION

2.5.01 Optimization of the lentiviral construct

One major challenge facing the field of lentiviral vector gene therapy is scaling LV production, so we created multiple lentiviral constructs with the intent of producing a superior and scalable vector (Figure 2A). Each construct was reliably and efficiently produced, despite the large size of the *UNC13D* cDNA (3273 bp) (US National Library of Medicine). This was important because compared to other FHL related proteins, Munc13-4 is large, and previous studies have shown diminishing titers as a result of increasing vector length [349].

We experimented with using the Ef1α and MND promoters to promote transgene expression, but found that the MND promoter to be significantly inferior at inducing the expression of transgenic Munc13-4 protein (Supplemental Figure 4). The other FHL3 gene therapy research groups report effective transgene expression using the Ef1α or EFS promoters [166, 168]. Therefore, the Ef1α promoter seems like a good choice for concurrently developing HSPC and T cell based gene therapies. Future studies might instead seek to use cell-specific promoters such as the CD3 promoter in T cells in order to generate even higher levels of Munc13-4 expression.

Western blot analysis of lysates from transduced NIH 3T3 cells showed that the codon-optimized version produced the highest expression of Munc13-4, which was comparable to constitutive expression levels in human PBMCs, even at relatively low MOIs (Figure 2C). This result is consistent with previous work from our lab which described how liver codon optimization produces superior transgene expression in multiple cell types [344]. We note that the other FHL3 gene therapy research groups have also codon-

optimized their lentiviral constructs, though the specifics as to how this codon optimization was performed are not specified [166, 168]. We therefore expect that a head to head comparison of the different lentiviral vectors would yield slightly different results owing to the differences in codon optimization. Future studies might include optimization of transgene expression in different FHL3-related cell types using alternative codon optimization strategies.

For our lentiviral construct we used the cDNA for isoform for the canonical Munc13-4 sequence, as listed on UniProt. To date, there are three described isoforms of Munc13-4 which result from alternative splicing, but computational mapping indicates as many as seven possible isoforms exist. For the work presented here, we have assumed both that the canonical sequence is responsible for mediating the degranulation response that is absent in FHL3 patients, and that supplementing FHL3 patients with this missing protein will correct their disease. The literature from other FHL3 gene therapy labs does not indicate which isoform was used in the construction of their gene therapy vectors [165, 166, 168, 274, 275], so we can only assume that these research groups also chose to base their gene therapies on the canonical *UNC13D* sequence. Mice have two described isoforms of Munc13-4 protein and a potential third isoform according to computational mapping (UniProt). Murine isoforms 1 and 2 differ only by one nucleotide and luckily, the *Jinx* mouse model is deficient in all described isoforms of murine Munc13-4 protein, owing to the pre-mature termination of Munc13-4 mRNA and the exclusion of the second MHD2 domain and second C2 domain [170]. Future work might examine the role of the other Munc13-4 isoforms in different cell types and their role in disease.

2.5.02 Optimization of HSPC isolation

We compared three commercially available HSPC isolation kits: MACS Miltenyi' Biotec's Sca-1[25] positive isolation kit, StemCell Technology's Sca-1 positive isolation kit, and StemCell Technology's lineage negative isolation kit. While Sca-1 positive selection kits have been used in many studies of mouse HSPCs [353, 354], it is worth noting that Sca-1 expression is not perfectly restricted to murine HSPCs. While it is true that murine HSPCs express Sca-1 on their cell surface, and that expression fades as those HSPCs differentiate into common myeloid progenitors [355] and common lymphoid progenitors [356], Sca-1 expression has also been observed on activated lymphocytes [357], mouse B cells [358], and rare leukocyte populations [359]. Therefore, it is possible that Sca-1+ cells could contain additional cell types beyond our desired murine HSPC population.

Similarly, while murine HSC lineage depletion kits have also been used in numerous studies [360], the purity of these kits is reportedly on 60-84% [StemCell Technologies]. From our own work we observed morphological heterogeneity in the isolates from the HSC lineage depletion kit as compared to the Sca-1 kits (Figure 3F). On

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[25] Importantly, different mouse strains will have different expression profiles for Sca-1. While all Ly6.2 strains such as C57/Bl/6 mice express Sca-1 on all of their HSPCs 350. Uchida, N. and I.L. Weissman, *Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow.* J Exp Med, 1992. **175**(1): p. 175-84., only about 25% of the HSPCs from Ly6.1 mouse strains like BALB/c mice express Sca-1 351.
Spangrude, G.J. and D.M. Brooks, *Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells.* Blood, 1993. **82**(11): p. 3327-32, 352.
Holmes, C. and W.L. Stanford, *Concise review: stem cell antigen-1: expression, function, and enigma.* Stem Cells, 2007. **25**(6): p. 1339-47.. *Jinx* mice, being derived from the C57/Bl/6 mouse strain, should have Sca-1 expression on all of their HSPCs.

average, about 10% of the cells made it through each isolation method, a percent which is still high when compared to estimates that only 0.01% of the total nucleated cells in the whole bone marrow populations are HSCs [360]. Therefore, future studies might include more rigorous selection methods to gain more purified HSPC populations.

Given that we expected the negative lineage marker kit to have the lowest purity of HSPCs, we were surprised to observe the greatest expansion in these samples from both WT and *Jinx* mice (Figure 4B). However, after transduction with either the EKCOU or GFP LVs, these isolates did not have significantly larger numbers of HSPCs nor viability of those HSPCs compared to the other samples (Figure 4C and D). Notably, no single mouse or isolation method yielded more than 2 million healthy HSPCs, meaning that future transplantation studies will be restricted to having a 1:1 ratio of donor to recipient mice. While this finding is consistent with our observations from previous transplantation studies, this small donor to recipient ratio could potentially be a limiting factor in completing large transplantation studies in the future.

While we observed substantial variation in the number of CFUs, we observed that EKCOU and GFP transduced *Jinx* HSPCs produced the most colonies if those cells were isolated using the MACS Sca-1 isolation kit. Furthermore, cells isolated with this method had higher expression of GFP expression as assessed by flow cytometry. Therefore for all subsequent HSPC studies, these cells were isolated using the HSPC kit.

2.5.03 Design and validation of the codon-optimized-tCD271 lentiviral construct

Previous studies have shown that truncated tCD271 can act as a marker for the identification and magnetic selection of transduced cells [334]. The aforementioned studies focused on generating a HSPC-based gene therapy, and because HSCPs divide very slowly and should be transplanted into recipient mice immediately after transduction, including a marker for tCD271 would not be practical. However, as we shifted our focus towards developing an FHL3 T-cell mediated LV gene therapy, including tCD271 in the construct made sense because of how T cells can be transduced and then expanded for sufficient time periods that tCD271 can be expressed as a result of bonafide transduction (as opposed to pseudo-transduction). We therefore cloned the sequence for tCD271 into our construct and observed that we could get expression of both CD271 and Munc13-4 expression in transduced cell lines—even after pro-longed periods in culture. Furthermore, tCD271 could be used to enrich the transduced Jurkat cell population using a simple magnetic beads procedure. Given the successful results attained in cell lines, we concluded that this construct was most suited for future works that involved the transduction of primary human T cells.

2.5.04 The effect of transfection plasmid ratios on LV producer cells and transduced target cells

Our lab uses a four plasmid transfection system consisting of Gag/Pol, Rev, VSV-G, and the cloned plasmid for our transgene of interest to generate self-inactivating second generation lentiviral vectors. This strategy has consistently produced high titered LVs that have been used in numerous studies [336, 361]. Previous optimization studies concluded

that a respective 1:1:1:3 molar ratio for the aforementioned plasmids produced the highest titers of virus (un-published data). Nonetheless, research indicates that altering this plasmid ratio yields different titers of LVs [362, 363], and so we hypothesized that different plasmid ratios would produce different titers of the EKCOU and EKCOU-tCD271 constructs. We observed no such difference in titer for the subsequently generated LVs, but we did notice that LVs produced from a 1:1:1:1 molar ratio produced significantly more robust CD271 expression in transduced Jurkats. Interestingly, this was true even with comparable numbers of infectious units. There are several possible explanations for this phenomena. First, it is possible that transfecting producer cells with less DNA induces less of an immune response in producer cells, and that subsequently fewer inflammatory factors are packaged into the LVs. It has been shown that dsDNA can induce a strong a STING mediated Type I interferon response in HEK 293T cells [364], and stands to reason that this response would have a more potent effect on T-cell derived Jurkat cells compared to HEK 293T cells, which are derived from kidney fibroblasts. Alternatively, it is also possible that producer cells that were transfected with of equal plasmid molar ratios spend less time expressing transgene DNA and therefore express more VSV-G protein on the surface of the subsequently generated LVs. This would mean that the LVs have more receptors to bind LDLR, which may be the limiting step in Jurkat transduction but not HEK 293T transduction. Future studies will have to explore the mechanism at work, but for the purposes of moving our FHL3 gene therapy project forward, we concluded that constructs for FHL3 gene therapy ought to be produced using a 1:1:1:1 molar ratio of the four transfection plasmids.

2.5.05 A comparison of different lentiviral vector purification methods

Lentiviral vector preparations produced from transfected HEK 293T cells are often contaminated with tubulovesicular structures (TVS). As the name suggests, TVS are long tubular structures that are produced from HEK 293T cell LV transfections in which VSV-G is the envelope protein. TVS bind nucleic acid and co-purify with LVs through ultracentrifugation. In the final LV product, they are often more abundant than LVs. These TVS can stimulate innate immune sensors in transduced cells, in particular the TLR9 receptor via producer-cell genomic DNA and the TLR7 receptor via LV RNA genomes. The result is an inflammation response which can have varying degrees of impact on the health of the transduced cells and the ultimate success of the gene therapy [365].

As previously observed, transduction of HSPCs with our LV preparations exacted a heavy toll on cell viability, and suspecting this to be the result of TVS or other cellular debris, we aimed to optimize a protocol for purifying our LV preparations. We began by adding a 10% sucrose cushion to the ultracentrifugation purification step for the purposes separating cell debris and TVS from the final LV product. Previous studies have found this sucrose cushion to be very a very cost-effective concentrating and purifying LVs [366]. We hypothesized that this cushion would decrease the titer of the final LV product, but observed no such change. This indicates that HEK 293T cells which are used for titering are not negatively affected by debris which can be excluded using a ten percent sucrose cushion.

Still wanting to mitigate our risk for TVS and host cell contamination, prior to transduction, we used a table-top centrifuge to briefly spin down the LVs and noted a pellet at the bottom of the tube. This was surprising given that our SOP for LV preparation called

for filtration of harvested producer-cell supernatant using a 0.22 micron filter. Therefore, presumably the pellet at the bottom of the tube consisted entirely of cellular debris that was no larger than 0.22 micrometers. Although small, this filter size could still allow the passage of TVS. However, no difference in transduced cell viability was observed, indicating that perhaps the pellet was bits of producer cell membrane or other non-toxic debris, and that the TVS could still be suspended with the LVs even after brief centrifugation. Furthermore, this centrifugation produced much lower transduction efficiency in Jurkats compared to un-spun LV, indicating that perhaps a large percentage of our transduction comes from LVs that are still bound to fragments of producer cell plasma membrane. If true, this would imply that perhaps the majority of our transduction comes not from VSV-G mediated attachment of individual LVs to target cell membranes, but rather it is the result of endocytosis of the associated larger cellular debris.

To conclude, we observed no real benefit of using a sucrose cushion as an additional purification step, despite previous studies from our lab that indicate a benefit in producing other vectors [367]. Furthermore we concluded that additional spinning of the virus prior to transduction should be avoided in order to maximize transduction efficiency. We acknowledge that there are still many alternative methods of LV purification to be explored, including tangential flow filtration (TFF) and chromatography [368]. While for now our results yielded an LV isolate suited for research purposes, further work must be done to optimize and LV that can be used in definitive pre-clinical testing.

2.5.06 Primary human T cells do not maintain their CD271 expression over time

Primary T cells from healthy human donors showed up-regulation of CD271 five days post-transduction, but unlike Jurkats this up-regulation constituted an overall shift rather than the formation of distinct CD271+ and CD271- populations. We attempted to create a distinct population of CD271+ primary T cells using the same magnetic sorting procedure that we had previously described in Jurkats, with the only change constituting an increase in time post-sorting in order to accommodate the slower rate of T cell division compared to Jurkats. Surprisingly, by nine days the T cells had lost all tCD271 expression, even though copy number showed consistent integration. Several reasons might account for this. First, it is possible that the CD271 which we observed on the T cells five day post-transduction was either left over from the fusion events of CD271+ LVs with the T-cell membrane, or that the tCD271 was the result of expression of non-integrated DNA. If so, it would mean that CD271 expression would diminish with time as T-cell membrane turn-over occurred. The second possibility is that after a certain number of days transgene expression was lost, possibly as a result of T cell exhaustion. We concluded that while the copy number data indicated that transduction had indeed occurred, further studies will have to be conducted to characterize transgene expression over time.

2.5.07 Microfluidics based transduction

Given the monetary expense and the time investment necessary to produce LVs, a developing trend within the world of *ex vivo* gene therapy is to perform transductions in microfluidics devices. Previous studies estimate that at 37 °C, LVs in culture media only travel about 600 micrometers from their original starting point through Brownian motion

[296, 369, 370]. This small distance is insufficient for most conventional transduction protocols in which LVs must travel millimeters to centimeters in order to reach the target cell [296]. As the name suggests, microfluidics devices allow the loading of target cells and LVs into very small volumes (as little as 20 microliters) in order to maximize the likelihood of attachment and fusion between cells and LVs. This approach has worked well for transduction of human and mouse cell lines, T cells and HSPCs [296]. Therefore it was surprising when our primary human TCs that were transduced with the EKCOU-tCD271 vector did not show any increase in transduction efficiency. If anything, a dip in transduction efficiency was observed, although this difference was not significant and copy number analysis still showed that integration into microfluidic transduced cells occurred. We hypothesize that as previously discussed, our LV preparations were still contaminated with TVS and other cytotoxic cell debris, and therefore the concentrating of these factors into fluid around target cells caused cell death or a decrease in cell growth either directly or indirectly as a result of an inflammatory response. Future work might investigate the released cytokine and transcriptional profiles of microfluidics transduced cells, and of course transduction with cleaner viral preparations.

2.5.08 The impact of Munc13-4 expression on transduction efficiency

We wondered if *Jinx* HSPCs, which lack functional Munc13-4 protein, might have been inefficiently transduced as a result of some intracellular trafficking deficiency. Our LVs were pseudo-typed with VSV-G, and therefore these LVs attach to low density lipoprotein receptor (aka LDL receptor or LDLR) expressed on the plasma membrane of target cells [371]. The LVs are subsequently internalized via clathrin-mediated

endocytosis, and they release their genomic contents into the cytosol via pH-dependent fusion of the LV membrane with the endosomal membrane [346-348] . Several years ago it was discovered that plasma-membrane localized Munc13-4 concentrates in lipid rafts that are subsequently re-internalized by AP-2/clathrin dependent endocytosis [209]. Furthermore, it was discovered that endosomal maturation is regulated by the interactions of Munc13-4 syntaxin 7, and VAMP8, and that a lack of Munc13-4 protein resulted in increased numbers of enlarged late-stage endosomes [248]. Therefore, although most of the literature on Munc13-4 protein function have focused on its role in exocytosis, we hypothesized that Munc13-4 plays a role in endocytosis, and therefore the lack of functional Munc13-4 protein in our *Jinx* mice would negatively impact their cell's abilities to be transduced with VSV-G pseudotyped LVs.

Importantly, in our hypothesis, we conceded that this this block to cellular transduction was likely not absolute, given that our lab as well as other groups have transduced Munc13-4 deficient cells [165, 166, 168]. However, even at MOIs of 100, it is difficult to obtain robust transduction in Munc13-4 deficient cells, especially when comparing to the transduction efficiency of those same cells with a measles H/F pseudotyped lentiviral vector [165]. Such a discrepancy could possibly be explained by how H/F and VSV-G take advantage of different endocytic pathways to mediate entry to a cell. While VSV-G utilizes a clathrin-mediated, dynamin dependent endocytic pathway, H/F induces a dynamin-independent pathway akin to micropinocytosis [372].

To test our hypothesis, we used the Munc13-4 small molecule inhibitor Bexin-1 to determine if Munc13-4 exerts an effect on transduction efficiency. Bexin-1 is one of the benzothiazol exocytosis inhibitor family of drugs (aka Bexins), which are defined by their

inhibitory activity on Munc13-4 protein. Bexin-1 inhibits the C2 domain of Munc13-4, preventing the protein's association with the plasma membrane [241]. This drug has been shown to affect exocytosis in mast cell lines, and we were curious if it would exert a similarly dramatic effect on the transduction of Jurkat cells as a result of interference with clathrin-mediated endocytosis [241]. However, based on observations of GFP expression in transduced Jurkat cells, there was no statistically significant difference between Jurkats that were treated or un-treated with Bexin-1 during transduction. This allows us to conclude that Munc13-4 protein is not necessary for efficient transduction with VSV-G pseudotyped LVs in Jurkats. While the alternative result would have been interesting, overall this is great news for the future development of an LV-based gene therapy for FHL3 patients because it means that Munc13-4 does not counter-act its own replacement gene therapy.

2.5.09 A comparison of different transduction enhancers

Polybrene

Transduction of primary human T cells in the presence of polybrene exerted no significant increase in transduction efficiency. This was surprising given that polybrene's long-standing reputation as a transduction enhancer [305, 307]. We suspect that owing to the small volumes that were used for transduction (100 microliters) and that fifty percent of that transduction media was concentrated LV preparation, enough contact was already made between LVs and target cells. If that were the case, then adding polybrene to the reaction would have a negligible benefit towards enhancing transduction. If that is true, then we hypothesize that transduction enhancement would only be observed when larger transduction volumes are used. This also would explain why polybrene has been shown to

have no added benefit to microfluidic transduction (unpublished data). While polybrene did not have a deleterious effect on transduction efficiency, it has been shown to have negative impacts on the viability and proliferation of cells [307]. Therefore we concluded that polybrene was not necessary for our transduction protocols, and unless otherwise noted all subsequent transductions were executed without it.

Cyclosporine H

The cyclosporines are a family of immune-suppressants that act by complexing with cyclophilin in lymphocytes and inhibiting the phosphatase activity of calcineurin and subsequent activation of NFAT associated transcription factors [373]. It was recently discovered that cyclosporine H enhances VSV-G pseudotyped LV transduction of human T cells and human and mouse HSPCs [304, 333]. The proposed mechanism of action behind this transduction enhancement is that cyclosporine H inhibits interferon-induced IFITM3, which normally exerts antiviral activity by restricting the fusion of endocytosed virions with the endosome membrane. The result is that internalized virions on LVs remain trapped within the endosomes [332]. Cyclosporine H degrades IFITM3, thereby overcoming restriction of viral entry to the HSPCs [304].

A characteristic of HLH patients is that they have high levels of circulating IFN-gamma [88]. We therefore hypothesized that HLH patients would have particularly high levels of IFITM3 and therefore that an HLH patient's T cells might be particularly resistant to transduction. To test this hypothesis, it was first necessary to re-capitulate the cyclosporine H enhancement of transduction efficiency in healthy human T cells. However, treatment of primary human T cells with cyclosporine H exerted no effect on

transduction efficiency when compared to un-treated cells. We hypothesized that this might be because these cells did not have substantial IFITM3 expression, so in a subsequent experiment we treated some cells with IFN-γ. However, no statistically significant decrease in transduction efficiency was observed in cells that had been transduced in IFN-γ containing media, and no subsequent rescue of transduction efficiency was observed in cells treated with both IFN-γ and cyclosporine H. It is disappointing that in our hands cyclosporine H does not enhance transduction, but perhaps further optimization of the dosing and kinetics of drug, IFN-γ, and virus exposure might help us replicate the success reported by others.

2.5.10 Optimization of T-cell degranulation assay

Upregulation of CD107a is a classic marker of degranulation in T cells, and an assay that measures CD107a upregulation is a tool commonly used throughout HLH research, [165, 166, 168, 374]. However, many variations of this protocol exist (Table 5). In order to proceed with our own FHL3 gene therapy project, it was necessary to test the different parameters of this protocol and create an optimized version that would reliably work in our hands.

Table 5 Variations in TC degranulation protocols.

Note that similar protocols exist for NK cells [170, 210, 375], platelets [214] and neutrophils [248], but this table confines itself to only those relating to T cells.

Stimulant	**Hours of stimulation**	**Cell-trafficking blocker**	**Cell source**	**Observed increase in CD107a from un-stimulated to stimulated conditions**	**Reference**
CD3 antibody (3 μg/mL)	3 hours	None	Mouse splenic CD8+	35% in WT mice, 5% in *Jinx* mice (no data for un-stimulated cells is presented, but it is presumed to be 0%)	[166]
CD3 antibody (30 μg/mL)	4 hours	None	Human peripheral blood CD8+	55% for healthy human controls, 10% for FHL3 patients	[165]
CD2/CD3/CD28 beads	4 hours	None	Human CTLs	Un-specified for health controls, 5-10% for FHL3 patients	[168]
PMA/Ionomycin	4 hours	Golgi-Plug (Brefeldin A)	Mouse splenic CD8+ T cells	7.5% in WT, 0.5% in *Jinx*	[170]
Plate bound CD3 antibody (10 μg/mL) and plate-bound anti-CD28 antibody (40 μg/mL(	4 hours	None	Mouse splenic CTLs	35% WT, 10% *Jinx*	[376]

An important consideration for this degranulation assay and using the *Jinx* mouse as a model for FHL3 disease in general is that the Munc13-4 block to degranulation is not

perfect. As noted in Table 5, other studies have all observed consistent but small increases to stimulated *Jinx* T cells as a result of stimulation. This can be attributed to how mouse CTLs express both Munc13-1 and Munc13-4 protein, and in the absence of Munc13-4, Munc13-1 can compensate for some but not all of the lytic degranulation and cytotoxic activity prompted by CTL stimulation [376].

We elected to use CD3 and CD28 antibody for our degranulation assay because of how it caused a robust increase in CD107a expression in WT mice but not *Jinx* mouse CD8+ splenocytes. While PMA/Ionomycin also induced an up-regulation of CD107a in WT but not *Jinx* mouse CD8+ cells, the difference between these two sample groups was less pronounced. Interestingly, PHA-L induced an increase in CD107a expression in both WT and *Jinx* mice, potentially indicating that PHA-L stimulation prompts Munc13-1 biased degranulation.

We hypothesized that we could increase the difference in CD107a upregulation between WT (positive control) and *Jinx* (negative control) mice by increasing the time spent stimulating these cells. While the time course experiment did indicate a trend towards increasing CD107a expression over time, longer stimulation also exacted a hefty toll on cell viability. In light of this information and how others report stimulating their cells for as little as three hours with robust results [166], we concluded that stimulation for eight hours was sufficiently robust for our degranulation assay protocol and would exert less cytotoxicity.

Different T-cell degranulation assay protocols report varying uses of cell trafficking inhibitors such as Brefeldin A [170]. Brefeldin A blocks trafficking of proteins from the ER to the Golgi complex, leading to an accumulation of proteins in the ER/Golgi

complex. Consequently, brefeldin A normally is used to enhance intracellular staining protocols, though some protocols have also found that it can increase CD107a expression at the plasma membrane [377]. When we compared CD107a upregulation in cells treated or un-treated with monensin (aka GolgiStop)—a similar protein that inhibits intracellular trafficking and promotes accumulation of proteins in the ER/Golgi—we observed that monensin reduces the amount of CD69 expression and to a lesser extent CD107a expression. The extent of this effect depends at least in part upon if antibody is added before or after the eight-hour incubation period, with the latter option providing an extra boost to CD69 and CD107a expression. We concluded that both including an excluding monensin from a TC degranulation protocol are appropriate choices, but for our own experiments we elected to move forward with using monensin because it 1) produced very clean looking flow plots that looked similar to those of other FHL3 gene therapy researchers and 2) would allow us to examine intracellular protein expression in the future with minimal modification to our SOP.

Knowing that future studies would require the infection of *Jinx* mice to re-capitulate the FHL3 disease model [170], we hypothesized that LCMV-specific splenocytes would provide us with the most robust assay for comparing gene therapy modified mice to un-modified WT and *Jinx* controls. *Jinx* mice were infected with 2×10^5 PFU of LCMV Armstrong, and ten days after infection the mice were sacrificed and their splenocytes were subjected to the T-cell degranulation assay. We identified LCMV-specific T cells using CD44 (a memory T cell marker) and GP33 tetramers in quantities similar to those reported in other studies [378], but unfortunately this population was not consistently large enough to be reliably used for degranulation assays. However, we did conclude that CD44+ CD8+

cells, exhibited more robust degranulation responses to stimulation compared to the CD8+ CD44- T cell population, and therefore we decided to analyze CD44+ CD8+ cells in subsequent degranulation assays. We note that previous FHL3 gene therapy studies have only examined CD8+ T cells or bulk CTLs [165, 166, 168], and so we hope that this additional marker for memory CD8s will provide a more specific assay.

Finally, in anticipation of future gene therapy studies which would require monitoring gene therapy modified mice over the course of a minimum of twelve weeks, we sought to optimize the degranulation assay for murine peripheral blood. Such an assay would allow us to observe the kinetics of improvements to degranulation in conjunction with engraftment measured via copy number. While no other FHL3 gene therapy studies have performed the T-cell degranulation assay in mouse peripheral blood, the assay has been adapted to human peripheral blood [130]. Unfortunately we found that the same stimulation conditions which prompt degranulation and activation in mouse splenocytes do not have the same effect in CD8+ cells from the peripheral blood. Furthermore, sterile housed *Jinx* mice do not have many CD44+ CD8+ cells, making flow analysis less robust. While these results do not mean that a T-cell degranulation assay could not be optimized for mouse peripheral blood in the future (perhaps using blood from cardiac puncture as a source), for our purposes, we decided to restrict our analysis to the tried-and-true splenocyte T-cell degranulation assay.

2.5.11 Neutrophil degranulation assay

Although FHL3 disease is primarily characterized by dysfunction of CD8 T cells and natural killer cells, an examination of the clinical FHL3 literature shows that other cell

types are likely involved in the disease pathology. In particular, neutropenias (a sub-set of the characteristic cytopenias) often present in FHL patients, as do bacterial and fungal infections [72, 118, 245, 258]. This suggests dysfunctional neutrophils as another mediator of disease.

Munc13-4 has been shown to be essential in neutrophils for phagosomal maturation[219], the release of tertiary (gelatinase) granules [204], and bacterial cell killing [219]. Furthermore, neutrophils from the *Jinx* mouse model have already been characterized as having impaired degranulation in response to fMLF and LPS stimulation [220]. Given this information, we aimed to optimize a robust neutrophil degranulation assay that could be used as a measure of disease correction in gene therapy modified *Jinx* mice.

We began by isolating neutrophils from the bone marrow of WT and *Jinx* mice using a MACS neutrophil isolation kit from Miltenyi Biotec. Interestingly, though viability of the final neutrophil product was similar in WT and *Jinx* mice, a smaller fraction of the bone marrow from *Jinx* mice was present in the final enriched neutrophil product. The literature on neutrophil abundance in *Jinx* mice varies from study to study. In initial studies, *Jinx* mice were described as being more prone to neutrophilia rather than neutropenia [170]. Later reports found no difference in the abundance of *Jinx* neutrophils compared to that of WT mice [218]. Now we report a significant decrease in the abundance of neutrophils from our *Jinx* mice, possibly indicating that variations can occur between different regions and housing conditions.

In our subsequent flow analysis, we chose to define our neutrophils as being Ly6G positive. While this flow gating strategy is standard [379-381] we also recognize that

multiple flow gating strategies for neutrophils exist (Table 6) and that numerous subsets of neutrophils have been identified [382-385]. For our purposes we wanted to keep the flow gating strategy as minimal as possible in order to avoid selecting markers that require Munc13-4 mediated exocytosis. For markers of degranulation, we chose to examine CD63 (aka "LAMP3") as a marker for primary granule release[26] [204, 248, 338, 388], and CD11b and CD66a (CEACAM1A) as markers for secondary, tertiary, and specific granule release [383, 388]. These markers have been extensively used in neutrophil degranulation assays for human cells [383] as well as mouse cells [220, 248].

[26] Primary granules, also referred to as "azurophilic granules" are members of the lysosome-related organelle family 386. Dell'Angelica, E.C., et al., *Lysosome-related organelles.* FASEB J, 2000. **14**(10): p. 1265-78., contain myeloperoxidase, cathepsin G and other proteases and secretory proteins 387. Kuijpers, T.W., et al., *Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation.* Blood, 1991. **78**(4): p. 1105-11..

Table 6 Alternative flow gating strategies for neutrophils. We gated our neutrophils as being Ly6G+, but different murine neutrophils gating strategies exist.

Flow gating strategy for neutrophils	Reference
Ly6G	[379-381]
Ly6G, CD11b	[380, 389]
Ly6G+, F40/80-	[383]
$Ly6C^{+}Ly6G^{+}$	[389]
$CD45^{+}$ $F4/80^{-}$ $Gr\text{-}1^{+}$	[390]
$Ly6G^{Hi}SSC^{Int}$ or $Gr\text{-}1^{Hi}SSC^{Int}$	[391]
Gr1+, with further sub-setting in into PMN-I and PMN-II subsets based on expression of CD49 and TLRs	[385]

Table 7 A comparison of *in vitro* murine neutrophil degranulation assay protocols

Cell source	Stimulant	Stimulation period	Additives/ additional manipulations	Result	Ref
T and *Jinx* peripheral blood (cardiac puncture)	PS (100 ng/mL) and/or fMLF (1 μM)	30 minutes	None specified	fMLF: WT mice increase MPO expression 5 ng/mL, *Jinx* increase 1 ng/mL. No significant difference in upregulation of CD11a or CD11b for WT vs. *Jinx* PMNs. LPS: no observed increase in MPO secretion. No significant difference in upregulation of CD11a or CD11b for WT vs. *Jinx* PMNs. fMLF + LPS: WT increase MPO expression by 25 ng/mL, *Jinx* mice by 8 ng/mL. No significant difference in upregulation of CD11a or CD11b for WT vs. *Jinx* PMNs.	[220]
T and *Jinx* bone marrow derived neutrophils	1) fMLF (10 μM), with/without pre-incubation with LPS (10 ng/mL).	Up to 20 minutes with fMLF, 30 minutes with LPS.	Cytochalasin D in specified experiments	fMLF stimulation of WT PMNs significantly 1) increased the localization of $p22^{phox}$ vesicles to exocytic active zones, 2) increased the percent of vesicles integrated into the plasma membrane, 3) increased	[219]

	2) serum optimized *Pseudomonas aeruginosa* strain PK, (1 bacterium/10 PMNs) 3) 12-myristate 13-acetate (0.1 μg/mL)			ROS production compared to *Jinx* PMNs. fMLF + LPS increased extracellular ROS production in WT vs. *Jinx* mice. Stimulation with P. aeruginosa increased intracellular ROS production in WT vs. *Jinx* mice. *Jinx* PMNs showed reduced killing abilities of P. aeruginosa compared to WT PMNs.	
T and *Jinx* peripheral blood (cardiac puncture)	PMA/ionomycin (0.1 μg/mL)	1 hour, terminated with cold-shock	None	Both WT and *Jinx* neutrophils upregulated CD11b expression.	[218]
T and *Jinx* bone marrow derived neutrophils	fMLF (10 μM) CpG (5 μM)	fMLF: 10 minutes CpG: 1 hour	+/- GM-CSF (10 ng/mL) +/- CQ	fMLF: 1) No significant increase the number of LEs/cell in either WT or *Jinx* PMNs. 2) WT PMNs increased in CD107a granule diameter, while *Jinx* PMNs did not. 3) Both WT and *Jinx* PMNs up-regulate CD11b as a result of fMLF stimulation CpG stimulation: WT PMNs increase CD11b expression, but *Jinx* PMNs do not.	He, Johnson [248]
T and *Jinx* PMNs, bone marrow and cardiac puncture	LPS (100 ng/mL)	30 minutes	None	No increase in CD44 expression was observed in either WT or *Jinx* PMNs as a result of LPS stimulation.	[217]
T and *Jinx* PMNs, bone marrow	LPS (100 ng/mL) + fMLF (10 μM) OR PMA/Ionomycin (concentration un-specified) Or IL-8 (250 ng/mL)	LPS for 1 hour, fMLF stimulation time unspecified PMA/ionomycin stimulation unspecified. IL-8 for one hour, followed by a 30 minutes or 60 minute recovery time, cold stop	None	LPS + fMLF stimulation produced not change in BSA endocytosis in either WT or *Jinx* PMNs. PMA/ionomycin stimulated WT PMNs upregulated PM Rab11a, but *Jinx* PMNs did not. IL-8 stimulated WT and *Jinx* PMNs both experienced increases in CXCR2 endocytosis	[392]

We stimulated murine bone marrow derived neutrophils using fMLF, PMA/ionomycin, or lysates from live *E. coli* bacteria, and examined their subsequent degranulation marker expression over time. Interestingly, no stimulant induced an increase in the degranulation of primary or secondary granules in exclusively WT mice. Stimulation with fMLF or PMA/ionomycin prompted both WT and *Jinx* neutrophils to degranulate at similar levels, while interestingly the *E. coli* lysates induced no up-regulation in degranulation in either mouse. Similar to the results from other studies, we observed that ten minutes was the time point which produced the most robust upregulation in degranulation markers [248], and that by 60 minutes, most stimulated neutrophils experienced a decline back to baseline levels.

Knowing that neutrophil degranulation responses are dependent on the presence of extracellular calcium [393], we hypothesized that supplementing the neutrophil media with 160 micromolar concentration of calcium chloride might enhance the degranulation response, particularly in the languid *E. coli* lysate stimulated neutrophils. However, no such enhancement was observed, leading us to conclude that a lack of calcium was not inhibiting neutrophil degranulation.

Neutrophils are very easy to stimulate, and so we hypothesized that we were inadvertently stimulating our non-stimulated samples. Freshly isolated neutrophils that were simply place in cold PBS until the time of flow analysis showed reduced levels of CD11b, CD63, and CD66a compared to neutrophils that were unstimulated but cultured in media. Therefore, we concluded that the manipulation of neutrophils for the purposes of

this assay induces unwanted upregulation of degranulation markers. Hoping that we could simply isolate neutrophils, put them in PBS, and add fMLF to the stimulated sample groups, we performed this simplified protocol, but observed that none of the samples showed signs of being stimulated. Therefore we concluded that PBS alone does not provide the conditions necessary for neutrophil degranulation in response to fMLF.

Having established that we were inadvertently inducing degranulation in our neutrophils as a result of protocol manipulations, we sought to determine if there was a particular step that was causing this problem. First, we hypothesized that the pre-stimulation step in which neutrophils are incubated in HBSS media containing cytochalasin B and BSA was causing this unwanted degranulation. We systematically excluded each of these reagents from the pre-stimulation media, and looked to see if the subsequent degranulation markers maintained low expression compared to the PBS-incubated control. Exclusion of each component resulted in only very slight shifts in secondary granule expression, but no condition reduced the upregulation in CD63 expression. Therefore, we concluded that no single media component was activating the neutrophils, and instead it was probably the cumulative effect of pipetting, washing, and other necessary experimental manipulations.

Knowing that neutrophils required calcium from the media but also that they were easily activated from experimental manipulations, we hypothesized that removing cytochalasin B from the media would differentially dampen the degranulation response in WT and *Jinx* neutrophils. Cytochalasin B is used in some protocols to inhibit actin polymerization and enhance the uptake of calcium ions [394-396]. We therefore reasoned that eliminating it might prevent *Jinx* neutrophils from degranulating while allowing the

presumably more-degranulation competent WT neutrophils to still perform exocytosis. We tested samples with and without cytochalasin B, but found that neither WT nor *Jinx* neutrophils could degranulate without this additive. We concluded that even if sufficient calcium is present for neutrophil degranulation, cytochalasin B is necessary to facilitate calcium uptake and subsequent degranulation of neutrophils in response to fMLF.

Multiple studies have identified subsets of neutrophils that more readily mobilize to sites of inflammation and activate their effector immune functions [338, 385]. Cystic fibrosis researchers at Emory University characterized and developed an *in vitro* protocol to generate a subset of neutrophils dubbed "GRIM" neutrophils, so named for their enhanced granule release, immuno-regulatory function, and metabolic activities [338]. This GRIM phenotype is only adopted after the neutrophils migrate through layers of epithelial cells, and results in the hyper-exocytosis of neutrophil-elastase rich granules (primary granules). We hypothesized that transmigration of WT bone-marrow derived neutrophils would induce these cells to adopt the GRIM phenotype, and as a result would show robust CD63 degranulation. However, transmigrated neutrophils that were stimulated with fMLF did not show any increase in CD63 expression nor any other degranulation marker over those that were un-stimulated. However, the un-stimulated transmigrated WT neutrophils showed a substantial increase in CD63 expression compared to un-stimulated WT neutrophils that were not transmigrated. This could indicate that primary granules are released as a result of the transmigration process. A similar change was not observed in *Jinx* neutrophils, and this could therefore indicate that the transmigration assay alone (not transmigration in conjunction with stimulation) could be used to observe differences between WT, *Jinx*, and gene therapy modified *Jinx* mouse neutrophils.

We are not the first to observe that *Jinx* neutrophils still can degranulate despite not having functional Munc13-4 protein. Researchers from the Scripps Institute observed that stimulating bone marrow-derived neutrophils increased CD11b expression in both WT and *Jinx* neutrophils, with no significant difference between the two groups [218, 248]. This indicates that Munc13-4 either does not play a significant role in the exocytosis of secondary and tertiary granules, or that an alternative mechanism exists. This is surprising given that in human neutrophils, Munc13-4 is a limiting factor for the degranulation of secondary and tertiary granules [204].

In terms of primary granule degranulation, WT neutrophils exhibited a slightly more distinct upregulation of CD63 expression as a result of PMA/ionomycin and fMLF stimulation compared to neutrophils from *Jinx* mice. *Jinx* mouse neutrophils showed a tendency towards higher endogenous levels of CD63 and a slight increase in that expression after stimulation. This result corroborates with studies from the Scripps Institute which indicate that a lack of functional Munc13-4 protein inhibits azurophilic granule exocytosis [218, 220]. Unfortunately, this inhibition is not absolute. Therefore, we concluded that using CD63 expression in neutrophils was not robust enough to compare phenotypic correction in gene modified *Jinx* mice to un-modified WT and *Jinx* controls. Alternative mechanistic pathways that circumvent Munc13-4 mediated exocytosis have already been described in lung epithelial cells [206] and T cells [376], so it is likely that a similar redundant mechanism for neutrophil degranulation is responsible for our observed results.

Future attempts to develop a robust neutrophil-based assay to test FHL3 correction in *Jinx* mice might examine CD107a granules, which are expressed on late endosomes in neutrophils [248]. These LEs reportedly increase in size and number in

bone-marrow derived neutrophils from WT mice upon stimulation with fMLF, especially when compared to the LEs in neutrophils from *Jinx* mice which have high basal levels for both these metrics, but do not as robustly upregulate expression of degranulation markers [248]. For the purposes of the work presented here, we ultimately concluded that a neutrophil degranulation assay was not robust enough for characterization of future gene therapy modified neutrophils.

2.6 CONCLUSIONS

In summary, we created and characterized a lentiviral vector that we thought would be useful for continued characterization using *in vivo* testing and another for the transduction and enrichment of human T cells. To the former objective, we concluded that isolation of murine HSPCs was best done using the MACS Sca-1 isolation kit. To the latter objective, we discovered that our vector works well in the transduction and enrichment of cell lines, but that transduction of primary human T cells will likely require further work. Importantly, with regards to both gene therapy approaches, we found transduction enhancers or alternative transduction procedures were unnecessary given that these produce negligible increases in transduction efficiency. We also concluded that future experiments ought to manufacture LVs using a 1:1:1:1 molar ratio of transfection plasmids. We made brief mechanistic inquiries into the function of Munc13-4 protein, and concluded that secondary mechanisms likely help neutrophils compensate for Munc13-4 deficiency, and that Munc13-4 deficiency will not inhibit transduction with VSV-G pseudotyped lentiviral vectors. Finally, we optimized a T-cell degranulation assay for future use in characterizing gene therapy modified *Jinx* mice. Taken together, we concluded that using the *Jinx* mouse

model provided no additional hurdles towards developing an LV-based gene therapy for FHL3. At the start of these studies this was not obvious given that Munc13-4 was known to have multiple functions in different intracellular trafficking pathways, and because no previous research reports had described transduction of *Jinx* mouse cells. As we were completing these ground-work studies, researchers from the Paris Descartes University published the first papers on FHL3 gene therapy which described LV gene transfer into Munc13-4 deficient cells [165, 166]. We therefore chose to move forward with our own gene therapy studies and begin transplantation of *Jinx* mice with LV modified HSPCs.

2.7 AKNOWLEDGEMENTS

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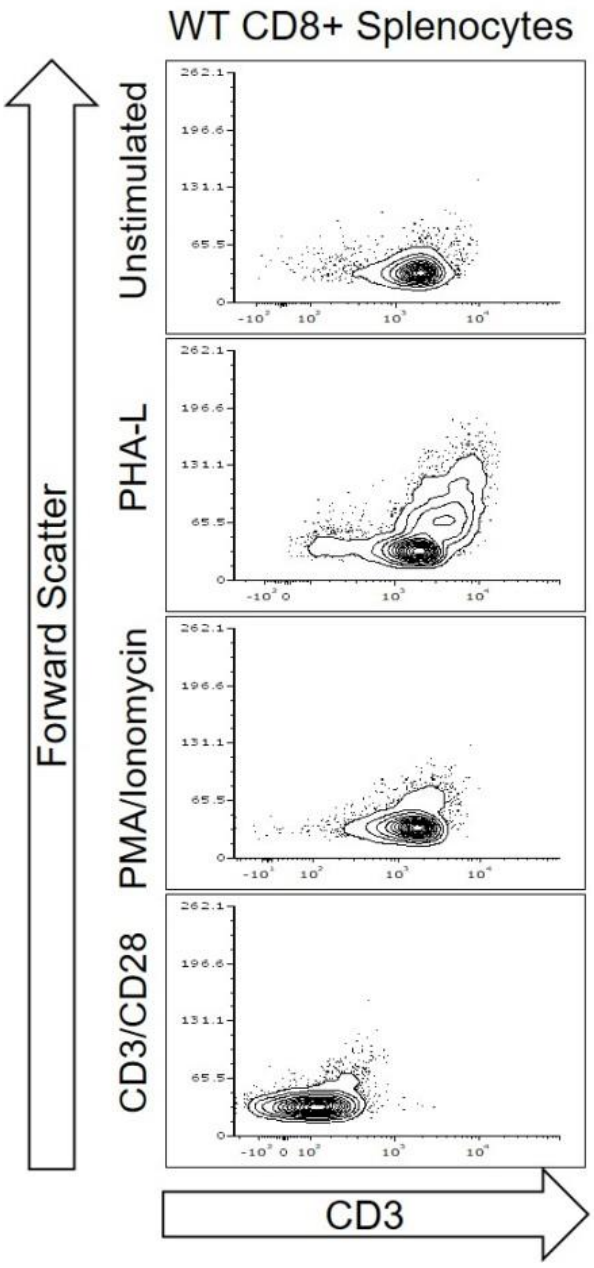
CBD and HTS are co-founders Expression Therapeutics and own equity in the company. Expression Therapeutics owns the intellectual property associated with ET3. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

2.8 Supplemental Figures

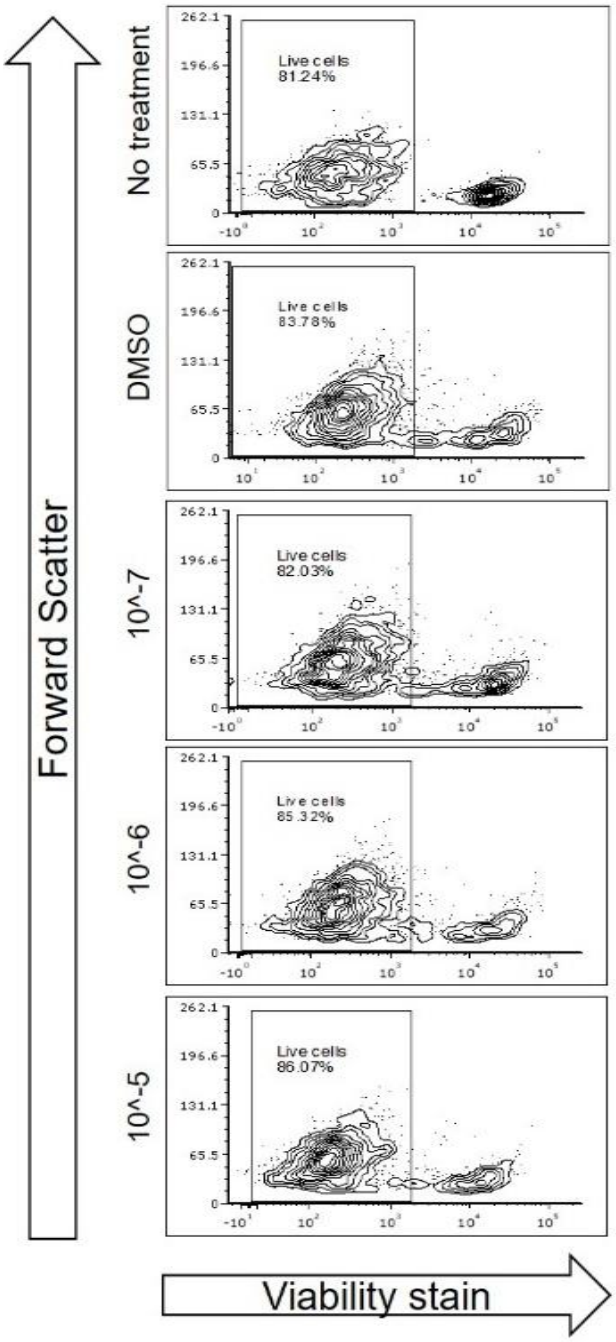
Supplemental Figure 1

CD3/CD28 stimulation causes internalization of plasma membrane CD3.



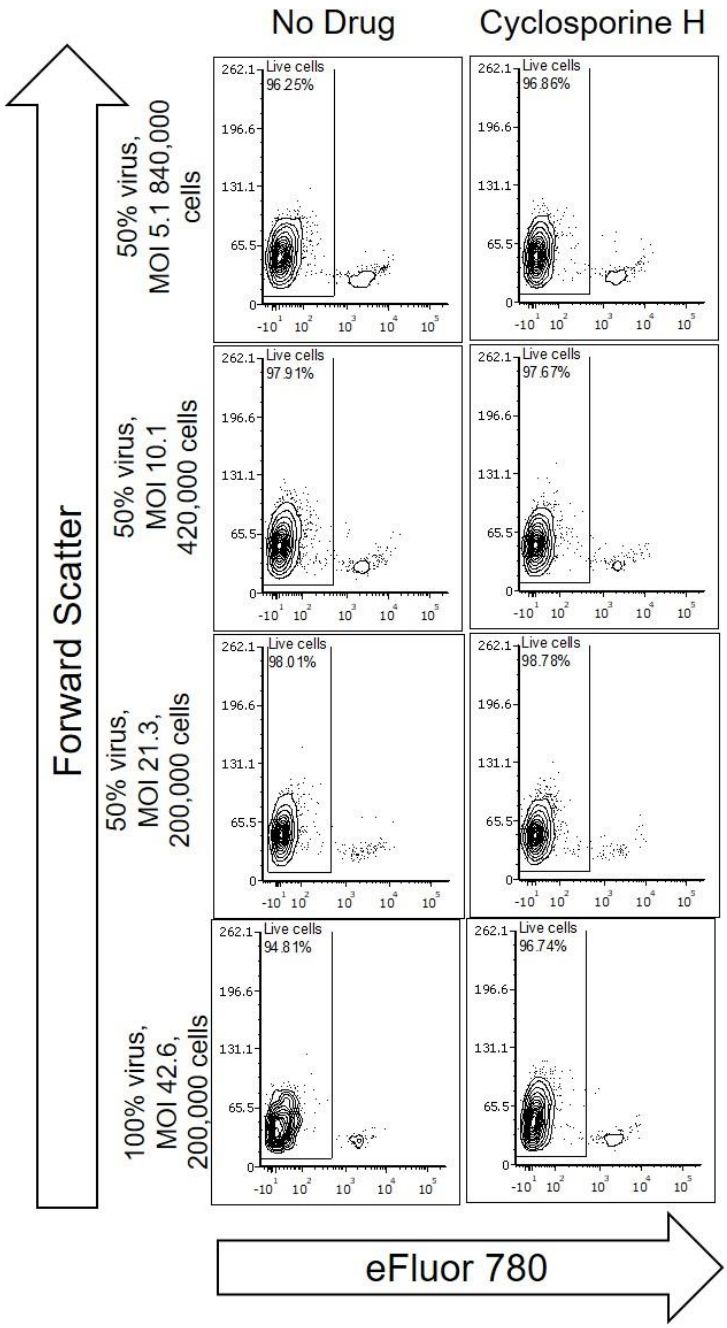
Supplemental Figure 2

The effects of cyclosporine H concentration on the viability of healthy donor T cells.



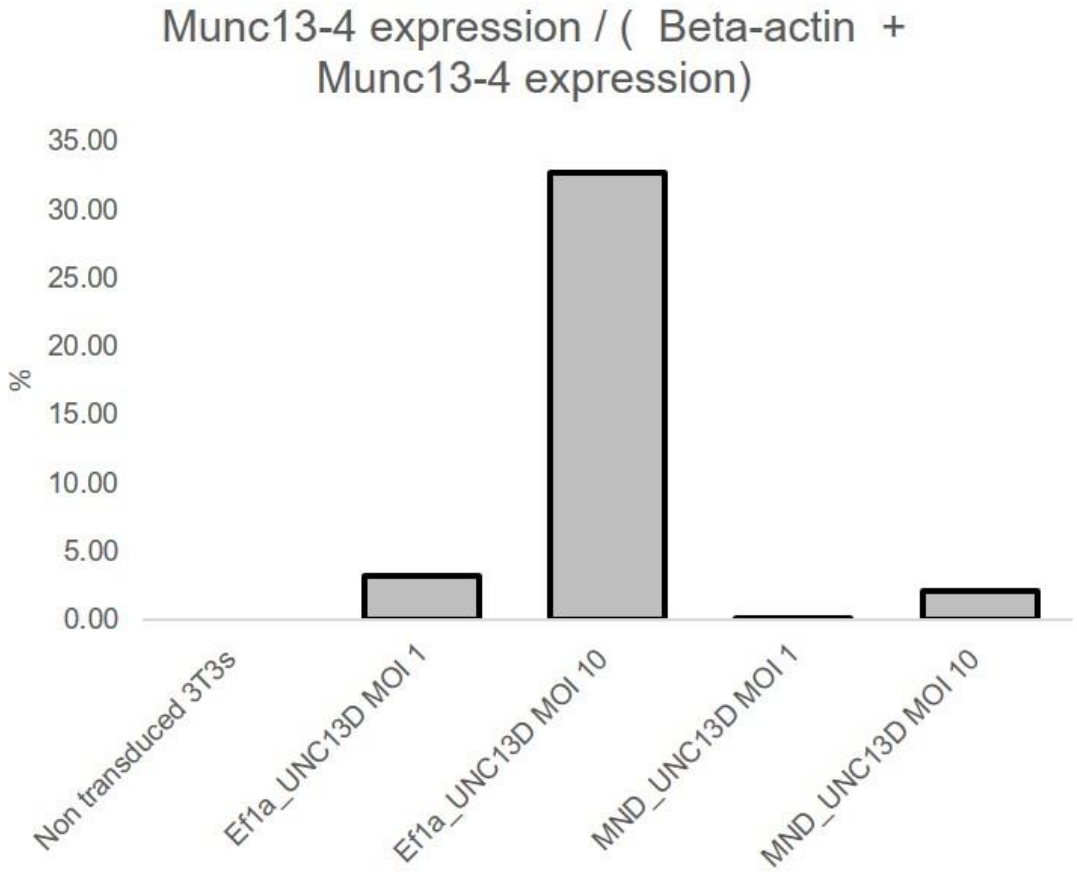
Supplemental Figure 3

Cyclosporine H does not exert an effect on the viability of transduced primary human T cells, even with varying transduction conditions.



Supplemental Figure 4

NIH 3T3 cells that were transduced with LV constructs that used an MND promoter as opposed to an EF1α promoter showed reduced expression of Munc13-4 protein as assessed by Western blot.



Chapter 3

Lentiviral gene therapy for familial hemophagocytic lymphohistiocytosis type 3, caused by *UNC13D* genetic defects

This work was published in *Human Gene Therapy*

Takushi, S., Paik, N. Y., Fedanov, A., Prince, C., Shields, J., Doering, C. B., Spencer, H. T., Chandrakasan, S.

Lentiviral gene therapy for familial hemophagocytic lymphohistiocytosis type 3, caused by UNC13D genetic defects

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Nayoon Paik and Sarah Takushi designed and cloned lentiviral vector plasmids. Andrew Fedanov produced and characterized the lentiviral vectors. Chengyu Prince and Shanmuganathan Chandrakasan performed the experiments depicted in Figures 21 and 22, and Sarah Takushi performed the experiments depicted in Figures 23-25. Jordan Shields transplanted the chimeric mice. Sarah Takushi, Chris Doering, Trent Spencer, and Shanmuganathan Chandrakasan conceived the experiments and edited the paper

3.1 ABSTRACT

Currently, the only curative treatment for familial hemophagocytic lymphohistiocytosis type III (FHL3) is hematopoietic stem cell transplantation (HSCT), and eligibility and response to this treatment is largely dependent on the ability to control inflammation prior to HSCT. Therefore FHL3 patients could greatly benefit from both a T cell based gene therapy that would involve the autologous adoptive transfer of gene therapy modified cells, and also from an HSPC based gene therapy which would provide long term correction of Munc13-4 deficiency in multiple cell types. In the previous chapter we designed and validated lentiviral vector plasmids with a codon optimized *UNC13D* gene—the gene absent or defective in FHL3 patients. Furthermore, we optimized protocols for target cell transduction and endpoint assays for testing an FHL3 gene therapy. In the following chapter we will apply this work towards characterizing a new, unique FHL3 gene therapy in both human T cells and murine hematopoietic stem and progenitor cells. In so doing we further validate the potential for FHL3 gene therapy, establish a target chimerism level, and show evidence of functional correction in both the hematopoietic stem and progenitor cells of the FHL3 mouse model and primary FHL3 patient T cells.

3.2 INTRODUCTION

Infants with FHL3 are born seemingly healthy, but upon encountering an immunological insult such as a viral infection (e.g. EBV), their lymphocytes cannot execute cytotoxic degranulation. This leads to a positive feedback loop of massive inflammation without clearing the infectious trigger [397-399]. Left untreated, the disease is fatal [119, 161]. To date, the only curative treatment available to FHL3 patients is allogenic hematopoietic stem cell transplantation (HSCT). However, inflammation must be adequately controlled prior to HSCT, and despite the new guidelines for intensification therapy made by HLH-2004, pre-HSCT mortality remains high at 19% [119]. For patients that do receive HSCT, the 5-year survival-rate has improved from 54% to 61%, with some variation occurring between different conditioning protocols [115, 132, 137-139, 154, 400-405].

Familial forms of HLH have been proposed as an excellent candidate for gene therapy intervention for several reasons. First, modification of a patient's own HSPCs would reconstitute the immune system with functional cytotoxic cells, theoretically correcting the HLH phenotype for life. Second, this autologous HSPC based gene therapy could eliminate the risk of graft vs. host disease compared to an allogenic HSCT. Third, patients that receive an HLH diagnosis would not have to wait to find a matching HSCT donor. Fourth, modification of a patient's own T cells could be used in autologous adoptive transfer to control infection and inflammation prior to HSCT [165, 166, 168]. Finally, FHL3 is one of the more common forms of primary HLH, second only to FHL2 [88, 406]. Consequently the pool of potential beneficiaries is expanded compared to other subtypes of familial HLH.

Although Munc13-4 is expressed in a variety of cell types (see Chapter 1), most of these are derived from hematopoietic stem and progenitor cells. To date, much of HLH research has focused on pathology arising from impairment of CD8 T cell and natural killer cell cytotoxic function [161], but it is worth noting that FHL3 patients also suffer from susceptibility to fungal infection [100], neurological symptoms [407], exacerbated response to lung infection [88, 407], and macrophage activation syndrome (MAS) [88]. In line with these clinical findings, Munc13-4 has been implicated in the exocytosis of lytic granules from cytotoxic cells [161, 210], tertiary and mucin-containing granules from neutrophils [204, 206], dense granules from platelets [232], and secretory granules from mast cells during regulated exocytosis [242]. All of these cell types could theoretically be modified by gene therapy corrected HSPCs. While additional cell types have been shown to express Munc13-4 protein the fact that HSCT results in FHL3 disease cure suggests that the critical importance of Munc13-4 throughout the body is confined to cells derived from hematopoietic stem and progenitor cells.

The first gene therapy study for FHL3 was recently published. These studies described transducing activated PBMCs from three FHL3 patients using either a vesicular stomatitis virus G protein (VSV-G) or a measles H/F pseudotyped lentiviral vector that encoded for the cDNA product from the human *UNC13D* gene. When NSG mice were transplanted with EBV-induced tumors and then infused with transduced FHL3 patient T cells, reduction in tumor size was observed [165]. The same group then showed transduction of hematopoietic stem cells from Munc13-4 null (*Jinx*) mice—the mouse model for FHL3—showed improvements to *in vitro* metrics and viral clearance as a result of gene therapy [166]. Shortly thereafter, a second group transduced FHL3 patient cells

with a gammaretrovirus based gene therapy construct and demonstrated that transduced cells had improved degranulation capacity and cytotoxic abilities [168]. Both groups used an Ef1α promoter to promote transcription of their transgenes, and both used a codon optimized version of the gene [166, 168]. Here, we describe several new lentiviral vectors, establish the minimum chimeric threshold needed for restoring adequate degranulation, and demonstrate that our described vector can be used to transduce both patient T cells as well as HSPCs from the FHL3 mouse model.

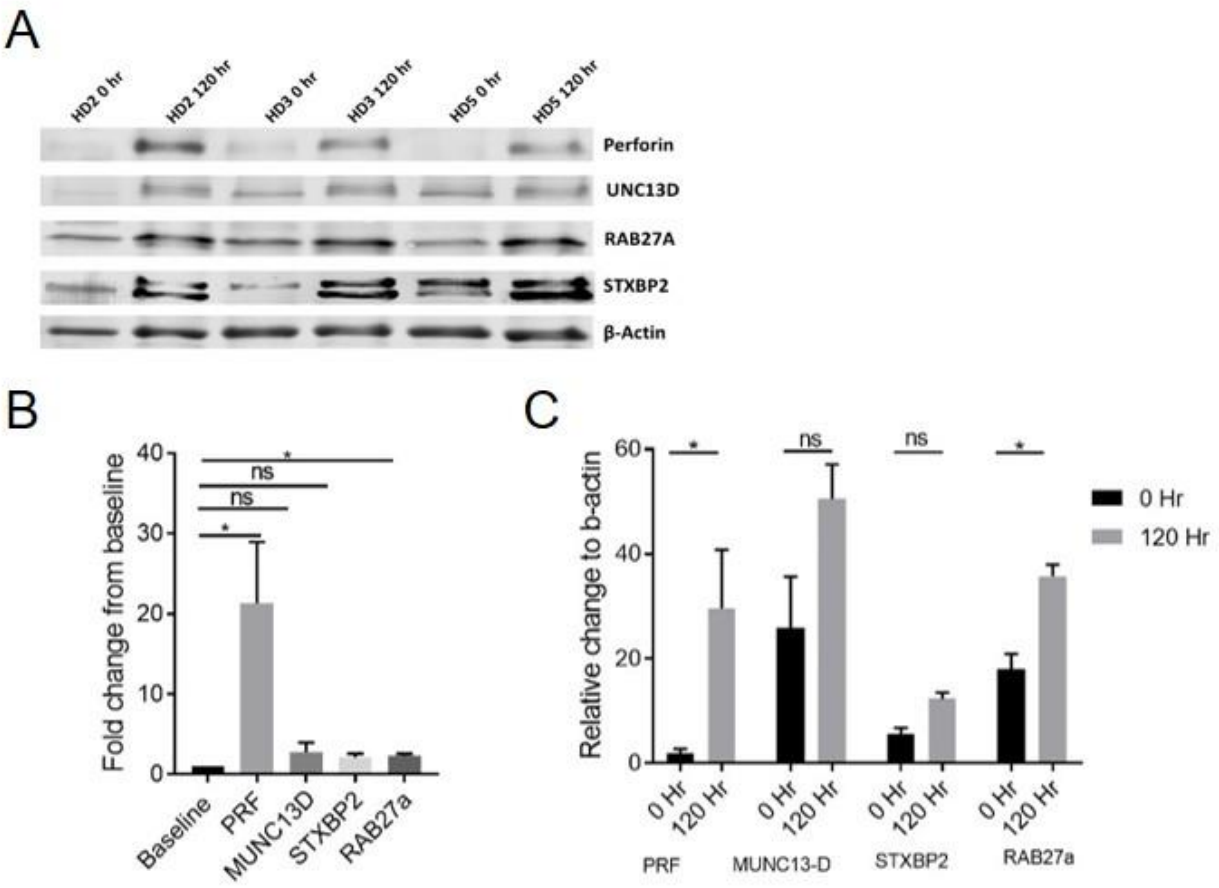
3.3 RESULTS

3.3.01 Stable expression of Munc13-4 in healthy donor cells

To begin, we set out to assess the dynamic change in expression of degranulation proteins from baseline to after immune activation. Western blot was performed on PBMCs from healthy donors to assess the expression of perforin, Munc13-4, Rab27a, and STXBP2 before and 120 hours after stimulation with CD3/CD28 beads (Figure 21A-C). The results indicate that perforin upregulates nearly 20 fold from its baseline levels in response to stimulation and activation, but that Munc13-4, Rab27a, and STXBP2 proteins were either not significantly different from baseline (Munc13-4 and STXBP2) or only 2 fold increased from baseline (Rab27a). This suggests that a constitutive promotor driven LV expression system would be appropriate for correcting FHL3-related degranulation defects.

Figure 21

A) Frozen PBMCs from three healthy donors (HDs) were thawed and cultured for five days in IL-2 supplemented media. Western blot was performed on lysates from these cells before and five days after stimulation with CD3 and CD28 beads. The blot was probed for proteins used in cytotoxic degranulation: perforin, Munc13-4, STXBP2, and Rab27a. β-actin was used as a loading control. Fold changes from baseline protein expression were calculated (B) as well as change relative to beta-actin (C). * indicates $p < 0.05$.



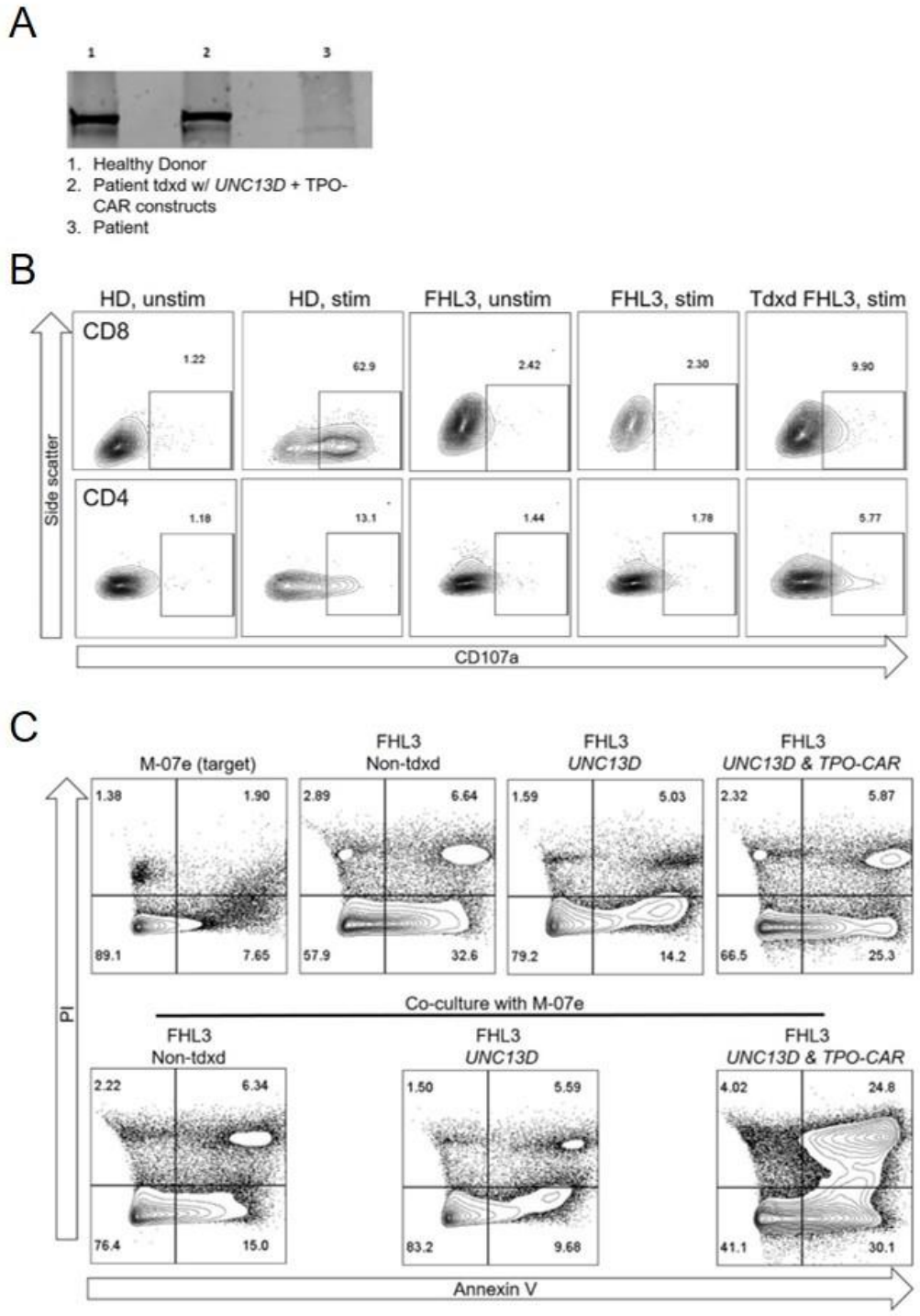
3.3.02 Transduction of FHL3 patient T cells

A lentiviral construct that expresses the cDNA product from the human *UNC13D* gene (GenBank Accession Number AJ578444.1) under the control of the Ef1α promoter was designed (Figure 2A). Stimulated PBMCs from an FHL3 patient were transduced

using a standard polybrene transduction protocol, and it was observed that these transduced cells expressed Munc13-4 (Figure 22A). Stimulation of the transduced cells with CD3 and CD28 induced increased expression of the exocytosis marker CD107a (LAMP-1) at the plasma membrane as compared to non-transduced patient cells, indicating functionality of the transgenic Munc13-4 (Figure 22B). The patients T cells used in this study was naïve to EBV or CMV infections, hence we could not assess cytotoxicity against viral antigen-producing cell lines. In order to assess the cytotoxic abilities of the transduced cells, we double transduced FHL3 patient PBMCs with both the *UNC13D* construct as well as a human thrombopoietin (TPO) chimeric antigen receptor (CAR) construct. This subsequently rendered the double-transduced cells cytotoxically competent and able to recognize cells expressing thrombopoietin receptor (MPL). A cytotoxicity assay performed by co-culturing these cells with MPL expressing target cells (M-07e cells) demonstrated an increase in cytotoxic function in transduced patient cells compared to non-transduced patient cells (Figure 22C).

Figure 22

A) PBMCs from an FHL3 patient were transduced using the *UNC13D* lentiviral construct, and subsequent Munc13-4 expression levels were assessed via Western blot five days after transduction. B) Stimulation of these cells using CD3 and CD28 antibody for four hours induced higher surface expression of CD107a (Lamp-1) in transduced patient cells compared to non-transduced patient cells. C) A cell killing assay was performed by culturing MPL-expressing M-07e target cells with either healthy donor, non-transduced patient, or patient T cells that had been double-transduce using the *UNC13D* and TPO-CAR constructs. Killed cells were identified as being positive for both propidium iodide (PI) and Annexin V.

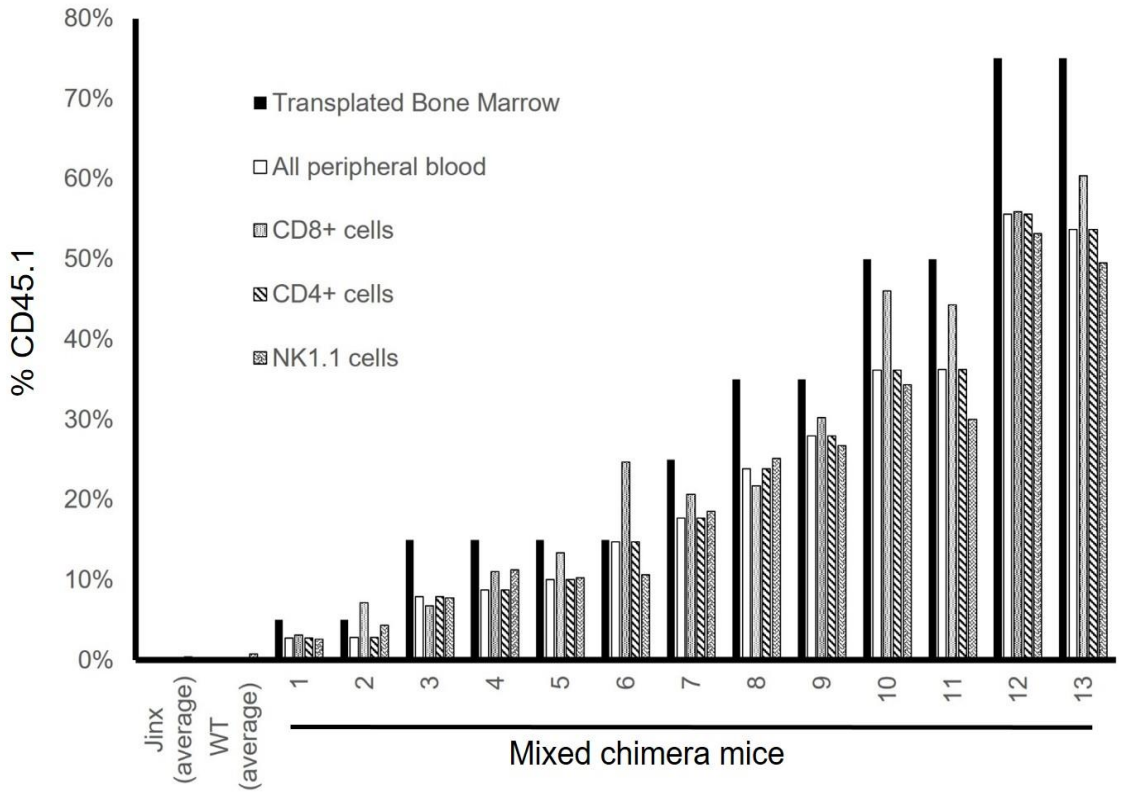


3.3.03 Identification of potentially therapeutic Munc13-4 expression levels

Jinx mice were irradiated twice with 550 rads of gamma radiation and transplanted with different mixtures of *Jinx* whole bone marrow (CD45.2+) and WT bone marrow (CD45.1+). Flow cytometry analysis showed that six weeks after transplant both WT and *Jinx* bone marrow engrafted in the recipient mice in proportions consistent with the original transplant. Furthermore, the ratios of CD45.2 to CD45.1 positive cells remained consistent across specific CD8+, CD4+, and NK1.1+ cell sub-populations (Figure 23).

Figure 23

Six weeks after transplant, flow cytometry analysis indicated that the chimerically transplanted mice maintained their chimerism within the bulk peripheral blood and also across the CD8+, CD4+, and NK1.1+ cell populations.

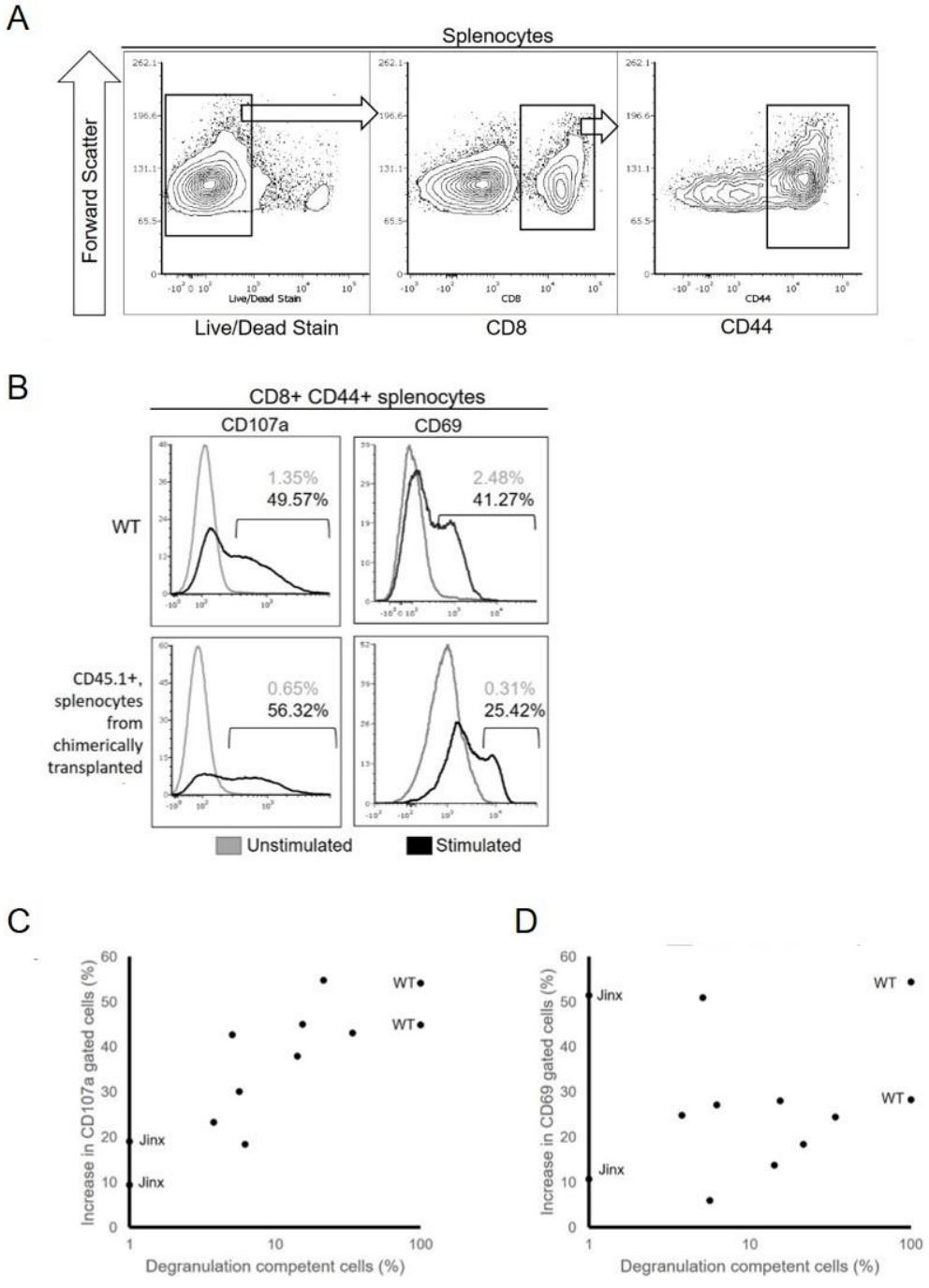


Chimerically transplanted mice were infected intraperitoneally with 2*10^5 PFU LCMV Armstrong to re-capitulate the FHL3 disease model. Eight days after infection, the mice were sacrificed, and their CD44+ CD8+ splenocytes were assessed for the ability to degranulate and activate when stimulated with CD3 and CD28 antibodies. The virulence of LCMV Armstrong stocks were previously confirmed (see Section 2.4.10 and Supplemental Figure 5). CD8+ and CD44+ cells from the mice transplanted with WT CD45.1+ bone marrow degranulated similarly to those from non-transplanted WT mice, indicating that HSCT did not affect degranulation capacity (Figure 24B). Consistent with previous

research, splenocytes from WT, *Jinx*, and chimeric mice all increased CD69 expression after stimulation (Figure 24D), corroborating the evidence that the presence or absence of Munc13-4 expression does not affect the mechanism of T-cell activation [170]. Mice with 15% of CD45.1+ mouse bone marrow showed CD107a upregulation comparable to that of a WT mouse (Figure 24C). Together, these data show that similar to studies done in the FHL2 gene therapy mouse model [408], a 15% correction should be sufficient to recapitulate the WT degranulation phenotype in FHL3 disease model mice.

Figure 24

A) Flow gating strategy. B) CD8+ CD44+ splenocytes from non-transplanted C57BL/6 mice and the CD45.1+ engrafted cells from chimerically transplanted *Jinx* mice showed similar levels of CD69 and CD107a expression before (gray) and after (black) stimulation with CD3 and CD28 antibodies, indicating no loss of cell activation or degranulation capabilities as a result of mixed chimerism conditions (Mann Whitney test, p = 0.2253 for CD69 expression, p = 0.8846 for CD107a expression.) Thirty weeks after transplant, the chimerically transplanted mice were infected intraperitoneally with 2*10^5 PFU LCMV Armstrong to re-capitulate the FHL3 disease model. Eight days after infection, the mice were sacrificed, and their CD44+ CD8+ splenocytes were assessed for the ability to degranulate (C, Spearman's rank-order correlation, two tailed: r = 0.814, p = 0.002) and activate (D, Spearman's rank-order correlation, two tailed: r = 0.2105, p = 0.508) as compared to the CD44+ CD8+ splenocytes from LCMV-infected WT and *Jinx* mice.

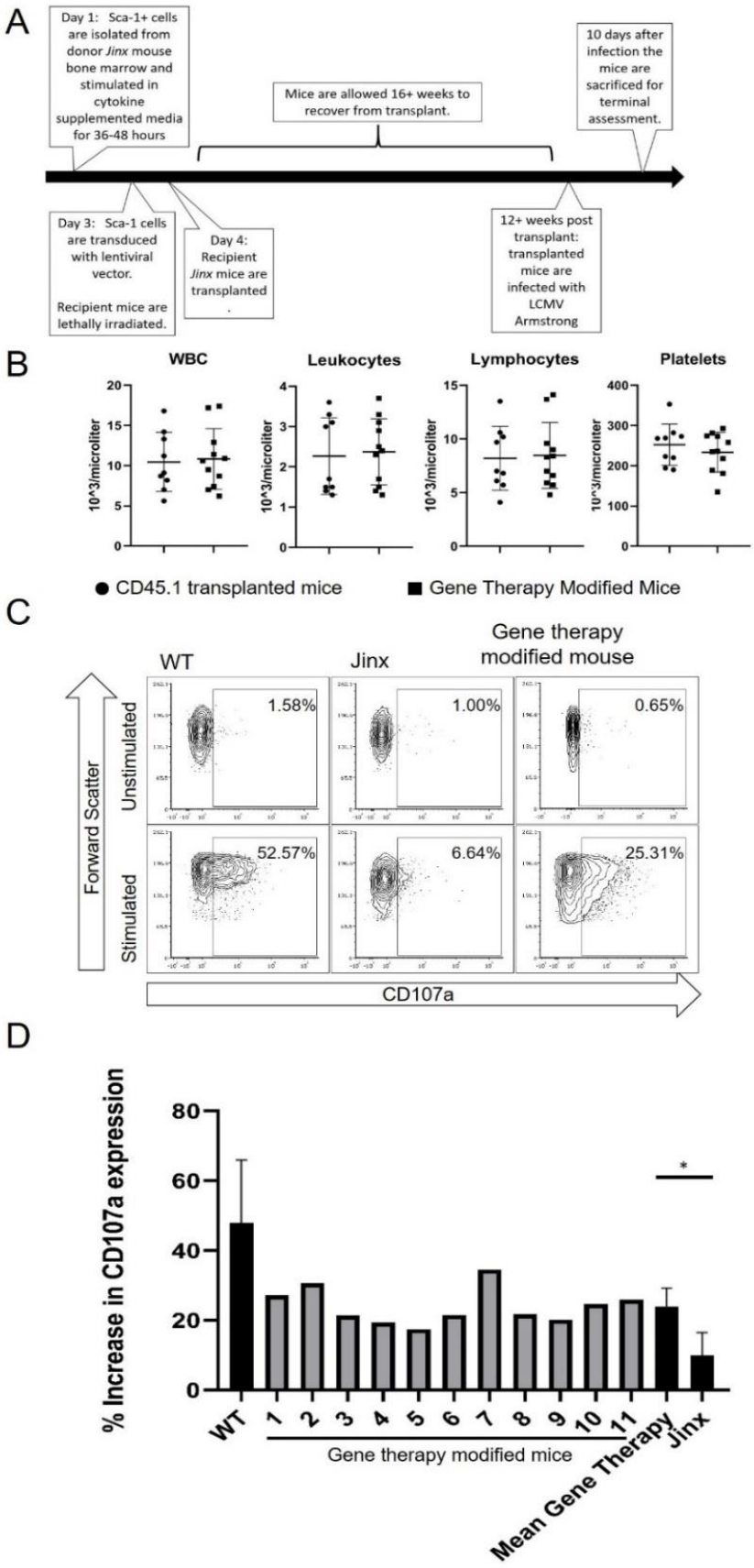


3.3.04 Gene transfer into the FHL3 disease mouse model

To determine *in vivo* efficacy of our gene therapy approach within the FHL3 mouse model, Sca-1 cells were isolated from donor *Jinx* mice and transduced with the Ef1α_Kozak_CodonOptimized*UNC13D* lentiviral vector. Subsequently, one million of these cells were transplanted into *Jinx* mouse recipients according to our previously published protocols (Figure 25A) [296]. Within twelve weeks mice had normal CBCs (Figure 25B) and an average copy number of 0.15 in the peripheral blood. Twenty weeks after transplantation, the mice were infected with $2 * 10^5$ PFU LCMV Armstrong in order to induce the FHL3 disease phenotype [170]. Ten days after infection, the mice were sacrificed, and degranulation assay results ascertained a statistically significant increase in CD107a expression in stimulated cells from gene therapy modified mice compared to non-transplanted *Jinx* mice (Figures 25C-D).

Figure 25

A) Diagram of experimental layout. B) After twelve weeks post-transplant, CBCs showed no difference between gene therapy modified mice and mice that were transplanted with CD45.1 Sca-1 cells in the WBC, leukocyte, and lymphocyte populations (unpaired, two-tailed t-test, p = 0.82, 0.84, 0.79 and 0.42 for WBCs, leukocytes, lymphocytes, and platelets respectively). C and D) Gene therapy-modified mice showed significantly greater up-regulation of surface CD107a after stimulation with CD3/CD28 antibody (two-tailed Mann Whitney U test, p = 0.005).


A
Day 1: Sca-1+ cells are isolated from donor Jinx mouse bone marrow and stimulated in cytokine supplemented media for 36-48 hours
Mice are allowed 16+ weeks to recover from transplant.
10 days after infection the mice are sacrificed for terminal assessment.
Day 3: Sca-1 cells are transduced with lentiviral vector.
Recipient mice are lethally irradiated.
Day 4: Recipient Jinx mice are transplanted.
12+ weeks post transplant: transplanted mice are infected with LCMV Armstrong
B
WBC
Leukocytes
Lymphocytes
Platelets
10^3/microliter
CD45.1 transplanted mice
Gene Therapy Modified Mice
C
WT
Jinx
Gene therapy modified mouse
Unstimulated
Stimulated
Forward Scatter
1.58%
1.00%
0.65%
52.57%
6.64%
25.31%
CD107a
D
% Increase in CD107a expression
WT
Gene therapy modified mice
Jinx
Mean Gene Therapy
*

3.4 DISCUSSION

Current treatment for HLH entails an allogenic HSCT, but the success of this procedure is greatly influenced by the ability to control disease-related inflammation prior to transplantation and availability of HLA-matched HSCT donors. Inadequate inflammation control increases the risk of a patient experiencing transplant related mortality (TRM), usually caused by infections, veno-occlusive disease (VOD), pneumonitis, graft failure and/or GVHD [78]. Only 53% of HLH patients achieve full resolution of their inflammation prior to HSCT, and of these patients, 13% experience disease reactivation [119]. Furthermore, the inability to control inflammation prior to HSCT accounts for 22% of diagnosed HLH patients dying before HSCT [119]. In light of sub-optimal outcomes, we and others have proposed that a gene therapy treatment by which a patient's T cells would be isolated, transduced with a corrective construct, and transfused back into the patient such that the transduced cells could function normally to resolve the cause of inflammation and if necessary, allow for additional HSPC based autologous gene therapy or allogeneic HSCT [165, 168].

3.3.01 Stable expression of Munc13-4 in healthy donor cells

LV gene therapy utilizing a constitutive promoter is better suited for genetic defects where the protein is consultatively expressed without much dynamic change upon activation. Our results showed limited to no significant change in expression levels of degranulation proteins Munc13-4, STXBP2, and Rab27a after TCR stimulation. Thus, these genetic defects are ideal candidates for constitutively expressing LV based gene therapy strategies. In contrast, perforin seems to be dynamically regulated, and therefore

perforin deficiency may be more adequately corrected though gene editing rather than gene addition strategies.

3.3.02 Transduction of FHL3 patient T cells

We were able to successfully augment Munc13-4 expression in activated patient T cells (Figure 22A), and restore their *in vitro* degranulation (Figure 22B). We note that our results corroborate those from other studies that have shown that T cells from FHL3 patients, despite having been subjected to a hyper-inflammatory environment, are permissive to lentiviral transduction [165, 168, 274]. Furthermore, using a dual transduction protocol we were able to restore cytotoxic capacity in FHL3 T cells, demonstrating the effectiveness of our lentiviral vector (Figure 22C) and the potential of a dual *UNC13D* and CAR-T transduction protocol as a therapeutic option to reduce viral-induced inflammation prior to a patient receiving HSCT.

3.3.03 Identification of potentially therapeutic Munc13-4 expression levels

Studies of HLH patients that received HSCT reported that between 20-30% bone marrow chimerism is protective against disease reactivation, although the correlation between donor chimerism and patient outcome is by no means perfect [156]. Similarly, gene therapy studies in the perforin-deficient FHL2 mouse model showed that as little as 10-20% engraftment of WT cells within the bone marrow of transplanted mice was sufficient to restore immune function [408]. We sought to determine what percent correction in a Munc13-4 null mouse model (*Jinx* mouse model) would be sufficient to

correct the Munc13-4 deficient degranulation defect. To this end, varying donor chimerism was established in *Jinx* mice using WT (CD45.1) and donor *Jinx* (CD45.2) bone marrow. This produced a gradient of intermediately degranulation-competent mice which maintained their relative proportions of WT vs. *Jinx* descendent cells across the CD8+, CD4+, and NK1.1+ cell compartments (Figure 23). We then infected these mice with LCMV Armstrong in accordance with the protocol from previous studies [170]. Degranulation assay results revealed that engrafted CD45.1+ cells up-regulated CD107a expression just as well as splenocytes from non-transplanted WT mice (Figure 24B), which allowed for the assumption that any increases in CD107a expression could be attributed to an increased proportion of CD45.1 cells. Subjecting splenocytes to the same degranulation assay showed a positive correlation between the proportion of engrafted CD45.1 cells and degranulation capacity (Figure 24C). No correlation was observed between the proportion of engrafted CD45.1 cells and cell activation (indicated by plasma membrane-bound CD69), which is to be expected given that both CD45.1 and *Jinx* cells are capable of activation in the presence of CD3 and CD28 antibody (Figure 24D) [170]. While the overall trends of this experiment are not surprising, what is important is the point at which the degranulation of the mixed chimera mice becomes comparable to that of WT mice. We assess that this occurs when about 15% of the peripheral blood consists of WT cells. This result is consistent with clinical data [156], as well as the finding that engraftment of 10-20% WT bone marrow in perforin$^{-/-}$ mice was enough to reduce serum levels of IFN-γ after LCMV infection [408], and that a perforin$^{-/-}$ mouse with as little as 8-15% of gene therapy modified CD8 T cells or greater than 30% gene therapy modified HSCs is protective against LCMV [92, 409]. While it would be an overreach to definitely say at this point

that FHL3 gene therapy need only strive for 15% correction in gene therapy modified autologous transplant, the existing body of evidence suggests that a subset of immunocompetent cells can compensate and adequately regulate the immune system.

3.3.04 Gene transfer into the FHL3 disease mouse model

Having investigated the percent of gene corrected HSPCs needed to engraft in a *Jinx* mouse, we sought to use our lentiviral vector to supplement *Jinx* HSPCs with a working copy of the *UNC13D* gene. Lethally irradiated mice received one million Sca-1 cells which had been transduced with our *UNC13D* lentiviral vector. By twelve weeks post-transplant these mice had normal complete blood counts compared to control *Jinx* mice that had received one million CD45.1 Sca-1 cells (Figure 25B). This indicates that gene therapy modification does not negatively affect engraftment potential.

Twelve weeks after transplantation, mice were injected with LCMV Armstrong to induce the well-characterized FHL3 disease phenotype [170]. The mean VCNs in the peripheral blood and bulk splenocyte population were respectively 0.15 and 0.30 copies per cell. For our degranulation assays, we specifically observed changes in CD107a expression within the memory CD8 T cell population (CD44+ and CD8+ cells). Upon stimulation, these cells showed significantly greater expression of CD107a compared to non-modified LCMV-infected *Jinx* mice. While an improvement, CD107a upregulation was less than that observed in WT mice, perhaps as a result of transgene integration into transcriptionally inactive genome sites. CD107a expression on cytotoxic cells is both an important diagnostic marker for FHL3 patients and an indicator of cytolytic ability using the perforin/granzyme pathway [410]. Therefore, the restoration of CD107a expression in our

degranulation assays indicates successful gene therapy modification and potential for FHL3 disease correction.

It is worth noting that a more clinically relevant therapy model would involve first inducing illness in *Jinx* mice via LCMV infection and then correcting it with HSPC based gene therapy. Furthermore, comparing the work presented here with previous FHL3 gene therapy mouse studies may be difficult owing to how we elected to infect our mice with LCMV Armstrong rather than the more virulent WE strain of LCMV, which was used in previous FHL3 gene therapy studies [166]. The LCMV Armstrong strain was used because of the well described CD8 T cell response in *Jinx* mice [170, 267]. Indeed, we were able to recapitulate previous findings showing a robust CD8 T cell memory response upon LCMV Armstrong infection, and gene therapy clearly improved degranulation of these cells. Future studies will focus on treating the myriad of additional effects that LCMV Armstrong has on *Jinx* mice such as elevated IFN-γ, enlarged spleens, and cytopenia.

Overall, a *UNC13D* lentiviral vector was shown to effectively modify FHL3 patient T cells as well as hematopoietic stem cells from the FHL3 (*Jinx*) mice. Furthermore, we showed that having 15% degranulation-competent cells is still sufficient to produce degranulation response comparable to WT. Therefore, a significant body of preclinical data support the pursuit of autologous HSCT or T cell lentiviral vector gene therapy as a transformative approach to FHL3 disease management or cure.

3.5 MATERIALS AND METHODS

Cloning

All cloning was done into an expression vector which has been FDA approved for a hemophilia A gene therapy products and which was supplied to us by Expression Therapeutics. In humans, *UNC13D* is comprised of 32 of exons [161], therefore in constructing our *UNC13D* lentiviral vector we used the sequence from the Homo sapiens unc-13homolog D (UNC13D) mRNA (NCBI reference Sequence: NM_199242.2). When translated, this nucleotide sequence yields isoform 1 of the Munc13-4 protein (protein Sequence NP_954712.1, UniProt identifier Q70J99-1). Codon optimization was performed by GenScript according to the algorithm previously described in our lab [335]. All clones were screened using restriction digests and sequenced by Genewiz.

Lentiviral Vector Packaging and Titering

Third generation, self-inactivating, VSV-G pseudotyped lentiviral vectors were constructed using a standard HEK 293T transfection protocol [336]. Specifically, HEK 293 T/17 cells were transfected with a four plasmid system consisting of the cloned, transgene-expressing plasmid and packaging plasmids provided by Dr. Denning at Expression Therapeutics for VSVG, REV, and GAG-POL proteins. The following day the media was replaced with DMEM, 1X with 4.5 g/L glucose, L-glutamine & sodium pyruvate, supplemented with 10% FBS. Supernatant from these transfected cells were collected on days two and three post-transfection. Viral vector was pelleted out of the supernatant by

overnight ultracentrifugation at 10,000 g, 4°C. Pelleted viral vector was subsequently filtered through a 0.22 micron filter and re-suspended in StemPro media.

All lentiviral vector preparations were titered by applying polybrene (aka "hexadimethrene bromide," 8 μg/mL, Sigma) and 3, 9, or 27 microliters of the new lentiviral vector onto HEK 293T/17 cells, incubating overnight, and then culturing in fresh media for five days. DNA was subsequently isolated from these cells using a Qiagen DNA Micro kit (Qiagen; 56304). Quantitative PCR was performed using Power SYBR Green Master Mix (ThermoFisher; 4367659) with RRE primers (Forward primer: TGG AGT GGG ACA GAG AAA TTA ACA, Reverse primer: GCT GGT TTT GCG ATT CTT CAA) to determine the average number of copies of viral vector DNA that were integrated per cell.

Transduction

All transductions were done in the presence of polybrene-supplemented media (8 μg/mL, Sigma). Cell lines used in *in vitro* experiments were transduced once with a single overnight application of lentiviral vector using the specified MOIs. HSCs that were used for transplant studies were transduced twice in 50-percent viral media (mean MOI of 42), with the first hit of virus being incubated on the cells overnight and the second hit of virus lasting for six hours.

Transplants

Whole bone marrow was flushed from the tibias and fibias from donor *Jinx* ("Unc13d^{Jinx}, unc-13 homolog D, Jackson Laboratories) or CD45.1+ mice. Sca-1+ cells were isolated according to manufacturer protocols using Sca-1 antibody (BD Biosciences; 553334), biotin-labeled magnetic beads (MACS Miltenyi Biotec; 130-090-485), and MACS magnetic separation unit (Miltenyi Biotec magnet and stand; 130-042-303 and 130-042-109 respectively). These isolated Sca-1 cells were then cultured in stimulation media consisting of StemPro nutrient-supplemented media (Gibco; 10640-019), recombinant mouse IL-3 (20 ng/mL; R & D; 403-ML), recombinant human IL-11 (100 ng/mL; R & D; 218-IL), recombinant human Flt3/Fc (100 ng/mL; R & D 368-ST), mouse mCSF (100 ng/mL; R&D 455 MC), L-glutamine (HyClone; SH30034.01), and penicillin and streptavidin (Lonza; 09-757F). These cells were subsequently transduced with the specified lentiviral gene therapy vector. Prior to transplant, recipient mice were kept on antibiotic water for one week and were lethally irradiated using two 550 rad doses of gamma radiation. For the transplant, recipient mice received one million transduced Sca-1 cells via retro-orbital injection and kept on antibiotic water for two weeks post-transplant.

Viral Copy Number (VCN) Analysis

The same RRE primers, SYBR Green Power PCR Master Mix, and PCR protocol that were used for the tittering of new lentiviral vectors were also used in the qPCR-based copy number analysis of transduced cells.

Infection

Jinx mice were infected via intraperitoneal injection with 2 x 10^5 PFU of LCMV Armstrong (generously provided by the Ahmed lab).

CFU Assays

One thousand cells/mL of Methocult Culture media (StemCell Technologies; 03434) were plated and incubated for 7-10 days. Colonies were subsequently counted, assessed using a viability stain, and the number of successfully transduced CFUs was determined either by copy number assay or flow cytometry on the pooled cells.

Western Blot

A standard Western blot protocol [337] was performed by blotting onto a PVDF membrane (BioRad; 162-0261) and staining for Munc13-4 (Abcam; Clone ERP4914, ab109113), and Beta-actin (GenScript; Clone 2D1D10, A00702). Secondary antibodies (IRDye 800 CW and IRDye 680, Licor; respectively catalogue numbers 926-32213 and 026-68072) were used to clearly distinguish between the Munc13-4 and beta-actin bands, and the blots were imaged using a Li-Cor Odyssey CLx imaging device using Image Studio Version 4.0 software.

Degranulation Assay

Homogenized splenic tissue was passed through a 70 micron filter, washed with PBS containing 5% FBS, and plated at 300,000 cells/FACS tube in RPMI containing 10%

FBS and 1% Penicillin and Streptavidin and supplemented with mouse IL-2 (10 ng/mL, Biolegend; 575402). Cells were then incubated for 30 minutes in either the presence or absence of 3 μg/mL of CD3 (BioLegend; Clone 145-2C11, 100301) and 1 μg/mL of CD28 antibody (BioLegend; Clone 37.51, 102101) to produce "stimulated" and "unstimulated" sample groups respectively. Subsequently, monensin (Biolegend; 420701) and CD107a antibody (BV421, BD; 121617) were added to all of the cultures, and the cells were incubated for an additional eight hours. Staining for analysis consisted of a live dead cell stain (ThermoFisher; 65-0865-14), CD8 (PE, BioLegend; 108739), CD44 (UV379, BD; 740215), and CD69 (FITC, BD; 557392). The chimeric transplant studies also incorporated the use of CD45.1 and CD45.2 antibodies (PerCP-Cy5.5, BD; 560580 and BUV737, BD; 564880 respectively) to distinguish between cells derived from *Jinx* and CD45.1 engrafted Sca-1 cells.

Cytotoxicity Assay

Frozen PBMCs from an FHL3 patient were thawed and stimulated using CD3 and CD28 (7 μg /mL and 0.5 μg /mL respectively) for 24 hours. These cells were subsequently double transduced with an MOI of 10 for both TPO-CAR and *UNC13D* lentiviral vectors according to the aforementioned transduction protocol. Five days post-transduction, these cells were co-incubated with M-07e cells. Cell killing was assessed by flow cytometry by looking for cell death markers Annexin V and propidium iodide (PI).

Primary Cell and Cell Lines

Healthy donor T cells were isolated by negative selection from donor PBMC sourced from leukoreduction filters, as previously described [340, 341]. The following cell lines were used in this study: NIH 3T3 cells (ATCC number CRL-1658), Jurkat clone E6-1 cells (ATCC number TIB-152), M-07e cells (ACC 104). MUNC13-D deficient T cells were isolated from FLH3 patients treated in the Children's Healthcare of Atlanta (CHOA), Emory University School of Medicine, Atlanta, Georgia, USA. The patient had biallelic genetic defects: a c.551G > A (p.Trp184*) mutation on one allele and a 253 Kb inversion on the other allele. The result was a lack of Munc13-4 protein formation. Informed consent and IRB approval was obtained for all studies involving human samples.

Cell Culture

The following reagents were used for cell culture: DMEM (Corning; 10-017-CV), RPMI (Corning; 10-040-CV), StemPro (Gibco; 10640-019), FBS (Atlanta Biologicals; S11150H), penicillin/streptomycin (Lonza; 09-757F).

Flow Cytometry

The following reagents were used throughout the various flow cytometry based experiments: eFluor 780 viability staining (ThermoFisher; 65-0865-14), PE mCD8 (BioLegend; 108739), UV379 mCD44 (BD; 740215), FITC mCD69 antibody (BD; 557392), BV421 mCD107a (BD; 121617), PerCP-Cy5.5 CD45.1 (BD; 560580), BUV737 CD45.2 (BD; 564880), Annexin V, and propidium iodide.

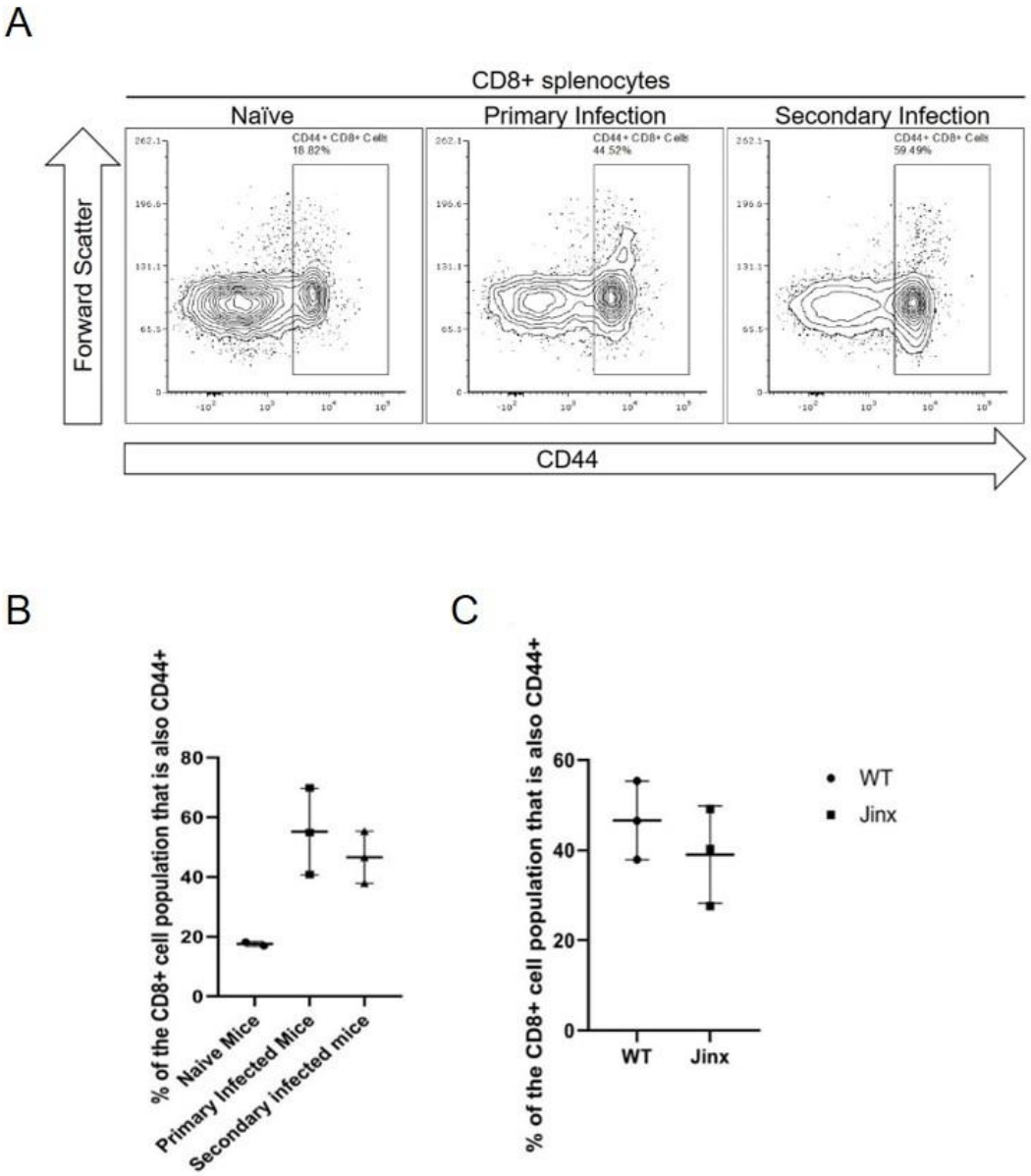
Statistical Analysis

All statistical tests (un-paired t-tests, Mann Whitney U tests, and Spearman rank-order correlation tests) were performed using GraphPad Prism Version 8.

3.6 SUPPLEMENTAL FIGURES

Supplemental Figure 5

A and B) WT mice that were infected with either one or two doses of LCMV Armstrong infection (2 x 10^5 PFU/mouse) developed greater numbers of CD44+ CD8+ cells in an antiviral immune response compared to LCMV naïve mice (unpaired t-test, p =0.04 and 0.02 for naïve to primary and naïve to secondary infections respectively). C) WT and *Jinx* mice do not differ in their increase in the number of CD8+ CD44+ cells as a result of a single 2 x 10^5 PFU/mouse infection of LCMV Armstrong (unpaired t test, p =0.396).



3.7 ACKNOWLEDGMENTS

Stocks of LCMV Armstrong were generously supplied by the Ahmed lab. Microfluidics devices where provided by the Lam Lab. Technical assistance for the Bexin-1 experiments was provided by Landon Clark.

Chapter 4

Conclusion

4.1 SUMMARY OF RESULTS

To date, there are cumulatively thousands of gene therapy clinical trials in some stage of development [411]. Taken together with the EMA and FDA approval for multiple gene therapy interventions, the field of gene therapy has regained the momentum lost from the setbacks of its early years. However, challenges such as the need for large quantities of vector, accommodating larger transgenes, limitations on transduction efficiency, obtaining sufficiently high levels of gene expression in target tissues, preventing off-target effects, complementation of gene therapy with other intervention protocols, as well as cost, still loom large.

Chapter 2 explores optimization strategies by which some of the aforementioned challenges could be overcome. First, we showed that LV production is optimized when the molar ratios of transfection plasmids are equal, and that additional centrifugation steps prior to transduction reduce transduction efficiency. Second, we compared methods of murine HSPC isolation and confirmed that MACS Miltenyi Biotec's Sca-1 isolation kit yielded the largest volume of healthy and transducible HSPCs. Subsequently, we optimized a streamlined transduction protocol by 1) comparing different volumes and vessels for transduction and 2) demonstrating that transduction enhancers did not bolster our transduction. We also showed that in human cell lines, the transduced cell population could be enriched by selecting for cell surface markers. In the interest of creating an LV gene therapy specifically for Munc13-4 deficiency, we also performed transductions using the Munc13-4 small molecule inhibitor Bexin-1, and concluded that Munc13-4 is not involved in the endocytic pathways that mediate the attachment and entry of VSV-G pseudotyped LV particles. Finally, we concluded that while the difference between WT and *Jinx*

neutrophil degranulation was not significantly different enough to provide a meaningful final measure for our gene therapy modified *Jinx* mice, a similar assessment of degranulation in murine CD44+ CD8+ splenocytes could be adapted to our experimental needs.

The work presented in chapter 3 applies some of the chapter 2 results towards creating an LV gene therapy product for either treating or curing FHL3. We first demonstrated that FHL3, FHL5, and Griscelli Syndrome Type II were ideal candidate diseases for developing gene therapies because of how the proteins which are deficient in these patients are all expressed constitutively in healthy T cells, even when those T cells are activated. We then transduced those FHL3 patient samples and demonstrated restoration of degranulation and cytotoxicity. Owing to the limited availability of these patient samples, we then re-focused our efforts on developing an HSPC based gene therapy solution for FHL3 using the Munc13-4 deficient *Jinx* mouse model. To this end, we transplanted lethally irradiated *Jinx* mice with different mixtures of *Jinx* and WT mouse whole bone marrow, creating a gradient of chimeric mice. LCMV infection of these mice and subsequent T-cell degranulation assays demonstrated that mice with at least 15% of WT bone marrow could re-capitulate a degranulation response similar to that of WT mice.

Now knowing that we would likely not need to modify one hundred percent of the bone marrow in order to correct Munc13-4 deficiency, we transduced HSPCs isolated from *Jinx* mice and transplanted them into lethally irradiated *Jinx* recipients. Twenty-two weeks after transplant these mice had all recovered from the transplantation procedure, and their memory CD8+ splenocytes showed an increased ability to degranulate. While preliminary,

we consider this to be a promising first step towards establishing a body of work to submit to the FDA as pre-IND studies.

4.2 IMPLICATIONS OF FINDINGS

The work presented here shows several lines of evidence that strengthen the case that FHL3 is a disease well suited for gene therapy intervention. The first piece of evidence came from how the Munc13-4 small molecule inhibitor Bexin-1 does not inhibit transduction of cell lines with VSV-G pseudotyped lentiviral vectors. While it was true that other research groups had achieved some success with transducing Munc13-4 deficient cells using VSV-G pseudotyped LVs, this success was limited, required large inputs of virus, and had one ninth the transduction efficiency of H/F pseudotyped lentiviral vectors [165]. Given that VSV-G and H/F pseudotyped lentiviral vectors utilize different mechanisms for entry into target cells [372], we hypothesized that Munc13-4 mediated the entry of VSV-G pseudotyped lentiviral vectors. However, inhibition of Munc13-4 protein did not reduce transduction efficiency. We therefore concluded that Munc13-4 does not play an essential role in the endocytic pathway that mediates VSV-G pseudotyped LV attachment and entry to target cells. Importantly for the field of FHL3 gene therapy, this means that a lack of Munc13-4 will not inhibit VSV-G pseudotyped LV gene therapy interventions.

The second piece of evidence came from our finding that Munc13-4 protein is not up-regulated in T cells as a result of stimulation. Unlike perforin expression which increases after stimulation with CD3/CD28 beads, Munc13-4 is expressed endogenously in T cells and remains constant even after stimulation. Therefore, as long as Munc13-4 can be

expressed endogenously in gene therapy modified cytolytic cells at a high enough baseline level, the mechanism for cytotoxic degranulation should be re-stored. Otherwise put, no additional work must be made to identify a promoter which must be dynamically regulated. This is fortunate given that we and the other FHL3 gene therapy research groups have restricted our research to using only the ubiquitous EF1α and EFS promoters [165, 166, 168, 169].

Thirdly in the case that FHL3 is an excellent candidate for gene therapy intervention is our finding that as little as having 10-20% of successfully gene therapy modified T cells ought to make a significant difference in the correction of FHL3 disease. We came to this conclusion after mixed chimerism studies found similar levels of degranulation in mice which received as little as 15% degranulation competent cells. This finding corroborates conclusions made by other FHL3 researchers [165, 166, 168], FHL2 disease research [408], and clinical findings [156]. While it is true that efforts should focus on yielding the maximum number of gene modified cells and insuring the highest level of engraftment possible for those cells, knowing that the target benchmark is attainable is a huge boost to morale in the world of FHL3 gene therapy.

In addition to these indirect inquiries into the feasibility of an FHL3 gene therapy, we also directly began proof-of-concept studies for the development of an LV based FHL3 gene therapy product. First, we transduced *Jinx* HSPCs with one of our optimized LVs, transplanted them into lethally irradiated *Jinx* recipients, and observed that the splenocytes of these cells exhibited partial rescue of CD107a degranulation. Furthermore, transduced primary FHL3 patient T cells also exhibited partial correction of degranulation and cytotoxic function. Thus, we have provided preliminary studies that set the groundwork for

both treating FHL3 with gene therapy modified T cells in adoptive cell transfer, and curing FHL3 using autologous transplant of gene therapy modified HSPCs.

4.3 LIMITATIONS AND FUTURE DIRECTIONS

The research presented here investigated two different approaches towards FHL3 gene therapy: the first being a long-term curative treatment mediated by LV gene therapy modified HSPCs, and the second consisting of a short-term intervention treatment for autologous transfer of gene therapy modified T cells. We believe that both of these therapies would provide substantial benefit to FHL3 patients, and therefore in the future we plan to move both of these projects forward. This discussion of limitations and future directions will therefore span both of these projects.

Limited availability of FHL3 patient samples remains one of the largest hurdles for developing an FHL3 gene therapy. As no doubt noted by the astute reader, we only had one sample of primary human T cells with which to perform experiments on in chapter 3. Since then, we have acquired two more FHL3 patient samples, and we hope that a partnership with the University of Cincinnati Ohio (the preeminent institution for HLH treatment) will give us access to even more patient samples. In the meantime, we intend to immortalize fractions of each currently available patient's samples in the hopes of being able to execute more experiments and replicates of those experiments than would otherwise be possible.

As noted in chapter 2, we also must unequivocally establish that we can induce lasting Munc13-4 transgene expression in transduced T cells from healthy donors before attempting to transduce our precious FHL3 patient samples. We were very surprised to

observe that despite robust expression of the tCD271 marker at the plasma membranes of transduced T cells five days post-transduction, this expression disappeared by nine days post-transduction. Our VCN analysis indicate that DNA from reverse-transcribed LV genomes was present in the cells, but the fading of tCD271 indicates that this DNA is not stably integrated. The observed tCD271 expression was therefore likely due to either the expression of this non-integrated DNA or the transfer of tCD271 from the membranes of fusing LVs or LVs fused to tCD271-expressing producer cells [412]. The therapeutic potential of non-integrating LVs have attracted attention for their ability to mediate short bursts of therapies [413], and FHL3 patients may indeed benefit from infusions of gene-therapy modified T cells targeted to a specific pathogen. However, our long-term goal is produce stable integration of the *UNC13D* transgene in T cells—particularly memory T-cells—such that a patient can have lasting protection against infectious triggers that contribute to flare-ups. We therefore will need to further optimize the T-cell transduction protocol either with simple adjustments like altering the time spent in transduction media, or by making larger strategy changes such as optimizing our integrase protein [414] or replacing viral integration machinery with non-viral mediators [415, 416]. Although frustrating given that transduction worked so well in cell lines and murine HSPCs (as evidenced by copy number analysis 22 weeks post-transplantation) we observe that others have successfully induced long-term transgene expression in gene-modified human T cells [417], and currently we see no reason why an LV gene therapy for Munc13-4 deficiency would be different in this regard.

Future studies with regards to developing an HSPC based gene therapy will absolutely require further optimization of the *Jinx* mouse model. As we discovered from

our own experimentation, infection with LCMV Armstrong did not produce a disease model that clinically resembled human FHL3, despite the fact that this was the originally proposed model for FHL3 disease [170]. Re-capitulating FHL3 disease in *Jinx* mice is not as simple as copying the methods that are used to induce FHL2 disease in perforin knock-out mice. For example, while infecting perforin knock-out mice with LCMV Armstrong leads to weight loss, progressive organ failure, and eventually death as a result of hyperinflammation [418], in hour hands, *Jinx* mice that are infected with LCMV Armstrong experience no weight loss and experience little to no clinical signs of distress as a result of the infection. Researchers from other laboratories have confirmed that LCMV Armstrong induces only a very weak disease phenotype (Michael B. Jordan, personal communication). A close inspection of the original study in which *Jinx* mice were interrogated with different infectious triggers reported the LCMV Armstrong infection induced anemia, thrombocytopenia, neutropenia, splenomegaly, high levels of IFN-γ in the serum, infiltration of CD8 T cells (particularly GP33 reactive CD8 T cells) and APCs in the spleen, macrophage infiltration in the bone marrow, greater levels of APC activation, and high viral loads in the spleen [170]. However, no comments on clinical or behavioral changes to LCMV infected *Jinx* mice are made [170]. Furthermore, these mice did not die from LCMV infection, indicating that perhaps there is some compensatory mechanism that keeps LCMV Armstrong infection in check. This is in stark contrast to human FHL3 disease in which the patients are obviously sick and can still die despite best intervention efforts [132, 419].

Other research groups produced a more robust FHL3 disease phenotype by infecting their *Jinx* mice with the WE strain of LCMV [166]. Specifically, they reported

significant differences in weight loss, body temperature, and liver enlargement between LCMV WE infected WT and *Jinx* mice. Michael B. Jordan, one of the leading researchers in HLH disease, also confirmed that the WE strain of LCMV produces a much more robust disease phenotype in *Jinx* mice than the Armstrong strain (personal communication). When we started our research this information had not yet been published, and the current understanding of the FHL3 mouse model was centered on the use of LCMV Armstrong [170]. Furthermore, we were able to easily obtain stocks of LCMV Armstrong. However, in moving forward, we must give serious consideration to switching to infecting our *Jinx* mice with LCMV WE strain in order to produce more clinically significant data.

An alternative infectious trigger that might be used to induce a more FHL3-like disease would be infection of *Jinx* mice using MCMV. *Jinx* mice were first identified in random mutagenesis studies as being particularly susceptible to MCMV infection [262]. Follow up studies found that infection with 10^5 PFU of MCMV Smith strain induces a strong cytokine response and death in these mice within six days of infection [170]. MCMV infection in BALB/c mice has already been used to create a mouse model of secondary HLH [420] and the response of these mice to MCMV is very similar to that observed in *Jinx* mice [170]. While most research with *Jinx* mice has focused on inducing FHL3 disease using different strains of LCMV [166, 170, 267], it is perhaps time to consider returning to the use of MCMV infections.

While the specifics of the infectious agent used in the FHL3 mouse model are paramount, we must also consider the more fundamental robustness of the *Jinx* mouse model. Our own experimental results as well as a review of the literature consistently show that *Jinx* T cells, despite being deficient in Munc13-4 protein, show low but noticeable

upregulation of CD107a as a result of stimulation (Table 5). This points to a potential imperfection in the *Jinx* mouse model that goes beyond matters relating to infectious triggers, and brings into question the further refinement of this model. We hypothesize that this is due to compensation by Munc13-1 protein, which is still present in *Jinx* mice and which has been shown to rescue degranulation defects in *Jinx* CTLs after transfection with Munc13-1 plasmids [376]. Whether or not Munc13-1 compensation also helps mitigate Munc13-4 deficiency in FHL3 patients has yet to be formally investigated, but seems very unlikely given that human T cells and natural killer cells do not express Munc13-1 protein [421] .[27] Therefore, ideally the FHL3 *Jinx* mouse disease model would also have some way to prevent Munc13-1 compensation. Unfortunately, knock out of Munc13-1 is lethal in mice, causing death five hours after birth [424, 425]. In contrast, heterozygous Munc13-1 deficient mice are indistinguishable from WT mice, indicating that even partial Munc13-1 expression is restorative [426]. Therefore adapting the FHL3 mouse model to be a double knock-out for both Munc13-1 and Munc13-4 is not an option. Instead, future research might cross breed *Jinx* mice with a Munc13-1 conditional knock-out mouse. We predict

[27] The reader might be curious to know if Munc13-1 deficiency ever results in human disease akin to HLH or if it could compound with Munc13-4 deficiency to contribute to FHL3 disease. As it would happen, very few documented cases of *UNC13A* deficiency have been documented. Currently available reports indicate that both homozygous and heterozygous mutations contribute to severe disease typified by movement disorders, cognitive impairment, microcephaly, cortical hyper-excitability, myasthenia and death 422. Lipstein, N., et al., *Synaptic UNC13A protein variant causes increased neurotransmission and dyskinetic movement disorder.* J Clin Invest, 2017. **127**(3): p. 1005-1018, 423. Engel, A.G., et al., *Loss of MUNC13-1 function causes microcephaly, cortical hyperexcitability, and fatal myasthenia.* Neurol Genet, 2016. **2**(5): p. e105.. Therefore Munc13-1 deficiency presents quite differently from typical HLH, and while it is theoretically possible that compounded mutations to Munc13-1 and Munc13-4 could produce a particularly nasty disease, this seems exceedingly unlikely. Furthermore, the resulting disease would likely be rapidly fatal owing to the severe nature of compounding both FHL3 disease and Munc13-1 deficiency.

that such a mouse would have impairment of its cytotoxic cells beyond what is observed in *Jinx* mice, and that these mice would be susceptible to LCMV Armstrong infection.

Breeding a new hybrid KO/conditional KO mouse strain is quite labor intensive, so another option that would allow us to investigate the compensatory role of Munc13-1 protein in LCMV-infected *Jinx* mice would be to inhibit Munc13-1 expression or function during our *in* vitro assays. For example, prior to stimulating T cells for the CD107a degranulation assay, we might be able to use siRNA knock-down to reduce their Munc13-1 expression. However, this would require extended culturing of the splenic T cells, and as noted previously in chapter 2, viability of these cells degrades over time. Alternatively, we could treat our T cells with resveratrol—a naturally occurring polyphenol which inhibits Munc13-1 by binding to the protein's C1 domain and inhibiting its translocation to the plasma membrane [427]. Munc13-4 lacks a C1 domain [161, 192], and therefore would be un-affected by resveratrol treatment. Whether by siRNA knockdown or drug interference, if the extemporaneous degranulation that we consistently see in stimulated *Jinx* T cells disappears with Munc13-1 inhibition, then we would conclude that this degranulation is Munc13-1 dependent. This would provide useful mechanistic insight into the FHL3 mouse model and help us to determine if further model refinement is warranted.

As a final note on future directions for the Emory Gene Therapy and HLH research labs, we would like to mention that we have begun efforts to develop LV based gene therapies for HLH caused by absent/defective *STXBP2* or *Rab27a* genes. Similar to the work described in chapter 2, we have cloned sequences for the cDNA for STXBP2 and Rab27a into expression plasmids and produced LVs using the aforementioned protocol. Western blots of NIH 3T3 cells show that this cell line endogenously expresses Rab27a, but

that transduction with an Ef1α_Rab27a construct can increase that expression (Figure 26A). As observed in experiments with the Munc13-4 constructs, using the Ef1α promoter was more effective than using the MND promoter.

We are excited by this early success with the Rab27a construct, and are in the process of codon-optimizing it and adding transduction markers. However, we are aware that substantial challenges exist with moving a Rab27a gene therapy project forward. First, as rare as other forms of FHL are, patients with Griscelli Syndrome type II (the disease caused by Rab27a deficiency) are even rarer [173]. Furthermore, the mean age of death for GSII patients is five years old, further complicating sample acquisition [428]. Therefore obtaining primary patient samples could be extremely difficult. Currently, we have samples from one Rab27a deficient patient, and we hope that partnering with other HLH treatment and research institutions will provide us access to more.

Another option for moving the Rab27a gene therapy project forward would be to obtain *Rab27a* KO mice, or ashen mice (MGI: 1861441), so named for their ash-gray coats, which closely resemble the gray hair coloring of GSII patients[28]. Infection of ashen mice with LCMV WE strain has been shown to robustly mimic GSII disease [429], and therefore could effectively be used to develop a GSII gene therapy. However, these mice have lower fecundity and on average produce litters only half the size as normal C57BL/6J mice [430]. Given the effort and time required to acquire either GSII patient samples or

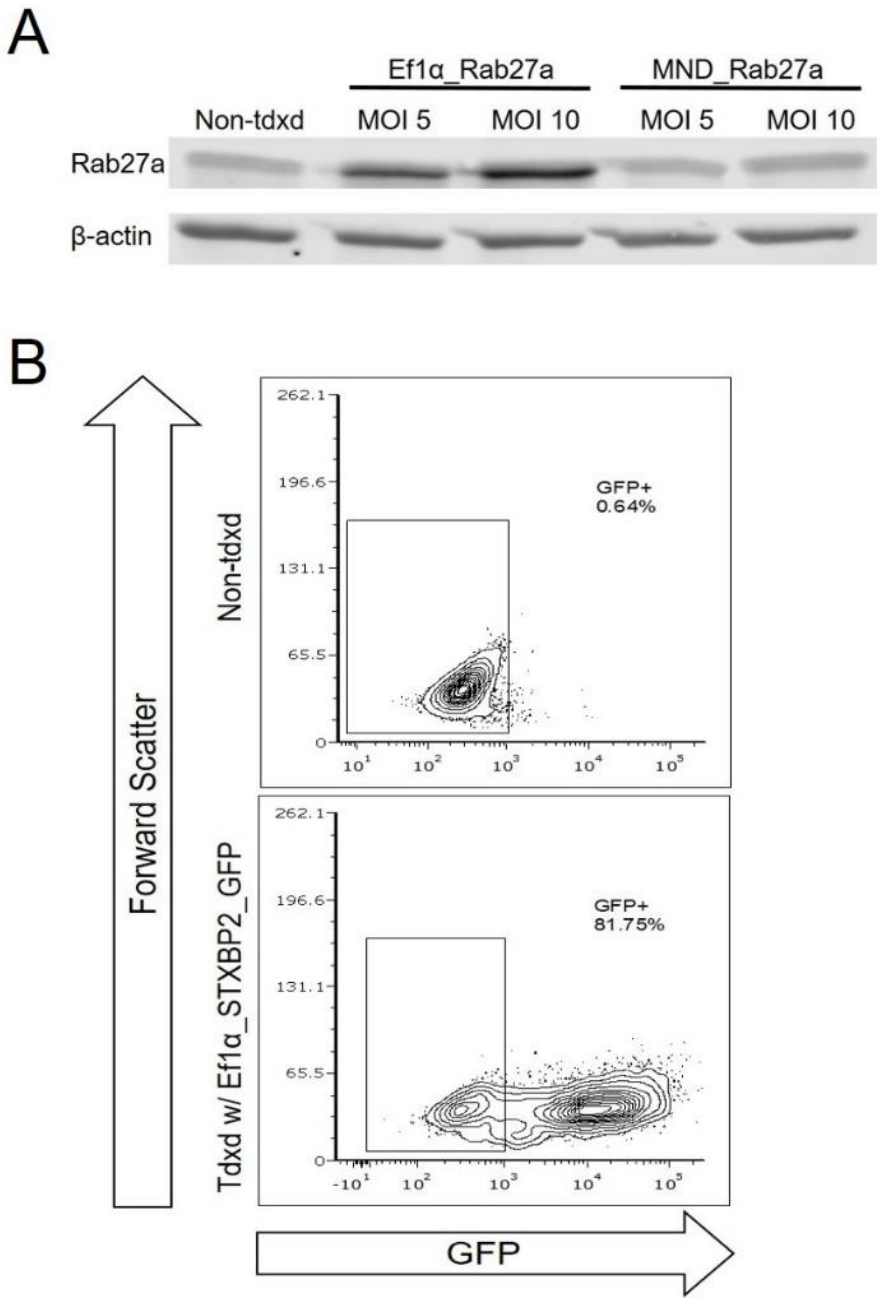
[28] It is important to note that "ashen" refers to a phenotype, and the ashen phenotype is confusingly not confined to mice with a mutation to the *Rab27a* gene. Several other strains of mice with other mutated genes are also referred to as "ashen," including the Rab27a/Rab27b double knockouts. Therefore care must be taken when reviewing the literature pertaining to “ashen” mice.

Rab27a null *ashen* mice, we are first characterizing our Rab27a LV constructs using *in vitro* assays to insure that an optimized LV is ready when either of these resources become available.

With regards to the STXBP2 gene therapy project, sequencing results showed that we successfully cloned in the sequence for STXBP2 cDNA into an LV transgene plasmid backbone, and we have used that plasmid to successfully create LVs. Unfortunately, transduction of NIH 3T3 cells showed no increase in STXBP2 expression as a result of transduction with an EF1α_STXBP2_GFP construct. However, flow analysis does confirm GFP expression in the transduced cells (Figure 26B). Efforts are currently under way to integrate a sequence for flag-tagged STXBP2 into the existing construct. In addition, we have begun efforts to use CRISPR/Cas9 to disrupt the first exon of the STXBP2 gene in KBM7 cells (the non-adherent form of the haploid Hap1 cell line), thus producing an STXBP2 KO cell line. In terms of mouse studies, homozygous knockout of STXBP2 protein as a result of mutation to the STXBP2 gene is lethal (MGI: 107370) [431]. A conditional STXBP2 KO mouse strain is available (MGI: 5428593) although only a few studies have utilized it [431, 432]. Once we can establish efficacy of our STXBP2 construct using cell lines, our next step will be to acquire this mouse strain for future studies.

Figure 26

A) Western blot revealed that transduction of NIH 3T3 cells with the Ef1α_Rab27a construct can induce expression of Rab27a protein five days post-transduction beyond the low endogenous levels. By contrast, it is unclear if transduction with the MND-Rab27a construct produces any increase in Rab27a expression in NIH 3T3 cells. B) Transduction of NIH 3T3 cells with the Ef1α_STXBP2_GFP construct yields GFP expression in cells five days post-transduction.



4.4 CONCLUSION

In summary, we have presented a body of work that describes the optimized production of unique lentiviral vectors designed to correct Munc13-4 deficiency, transduction into both primary human T cells and murine HSPCs, the benchmarking of the minimal requirements for engraftment of gene therapy modified cells, and preliminary results for the correction of Munc13-4 deficiency in *Jinx* mice. To our knowledge, including Emory University, there are currently three research groups actively working on creating gene therapies for FHL3 disease. Each of these groups has published unique gene therapy products, although clear similarities exist between the methods and reasoning used to create and test those products [165, 166, 168, 169]. We hope that this indicates a robustness in logic and methodology, and further strengthens the case that FHL3 is an excellent candidate disease for gene therapy intervention.

In addition to the work strictly devoted to developing an FHL3 gene therapy, we hope that the results of some of our other work—particularly that described in chapter 2—can help more broadly inform the field of LV gene therapy research and basic immunology. In particular, we hope that our review of different methods of isolating mouse HSPCs will provide other researchers with insights into improved methodologies for isolation and transduction of these cells. Furthermore, our results pertaining to the efficiency of commonly used transduction enhancers, microfluidics, or LV purification methods might help gene therapy researchers save time and resources optimizing their protocols for transduction using their own LV gene therapies. Finally, we contribute some insights into the role of Munc13-4 protein in trafficking of secretory vesicles in neutrophils and lack of

Munc13-4 involvement in the pathway that mediates VSV-G pseudotyped LV attachment and entry into target cells.

We hope that this work will convince the reader that an LV based gene therapy has potential for clinical translation towards treating FHL3 disease. Furthermore, we hope that the reader appreciates the rigor with which the Emory Gene Therapy Program is dedicated towards creating optimized protocols and systems for gene therapy production and testing. The next step towards developing a clinical product is to complete pre-IND studies. We are hopeful that the work presented here is a first step down the long road towards generating an efficacious clinical product.

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