Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Athena Liza Russell Date

Non-genotoxic anti-CD117 conditioning for hematopoietic stem cell transplantation and gene therapy applications

By

Athena Liza Russell
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Genetics and Molecular Biology

_____
Christopher B. Doering, PhD
Advisor

_____
Anthony Chan, DVM, PhD
Committee Member

_____
Judith Fridovich-Keil, PhD
Committee Member

_____
H. Trent Spencer, PhD
Committee Member

_____
Bing Yao, PhD
Committee Member

Accepted:

_____
Lisa A. Tedesco, PhD
Dean of the James T. Laney School of Graduate Studies

_____
Date

Non-genotoxic anti-CD117 conditioning for hematopoietic stem cell transplantation and gene therapy applications

By

Athena Liza Russell
B.S., University of North Florida, 2006

Advisor: Christopher B. Doering, 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,
Genetics and Molecular Biology
2021

Abstract

Non-genotoxic anti-CD117 conditioning for hematopoietic stem cell transplantation and gene therapy applications

By Athena Liza Russell

Nearly 1.5 million hematopoietic stem cell transplants have been performed world-wide since this life-changing and lifesaving procedure was first performed nearly 65 years ago. This powerful therapy can be used in the treatment or cure of a broad range of malignant and non-malignant hematologic diseases. However, despite its robust therapeutic potential, hematopoietic stem cell transplantation is underutilized in many non-malignant disease settings for which it can be curative. A critical component of the procedure is the pre-transplantation conditioning or preparative regimen. The conditioning regimen serves multiple functions: (a) ablation of endogenous bone marrow resident hematopoietic stem cells to make space for transplanted cells to engraft and restore hematopoiesis; (b) immune suppression, in the setting of allogeneic transplantation, to prevent graft rejection; (c) elimination of disease in the case of hematologic malignancy. Agents that are conventionally used for hematopoietic stem cell transplantation conditioning are DNA-damaging, genotoxic agents such as ionizing radiation or chemotherapeutic alkylating drugs. Though effective, these agents cause cytotoxicity not specific to the target cells, simultaneously inducing damage to non-target healthy cells and tissues. As a result, genotoxic conditioning agents are associated with many short- and long-term adverse effects and significant regimen-related toxicity and treatment-related mortality. These risks are more acceptable in the setting of active malignancy, however for patients with non-malignant disease for which alternative therapies exist, the risk-benefit ratio of enduring genotoxic conditioning is often deemed too great. Therefore, improving the safety profile of the hematopoietic stem cell transplantation conditioning regimen is a major unmet need in the field. Developing safer and less toxic conditioning agents would not only expand the patient population in whom traditional hematopoietic stem cell transplantation can be safely performed, but also broaden its applicability to providing safe, curative ex vivo lentiviral modified autologous hematopoietic stem cell gene therapy options for monogenic diseases.

The work presented herein represents a preliminary step towards realizing this objective. We evaluated the use of a non-genotoxic immunotoxin targeting the stem cell factor receptor CD117 and its utility in enabling pre-transplant conditioning in murine models of ex vivo lentiviral hematopoietic stem cell gene therapy for hemophilia A and allogeneic hematopoietic stem cell transplantation for ataxia telangiectasia. We hypothesized that a non-genotoxic immunotherapy could replace genotoxic radiation and chemotherapy in both allogeneic and autologous gene therapy hematopoietic stem cell transplantation settings. CD117 immunotoxin selectively depletes hematopoietic stem cells in bone marrow and allows donor cell engraftment and multilineage chimerism following transplantation, correcting or ameliorating disease phenotypes while sparing healthy tissues and minimizing adverse effects. This dissertation presents proof-of-concept data in support of efforts towards clinical translation of similar non-genotoxic antibody-based regimens for human application.

Non-genotoxic anti-CD117 conditioning for hematopoietic stem cell transplantation and gene therapy applications

By

Athena Liza Russell
B.S., University of North Florida, 2006

Advisor: Christopher B. Doering, PhD

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,
Genetics and Molecular Biology
2021

Acknowledgements

I would first like to thank my advisor, Dr. Chris Doering for adopting me into his lab and guiding my graduate school experience. Chris has been an outstanding mentor – he has supported me every step of the way, he always has my best interests in mind, and has played a massive role in helping me to become the scientist that I am today. For all his efforts and support, I could not be more grateful. I would also like to thank Dr. Trent Spencer who has also been an exceptional and influential mentor to me and has always supported, encouraged, and believed in me. I am so incredibly fortunate to have found this lab – thank you so much for having me these last four years. I am also so grateful to have had the opportunity to receive mentorship from Dr. Shan Chandrakasan. Shan has always been there for me and is a huge inspiration to me. I have learned so much from him and I feel extremely fortunate to have had the opportunity to work with him and his wonderful lab. I would like to sincerely thank my other committee members: Dr. Judy Fridovich-Keil, Dr. Anthony Chan, and Dr. Bing Yao. They have been an absolutely incredible support network for me. Each one of them has been there for me many times during the course of my graduate studies. Each one of them has been my cheerleader and has supported me from the beginning through to the end. Every one of these individuals, Chris, Trent, Shan, Judy, Anthony and Bing, are truly wonderful and inspirational people who I will always look up to greatly. They have all made a huge impact on my experience at Emory and have left a lasting imprint (…genetics pun about a concept I hope I will never be asked to explain again!)

I would like to thank my lab mates, especially Taran, Kristopher and Chengyu. Without them, I would not be writing this today. I truly could not have done this without them. They helped me with my experiments, they made buffers for me, they weaned mice for me, they coated plates for me, they purified protein for me, but most of all, they were there for me always. They listened to me, they answered my questions, they made me laugh. They were always amazing, and they will remain my friends for a lifetime without question.

Most importantly, I thank my family. I thank my husband, Scott, who supported the uprooting of our lives and our 4-year-old son away from everyone we knew and our entire support network in Florida so I could pursue this long-held dream. Though initially skeptical, it quickly became clear that coming to Emory was the best decision ever made for both of us. I thank my wonderful parents and my beautiful sisters. No one on Earth believes in me more than they. Their love and support have been never-ending (i.e., 40-year-old graduate student… their support of my academic endeavors has quite literally never ended). I have the best family in the world, and I will never take for granted how lucky I am to have been born into this wealth of love.

Last, but the farthest from least one could possibly be, I thank my precious son Elijah (now 8 years old!) I thank him for putting up with all he has had to put up with having a mother in graduate school during some of his most formative years. I thank him for being the absolute best thing EVER. I dedicate this dissertation to you. This is all for you.

Table of Contents

Abstract

Acknowledgements

Table of Contents

List of Figures and Tables

List of Abbreviations

Chapter 1 1

1.1 Hematopoietic Stem Cell Transplantation 2

1.1.1 History of Bone Marrow Transplantation 2

1.1.2 Current Platforms of Hematopoietic Stem Cell Transplantation 3

1.1.3 Conditioning for Hematopoietic Stem Cell Transplantation 13

1.1.4 Next generation Non-genotoxic Antibody- and Immunotoxin-based Conditioning Agents 16

1.1.5 Hematopoietic Stem Cell Gene Therapy 22

1.2 Hemophilia A 29

1.2.1 Hemophilia A Background 29

1.2.2 Factor VIII and Its Role in Blood Coagulation 30

1.2.3 Current and Developing Therapies for Hemophilia A 32

1.2.4 Gene Therapy for Hemophilia A 36

1.3 Ataxia Telangiectasia 40

1.3.1 Ataxia Telangiectasia Background 40

1.3.2 ATM Kinase: Central Role in DNA Double-strand Break Repair and Other Diverse Functions 42

1.3.3 Current and Developing Therapies for Ataxia Telangiectasia 47

1.3.4 Allogeneic Hematopoietic Stem Cell Transplantation for Ataxia Telangiectasia 51

1.4 Hypothesis 54

Chapter 2 56

2.1 Abstract 57

2.2 Introduction 57

2.3 Materials and Methods 60

2.4 Results 66

2.5 Discussion 88

2.6 Acknowledgements ... 96
2.7 Supplemental Information ... 98
Chapter 3 ... **112**
3.1 Abstract ... 113
3.2 Introduction ... 114
3.3 Materials and Methods ... 117
3.4 Results ... 121
3.5 Discussion ... 136
3.6 Acknowledgements ... 139
3.7 Supplemental Information ... 141
Chapter 4 ... **146**
4.1 Summary of Results ... 147
4.2 Implications of Findings ... 150
4.3 Limitations and Future Directions ... 152
4.4 Conclusions ... 157
References ... **158**

List of Figures and Tables

Figure 1. Bone marrow HSC differentiation and hematopoiesis 5
Figure 2. HSC bone marrow niche 6
Figure 3. Autologous and allogeneic hematopoietic stem cell transplantation 9
Figure 4. HSC mobilization and homing 12
Figure 5. Busulfan and its mechanism of DNA or protein alkylation 14
Figure 6. Classification of conditioning regimen intensity 15
Figure 7. Immunotoxin structure and mechanism of action of saporin-based immunotoxin 19
Figure 8. Ex vivo autologous hematopoietic stem cell gene therapy 24
Figure 9. Third-generation lentiviral vector system 26
Figure 10. Intrinsic and extrinsic pathways of the coagulation cascade (secondary hemostasis) 32
Figure 11. Mechanism of action of emicizumab 35
Figure 12. AAV vector gene delivery 38
Figure 13. Broad scope of ATM functional interactions and substrates 43
Figure 14. ATM and DNA double-strand break repair 45
Figure 15. ATM and maintenance of cellular homeostasis 47
Figure 16. CD117-saporin immunotoxin selectively depletes HSCs and spares non-hematopoietic tissues in hemophilia A mice 69
Figure 17. CD117-sap + mATG conditioning enables engraftment of CD68-ECO-ET3-LV transduced LT-HSCs and multilineage chimerism in a majority of recipients 73
Figure 18. HA mice engrafted with gene-modified HSCs following CD117-sap + mATG conditioning exhibited therapeutic levels of plasma ET3 activity while some non-engrafted mice developed ET3 inhibitors 76
Figure 19. Rabbit anti-mouse mATG binds mature and primitive hematopoietic cells in peripheral blood, spleen and bone marrow 79
Figure 20. CD117-sap + T cell mAbs conditioning enables engraftment of CD68-ECO-ET3-LV gene-modified LT-HSCs and multilineage hematopoietic chimerism in all transplant recipients 83
Figure 21. HA mice conditioned with CD117-sap + T cell mAbs and engrafted with CD68-ECO-ET3-LV gene-modified HSCs exhibited therapeutic levels of plasma ET3 activity and phenotypic correction 86
Figure 22. HA mice engrafted with ET3-modified cells after CD117-sap + T cell mAbs conditioning exhibit healthy tissue morphology and normal litter sizes 87
Figure 23. Multilineage chimerism and correction of T cell numbers, but not growth defect, is achieved after HSCT using CD117-sap 124
Figure 24. IgA levels and T cell-dependent immune responses corrected after HSCT using CD117-sap. 127
Figure 25. Host-derived T cell lymphoma detected in one *Atm*$^{-/-}$ recipient conditioned with CD117-sap alone 129
Figure 26. Multilineage chimerism and correction of T cell numbers is achieved after HSCT using CD117-sap plus lymphocyte depleting antibodies 133

Figure 27. Donor-derived GFP$^+$ microglia not detected in chimeric *Atm*$^{-/-}$ brain, however GFP$^+$ cells are present 136

Table 1. Diseases treated using autologous or allogeneic hematopoietic stem cell transplantation…….……10
Table 2. Agents commonly used in HSCT conditioning regimens……...….16
Table 3. Genetic diseases treated in HSC-GT clinical trials registered on ClinicalTrials.gov as of March 2021……...…27
Table 4. HSC-GT that have received market approval in the European Union…..……...29

Figure S1……...……98
Figure S2……...……99
Figure S3……...…..100
Figure S4……...…..…101
Figure S5……..…..102
Figure S6……..…..…..103
Figure S7……...…..104
Figure S8……...……..105
Figure S9…….……....106
Figure S10…….....……107
Figure S11……...108
Figure S12……...…110
Figure S13……...…141
Figure S14……...…142
Figure S15……...144
Figure S16……...…144

Table S1……...……111
Table S2……...145

List of Abbreviations

AAV	adeno-associated virus
AML	acute myeloid leukemia
APC	activated protein C
aPCC	activated prothrombin complex concentrate
ASO	antisense oligonucleotide
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
ATG	anti-thymocyte globulin
cDNA	complementary deoxyribonucleic acid
cGMP	current Good Manufacturing Practices
DNA	deoxyribonucleic acid
DSB	double-strand break
ECO	expression codon optimized
EHL	extended half-life
Fc	fragment crystallizable region
FcRn	neonatal Fc receptor
Fcγ1	Fc gamma 1
FDA	Food and Drug Administration
fI	Factor I (fibrinogen)
fIa	Activated Factor I (fibrin)
fII	Factor II (prothrombin)
fIIa	activated Factor II (thrombin)
fIX	factor IX
fIXa	activated factor IX
fVa	activated Factor V
fVIIa	activated Factor VII
fVIII	Factor VII
fVIIIa	activated Factor VIII
fX	Factor X
fXa	activated Factor X
fXIII	Factor XIII
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GVHD	graft versus host disease
GVL	graft versus leukemia
HA	hemophilia A
HB	hemophilia B
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSC-GT	hematopoietic stem cell gene therapy
HSPC	hematopoietic stem or progenitor cell
IND	investigational new drug
IgA	immunoglobulin A
IgE	immunoglobulin E

IgG1	immunoglobulin G type 1
IgG2	immunoglobulin G type 2
IgM	immunoglobulin M
IV	intravenous
kb	kilobases
kDa	kilodalton
LT-HSC	long-term hematopoietic stem cell
LV	lentiviral vector
LSK	lineage negative Sca-1 positive c-Kit positive
mAb	monoclonal antibody
mATG	anti-mouse anti-thymocyte globulin
MDS	myelodysplastic syndrome
MPP	multipotent progenitor
mRNA	messenger ribonucleic acid
NHP	non-human primate
PBSC	peripheral blood stem cell
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIKK	phosphatidylinositol 3-kinase-related protein kinase
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
RIC	reduced intensity conditioning
RNA	ribonucleic acid
SCF	stem cell factor
ST-HSC	short-term hematopoietic stem cell
TBI	total body irradiation
TFPI	tissue factor pathway inhibitor
UCB	umbilical cord blood
vWF	von Willebrand factor
WBC	white blood cell

Chapter 1

Introduction

1.1 Hematopoietic Stem Cell Transplantation

1.1.1 History of Bone Marrow Transplantation

Following the nuclear bomb explosions in Japan during World War II, new research was being directed at understanding the effects of radiation on human physiology and how to prevent radiation-induced organ damage. In 1949, Jacobson and colleagues demonstrated protection from lethal ionizing radiation by shielding the spleens and femurs of mice with lead.[1] Soon after, Lorenz et al. showed mice and guinea pigs could be protected from irradiation injury through intravenous bone marrow infusion.[2] There was disagreement among researchers regarding what the responsible factors were that could convey this protection. Many believed the protection must be attributable to some soluble humoral factor from the spleen or bone marrow, however in the mid-1950s multiple laboratories confirmed this protective effect could be ascribed to transplanted cells repopulating the marrow.[3, 4]

These discoveries ignited much excitement in the scientific and medical communities, primarily due to the potential implications for developing a new therapeutic approach to hematologic malignancies. Bone marrow transplantation could make it possible to deliver much larger doses of radiation and chemotherapy to destroy the cancer, after which the hematopoietic system could be rescued with healthy bone marrow. The first human bone marrow transplantation was reported in 1957 by E. Donnall Thomas, now regarded as the "father of bone marrow transplantation," who in 1990 won the Nobel Prize in Physiology or Medicine for his pioneering work in this field. Early transplantation procedures occurred primarily in patients with leukemia, and although they were largely unsuccessful at curing the underlying disease, these experiences paved the way towards a better understanding of bone marrow transplantation biology and immunology, including graft

versus host disease (GVHD; donor cells attacking the recipient) and the important graft versus leukemia (GVL; donor cells eliminating malignant cells) phenomenon. Still, since most preclinical research that led to these early human transplantations was performed in inbred mouse models, nearly all these early human transplantation efforts failed, and most investigators lost confidence that the immunological barrier in humans could be surmounted.

Continued research in animals led to the further understanding of histocompatibility systems. In 1968 the canine histocompatibility typing system was developed and bone marrow transplantation studies in dogs showed that littermates that were matched for dog leukocyte antigens (DLA) had significantly better outcomes than those that were not DLA-matched.[5] Further progress made in animal transplantation models and in the understanding of the importance of human leukocyte antigen (HLA) matching led to resumption of human trials, and in the same year the first successful allogeneic human bone marrow transplantation was performed by Robert Good in an infant with immune deficiency using donor marrow from an HLA-matched sibling.[6] Reports of other successful transplantations for primary immune deficiencies occurred in quick succession,[7, 8] followed by hematologic malignancies and aplastic anemia.[9] Although there was still much yet to discover over the course of six decades that would continue to revolutionize the practice, these early breakthroughs set the stage to transform bone marrow transplantation into one of the mainstays of hematological/oncological practice that it represents today.

1.1.2 Current Platforms of Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT), as it is now more accurately referred due to the various cell sources other than bone marrow currently exploited for the procedure, is the most

widely used cellular immunotherapy in clinical practice today.[10] More than 50,000 transplantations are performed at hundreds of centers worldwide each year,[11] and data registries such as the European Bone Marrow Transplant Registry (EBMT) and the Center for International Bone Marrow Transplant Research (CIBMTR) have collected information on nearly 1.5 million HSCT procedures to date.[9]

HSCT is a powerful procedure used for a large variety of hematologic diseases, malignancies, and immune deficiencies with the potential to be curative in many of these settings. Healthy hematopoietic stem and progenitor cells (HSPCs) are harvested and cryopreserved while the patient undergoes high dose chemotherapy or radiation to treat the underlying disease and/or ablate endogenous bone marrow cells to make room for the healthy HSPC graft (i.e., conditioning). Once these new cells engraft in the bone marrow, hematopoiesis is initially restored from more differentiated hematopoietic progenitor cells (HPCs) in the graft, while long-term repopulating hematopoietic stem cells (HSCs) replenish the HPC pool and reconstitute long-term hematopoiesis (**Figure 1**).

HSCs reside in highly specialized microenvironments within the adult bone marrow known as niches, which play critical roles in regulating HSC fate with regard to quiescence, differentiation, and mobilization into the peripheral blood (**Figure 2**). The HSC niche is comprised of a large variety of hematopoietic, stromal, endothelial, osteoclasts, and nerve/glial cells that provide signals to HSCs in the form of soluble factors, cell surface ligand interactions, and direct cell-cell interactions that regulate HSC maintenance.[12] HSCs express the surface receptor CXCR4, which interacts with the chemokine CXCL12 (SDF-1) that is secreted by various niche cells such as CXCL12-abundant reticular (CAR) cells, osteoblasts, and vascular endothelial cells.[13] The

CXCR4/CXCL12 axis plays a critical role in HSC retention within the bone marrow, mobilization from the bone marrow into the periphery, and homing back to the bone marrow niche after HSCT.

Figure 1

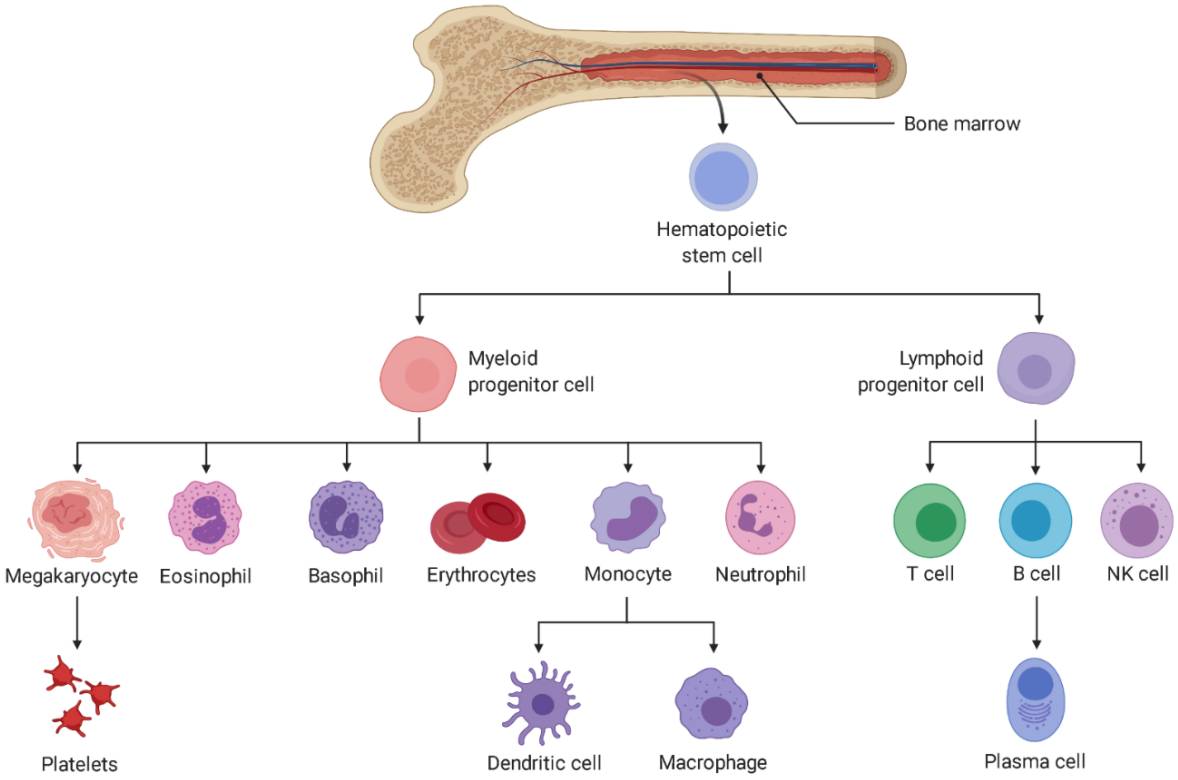


Figure 1. Bone marrow HSC differentiation and hematopoiesis
In the adult human, HSCs reside in the bone marrow in specialized microenvironments termed the HSC niche. HSCs self-renew and differentiate into all blood cells to supply life-long hematopoiesis. [Schematic created with Biorender.com]

Figure 2

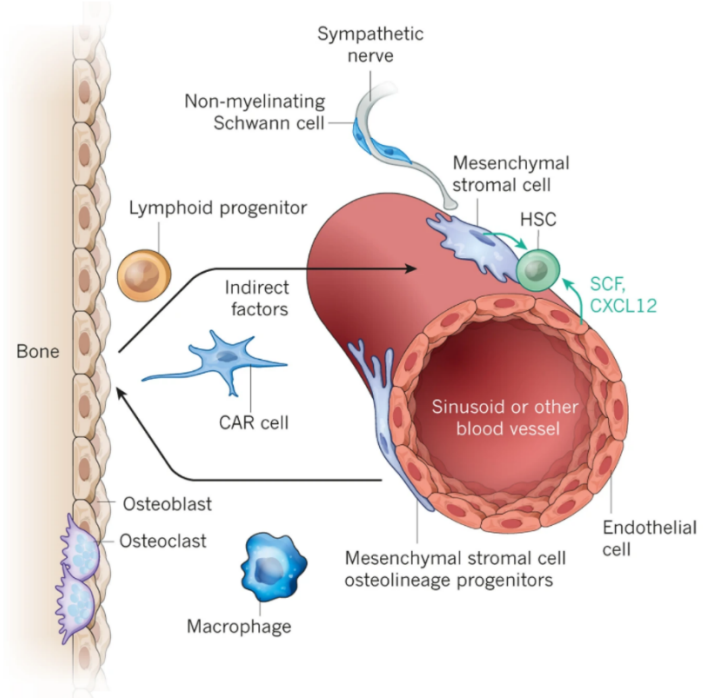


Figure 2. HSC bone marrow niche
The bone marrow is densely innervated by sympathetic nerves and vasculature. HSCs associate closely with this neurovascular network and typically reside adjacent to sinusoids. Endothelial cells and mesenchymal stromal cells produce stem cell factor (SCF) and CXCL12 (SDF-1) that promote HSC maintenance. Other cells that regulate HSCs niches include macrophages, megakaryocytes, lymphoid cells, osteoclasts, CXCL12-abundant reticular (CAR) cells, sympathetic nerves and non-myelinating Schwann cells. [Reprinted with permission from Nature Research: Morrison SJ & Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature **16,** 327-34 (2014). doi: 10.1038/nature12984. License #1115802-1]

There are two major forms of HSCT: autologous and allogeneic (**Figure 3**). In autologous HSCT, stem cells are collected directly from the affected patient and cryopreserved to be reinfused at a later time following chemotherapy and/or radiation treatment. Allogeneic HSCT relies on stem cells donated from an HLA-matched related or unrelated healthy donor. Since the patient's own cells are used in autologous HSCT, the risks of graft rejection, GVHD, and prolonged immunodeficiency are eliminated.[14]

The earliest HSCT conditioning regimens were fully myeloablative, such that the dose was high enough to ablate marrow hematopoiesis and not allow for autologous hematopoietic recovery.[15] A high single dose of total body irradiation (TBI) was initially used for the purpose of immune suppression (in allogeneic HSCT), making space in the bone marrow for the new cells in to engraft, and eradication of malignant disease.[16] However, in patients with active leukemia, high-dose TBI frequently caused fatal tumor lysis. Therefore, the chemotherapy drug cyclophosphamide was later given before TBI in an attempt to prevent this adverse response. In the 1980s, alternative regimens were explored, primarily due to the lack of access to TBI for some transplantation programs, and the combination of busulfan + cyclophosphamide later became an alternative myeloablative regimen of choice. It was eventually recognized that immunologic responses of donor cells in the graft towards residual malignant cells in the recipient made significant contributions to the effectiveness of HSCT (the so-called GVL effect).[15] This led to the development and implementation of lower intensity and less myeloablative regimens over the last 30 years with improved toxicity profiles, making HSCT available to older and infirm patients (discussed further in section 1.1.3). Though myeloablative regimens (TBI + cyclophosphamide or busulfan + cyclophosphamide) are still in use, these lower intensity regimens have gained popularity and use of "historic" conditioning regimens continues to decline.[16, 17]

Autologous HSCT for hematologic malignancy is associated with considerably lower treatment-related mortality, however this is offset by higher rates of disease relapse.[18] On the other hand, allogeneic HSCT for hematologic malignancy is associated with lower risk of disease reoccurrence, while treatment-related mortality is significantly higher. Recipients of allogeneic

transplants generally receive additional chemotherapy for prolonged immune suppression to prevent graft failure and control GVHD, making these patients more vulnerable to acute and chronic infection and regimen-related organ toxicities. The major advantage of allogeneic HSCT is the ability of donor lymphocytes in the graft to eradicate residual malignant cells in the recipient, the so-called GVL or graft versus malignancy effect. In addition, allogeneic HSCT can be used to cure certain congenital hematologic or immune diseases by replacing the recipient's bone marrow with normal bone marrow capable of reconstituting a new, functional hematopoietic system.[14] Selection of autologous versus allogeneic transplantation is based on which treatment modality has shown greater efficacy in clinical studies and clinical practice for a given disease.[11] The European Society for Blood and Marrow Transplantation (EBMT) provides guidelines and recommendations for the use of HSCT for particular disease indications, classifying them as standard of care, clinical option, developmental, or generally not recommended. **Table 1** outlines a non-exhaustive list of malignant and non-malignant diseases treatable with either autologous or allogeneic HSCT.

Figure 3

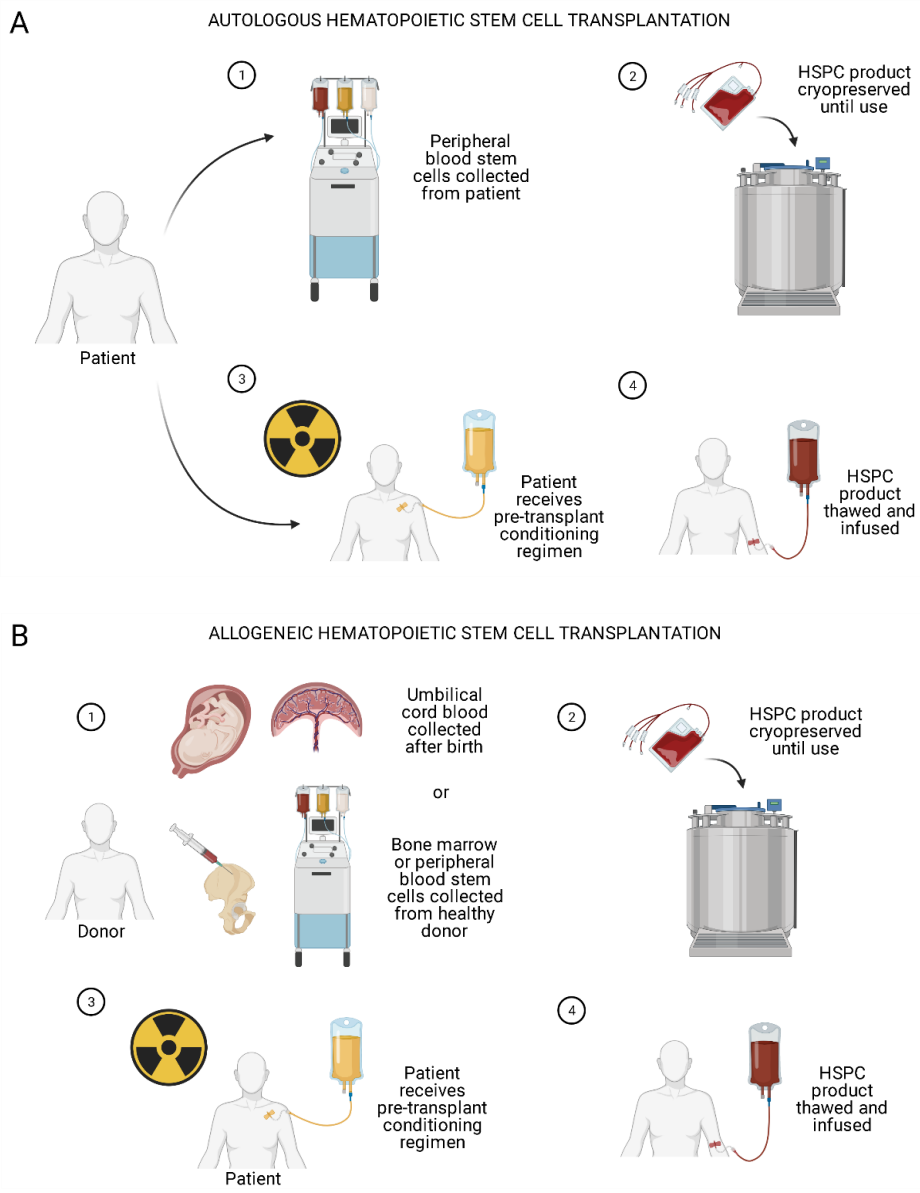


Figure 3. Autologous and allogeneic hematopoietic stem cell transplantation
(**A**) The patient serves as their own stem cell donor in autologous HSCT. The patient's HSPCs are first mobilized from the bone marrow using G-CSF with or without plerixafor. Stem cells are then collected from the peripheral circulation by apheresis. The HSPC product in processed and volume-reduced under sterile conditions and cryopreserved in liquid nitrogen until the patient is ready for transplantation. The patient then receives the conditioning regimen, which is typically high-dose chemotherapy and/or radiation. Soon after, the HSPC product is thawed and reinfused into the patient to re-establish hematopoiesis. (**B**) In allogeneic transplantation, the donor is HLA-matched to the patient, and the HSPC product may be bone marrow, peripheral blood stem cells from an apheresis collection, or umbilical cord blood. Once the HSPC source is secured, the patient undergoes conditioning and hematopoiesis is rescued with an intravenous HSPC infusion. [Schematic created with Biorender.com]

Table 1

Autologous	Allogeneic
Multiple myeloma	Acute myeloid leukemia
Non-Hodgkin's lymphoma	Acute lymphocytic leukemia
Hodgkin's lymphoma	Chronic myeloid leukemia
Acute myeloid leukemia	Myelodysplastic syndrome
Neuroblastoma	Myeloproliferative disorder
Ewing sarcoma	Non-Hodgkin's lymphoma
Germ cell tumors	Hodgkin's lymphoma
Amyloidosis	Chronic lymphocytic leukemia
Autoimmune disorders	Multiple myeloma
	Aplastic anemia
	Paroxysmal nocturnal hemoglobinuria
	Fanconi anemia
	Blackfan-Diamond anemia
	Thalassemia
	Sickle cell disease
	Severe combined immunodeficiency
	Wiskott-Aldrich syndrome
	Lysosomal storage diseases
	Osteopetrosis
	Chronic granulomatous disease
	Kostman syndrome

Table 1: Diseases treated using autologous or allogeneic hematopoietic stem cell transplantation Autologous HSCT and allogeneic HSCT are used to treat a wide variety of malignant and non-malignant disorders. [Table adapted from Copelan[19] and Yesilipek[20].]

Before the introduction of drugs that are now routinely used to mobilize HSPCs into the peripheral blood for easier collection, HSCs could only be obtained from donor bone marrow aspirated from the posterior iliac crest in the operating room under general anesthesia. Today, multiple sources of HSPCs are available for allogeneic HSCT, each with distinct advantages and disadvantages. Effective and safe techniques using granulocyte-colony stimulating factor (G-CSF) to mobilize these cells from the marrow compartment into the peripheral circulation for collection by apheresis were developed and a shift towards the use of peripheral blood stem cells (PBSCs) occurred in the

1990s.[10] Newer mobilization agents, such as plerixafor, a CXCR4 antagonist, were later developed that enable even higher yields of $CD34^+$ PBSCs for collection (**Figure 4**). PBSCs remain the most common source of HSPCs for HSCT in both autologous and allogeneic transplantation, in part due to ease of collection and donor preference. PBSC products provide the advantage of faster time to engraftment and recovery of white blood cell populations and lower incidence of graft failure.[19] However, in allogeneic HSCT these advantages are offset by increased incidence of GVHD due to greater numbers of mature T cells in the graft. For this reason, bone marrow has remained the preferred product for treatment of non-malignant diseases such as aplastic anemia and hemoglobinopathies.[9] Still, recent large studies have shown that PBSC transplants are associated with decreased incidence of relapse in hematologic malignancy and improved overall and disease-free survival in late-stage disease,[21] likely due to the GVL effect.

Figure 4

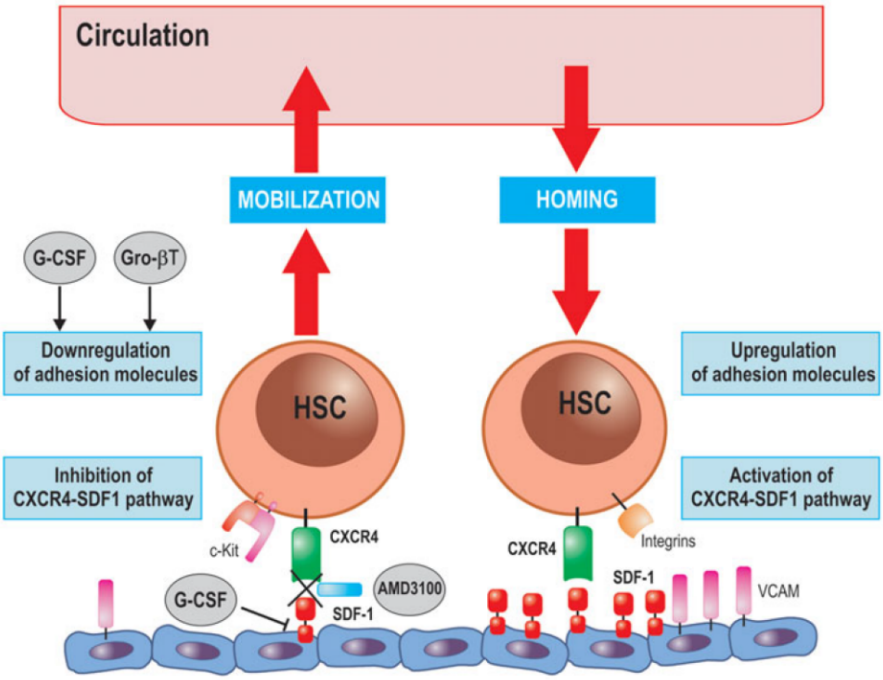


Figure 4. HSC mobilization and homing
Downregulation of adhesion molecules and chemokine receptors such as CXCR4 drive mobilization of HSCs from the bone marrow into the peripheral blood. HSC mobilization can be induced pharmacologically using agents such as G-CSF or plerixafor (AMD3100), which target the CXCR4/CXCL12 (SDF-1) axis. HSC homing is the reversal of this process in which CXCR4-expressing HSCs from the periphery return to the bone marrow in response to chemoattractant forces mediated by the SDF-1-expressing niche. Efficient HSC homing is critical for successful engraftment of donor HSCs during HSCT. [Reprinted with permission from Springer Nature: Suarez-Alvarez B, Lopez-Vazquez A, & Lopez-Larrea C. Mobilization and homing of hematopoietic stem cells. Adv Exp Med Biol **741,** 152-70 (2012). doi: 10.1007/978-1-4614-2098-9_11. License #5058911331758]

Only 35% of patients in need of allogeneic HSCT have HLA-matched siblings who can serve as bone marrow or PBSC donors. This limitation sparked the establishment of national and international registries in the 1980s, such as the National Marrow Donor Program (NMDP) in the United States. These registries recruit healthy unrelated volunteer donors and store their HLA typing information for potential future donations. They currently hold data on more than 36 million unrelated donors across the globe that can be matched with patients in need.[9] Despite this rich resource, great disparities exist regarding the likelihood of finding a suitable matched unrelated donor based on ethnic background, with the probability for non-Caucasian patients being dramatically lower. The availability of HSPC products sourced from umbilical cord blood (UCB) has assisted in overcoming these challenges. UCB was first used in a patient with Fanconi anemia in 1989[22] and became a viable alternative source of HSPCs in the 1990s with unique advantages. Because the cells present in UCB are primitive and immunologically naïve, greater disparities in HLA matching between the donor and recipient are tolerated, and UCB is less likely to cause GVHD. Cord blood units are readily available, have a low risk of viral contamination, and greatly expand HSCT access to patients from minority ethnic groups for whom a matched unrelated donor cannot be otherwise identified. The primary limitations of UCB include slow engraftment and

immune reconstitution, leading to infection susceptibility in the early post-transplant period, and low doses of nucleated cells and $CD34^+$ HSPCs. Transplantation using double cord blood units are a common strategy to overcome low HSPC doses, and newer protocols for ex vivo expansion of cord blood are being developed that can improve rates of cord blood engraftment.[23-26]

1.1.3 Conditioning for Hematopoietic Stem Cell Transplantation

The conditioning or preparative regimen is a critical component of the HSCT procedure. The purpose of the conditioning regimen is to (a) ablate endogenous bone marrow HSCs to make space for the incoming HSPC graft, (b) in the case of malignancy, eradicate the underlying disease by destroying as many cancer cells as possible, and (c) suppress the recipient's immune system in the setting of allogeneic HSCT to prevent allograft rejection. Traditional conditioning regimens involve chemotherapy and/or radiation, which impart cytotoxicity of target cells by eliciting damage to DNA, also known as genotoxicity. However, most conditioning agents conventionally used also affect non-target healthy cells and tissues, and for this reason are also responsible for causing significant regimen-related toxicities in HSCT recipients.

Choice of conditioning regimen depends primarily on the disease being treated as well as patient demographics and transplant center experience. Early regimens were designed to maximize dose of chemotherapy or radiation for maximum effect on disease eradication and immune suppression. These myeloablative regimens, including total body irradiation (TBI) and chemotherapeutic DNA alkylating agents (**Figure 5**), are profoundly cytotoxic and therefore associated with a wide variety of short- and long-term side effects including organ damage, infection, metabolic and endocrine dysfunction, and secondary malignancy.[11] TBI in children may cause growth failure and infertility

and is not recommended.[20] Additionally, elderly and medically infirm patients poorly tolerate intense myeloablative conditioning regimens, despite that many of the diseases treatable by HSCT are more common in older patients.

Figure 5

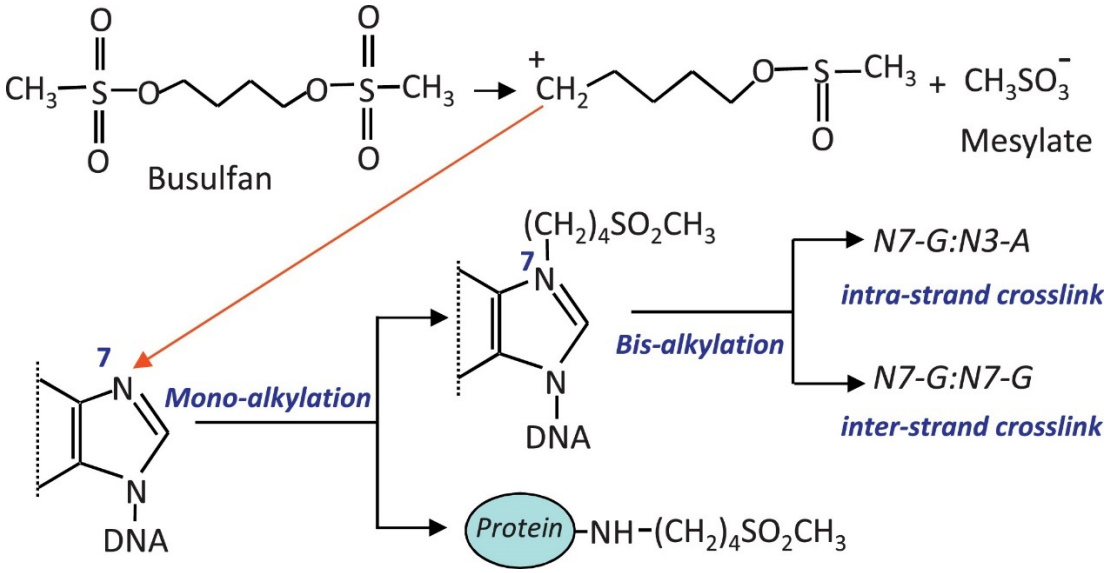


Figure 5. Busulfan and its mechanism of DNA or protein alkylation
Busulfan is one of the most common alkylating agents used for HSCT conditioning. Busulfan induces N7G:N3A intrastrand or N7G:N7G interstrand crosslinks in DNA, or protein-DNA crosslinks, which causes cytotoxicity. [Reprinted with permission from Elsevier: Puyo S, Montaudon D, & Pourquier P. From old alkylating agents to new minor groove binders. Crit Rev Oncol Hematol **89,** 43-61 (2014). doi: 10.1016/j.critrevonc.2013.07.006. License #5058971070804]

Once the important role of the GVL effect in successful allotransplantation for malignancy was realized, newer reduced-intensity and non-myeloablative regimens were developed (**Figure 6**) and access to HSCT could be more safely expanded to older patients and other vulnerable patient populations.[10] These lower intensity and lower toxicity regimens are now widely used in both malignant and non-malignant disease settings. Still, early and late complications persist due to the genotoxic nature of these agents. **Table 2** lists conditioning agents commonly used in HSCT.

Figure 6

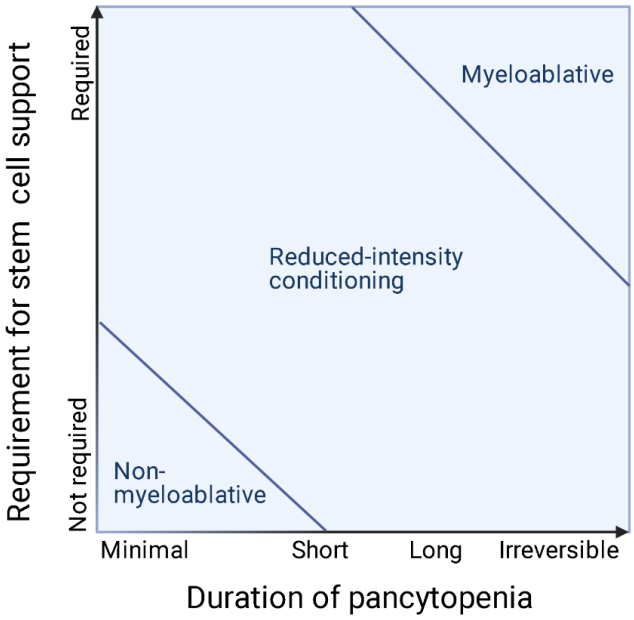


Figure 6. Classification of conditioning regimen intensity
There is significant variation in conditioning regimen intensity based on a large variety of possible combinations of agents and variable practices at different transplant centers. Several working groups have convened to better define and categorize the intensity of conditioning regimens based on the degree of marrow ablation, requirement for stem cell support, and degree of cytopenia imposed by the regimen. [Adapted from Bacigalupo et al.[17] Schematic created with Biorender.com]

Table 2

Agent	Class
Total body irradiation	Ionizing radiation
Total lymphocyte irradiation	Ionizing radiation
Busulfan	Alkylating agent, alkyl alcane sulfonate
Cyclophosphamide	Alkylating agent, oxazaphosphorine
Melphalan	Alkylating agent, nitrogen mustard
Treosulfan	Alkylating agent, dimethanesulfonate
Thiotepa	Alkylating agent, ethylene imine, polyaziridine
Bendamustine	Alkylating agent, nitrogen mustard
Carmustine	Alkylating agent, nitrosourea
Fludarabine	Antimetabolite, pyrimidine analog
Cytarabine	Antimetabolite, pyrimidine analog
Thioguanine	Antimetabolite, purine analog
Cladribine	Antimetabolite, purine analog Adenosine deaminase inhibitor
Idarubucin	Anthracycline
Etoposide	Topoisomerase II inhibitor
Anti-thymocyte globulin	Immune globulin, immunosuppressant
Alemtuzumab	Monoclonal antibody
Rituximab	Monoclonal antibody

Table 2: Agents commonly used in HSCT conditioning regimens. Conditioning agents used most often for HSCT include ionizing radiation, DNA alkylating chemotherapy drugs and antimetabolite chemotherapies. Antibody products are also commonly used for immune suppression.

1.1.4 Next generation Non-genotoxic Antibody- and Immunotoxin-based Conditioning Agents

There are substantial risks of complications and treatment-related morbidity and mortality in conventional allogeneic HSCT that are largely attributable to the genotoxicity and non-specificity of standard conditioning agents. Although supportive care and treatments for transplant-related toxicities have improved over the last two decades, HSCT under myeloablative conditioning is associated with a 5-10% increased risk of mortality compared with living with chronic non-malignant disease.[27-30] Therefore the risk-benefit ratio of this treatment modality for many non-malignant genetic diseases is often percieved by patients, families and caregivers to be too high

for its widespread adoption in these settings. The development of safer alternative strategies to bone marrow pre-conditioning with reduced toxicity would lead to better outcomes, and would expand access to this potentially curative therapy to patients who are poor candidates for HSCT due to the toxicity of current conditoning protocols. In order to address this major unmet need in the field of HSCT, many promising research efforts are currently being directed toward preclinical and clinical development of non-genotoxic antibody-based HSC-specific agents for conditioning.

Much of the prominent pioneering work studying the feasibility of antibodies as conditioning agents for HSCT was performed by research groups at Stanford University led by Irving Weismann and Judith Shizuru. In 2007, a landmark study published in Science Magazine described the use of a naked antibody directed against the CD117 stem cell factor (SCF) receptor (clone ACK2) present on mouse HSPCs to accomplish greater than 98% depletion of endogenous HSCs and donor chimerism up to 90% after HSCT in immunodeficient mice.[31] These findings were encouraging, however advancing this approach to achieve similar successes in immunocompetent mouse models proved more challenging. Although ACK2 antibody as a single agent was used successfully in an immunocompetent in utero HSCT model to achieve HSC depletion and engraft congenic bone marrow in fetuses, efficacy decreased when pups were conditoned postnatally.[32] Moreover, studies in adult immunocompetent mice demonstrated the need for additional conditioning agents such as low-dose irradiation or CD47 blocking agents to potentiate depletion and engraftment using ACK2.[33, 34] Nevertheless, recent findings reported by George et al. demonstrate that combinatorial approaches using cocktails of naked antibodies targeting CD117, CD47, T cells and NK cells can enable fully MHC-mismatched HSCT and donor-specific

immunological tolerance, highlighting the robust potential of a completely antibody-based conditioning protocol for HSCT.[35]

Immunotoxins or antibody-drug conjugates (ADCs) are leading novel strategies for increasing the specificity and potency of antibody-based cytotoxicity towards specific target cells, primarily in cancer therapeutic development. Immunotoxins and ADCs are bioconjugate drugs consisting of a targeting moiety, typically a monoclonal antibody, covalently linked to a cytotoxic small molecule drug or protein toxin (**Figure 7A**). Most immunotoxins or ADCs exert their cytotoxic effects by binding to a cell surface receptor, which is ideally specific for the cell type of interest, via the antibody targeting moiety and transporting the cytotoxic payload to the intracellular environment by receptor-mediated endocytosis. After entering the endocytic pathway, the payload will eventually be released by hydrolysis caused by decreases in the endosomal or lysosomal pH or cleavage by lysosomal proteases. Once released, the payload can escape the endolysosomal compartment and exert specific cytotoxic effects depending on the characteristics of the payload (**Figure 7B**). Small molecule drugs commonly used as ADC payloads include microtubule-disrupting agents (e.g., auristatin and maytansinoids) and DNA damaging agents (e.g., calicheamicin and doxorubicin). Protein toxins commonly used in immunotoxin development include ribosome-inactivating proteins (e.g., saporin, ricin A chain, gelonin), bacterial toxins (e.g., Diphtheria toxin, Pseudomonas exotoxin A), and RNA polymerase inhibitors (e.g., amanitin).[36-38]

Figure 7

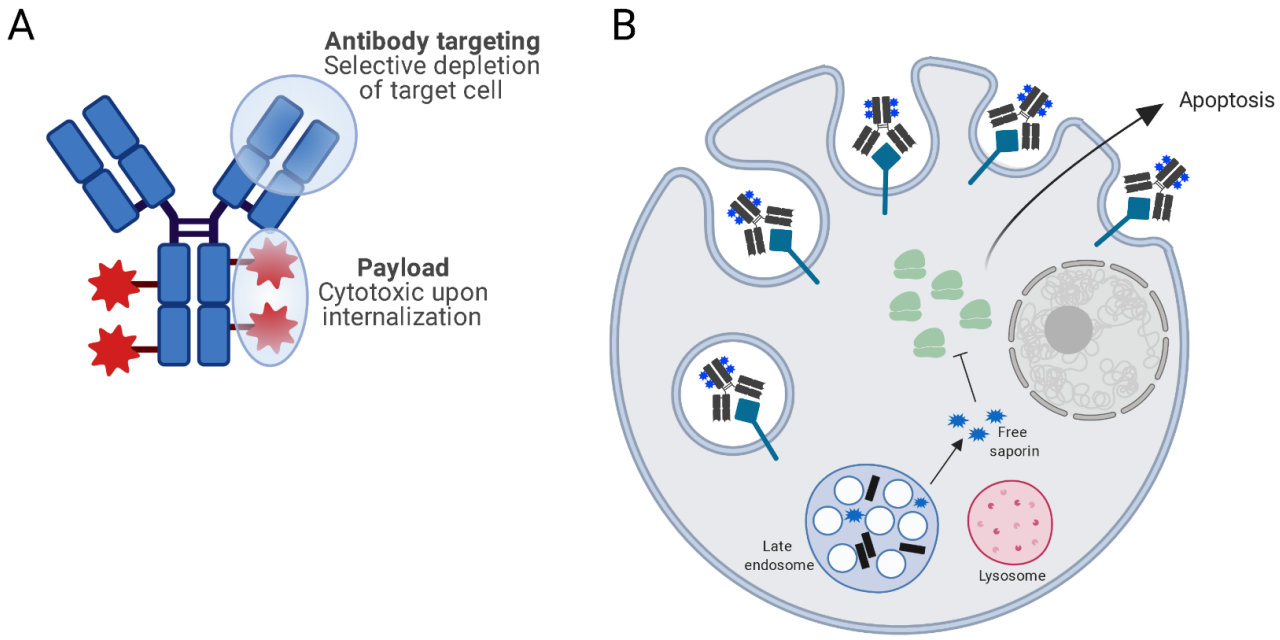


Figure 7. Immunotoxin structure and mechanism of action of saporin-based immunotoxin
(**A**) Immunotoxins and ADCs consist of a monoclonal antibody that is specific for a surface receptor on a cell of interest, biochemically conjugated to a cytotoxic drug or toxin payload. (**B**) Immunotoxins and ADCs exert their cytotoxic effects primarily by gaining access to the intracellular environment through receptor-mediated internalization. Payload linkers are hydrolyzed within the endocytic pathway, allowing the drug or toxin to escape the endolysosomal compartment and target various intracellular components depending on the particular mechanism of action of the payload. [Schematic created with Biorender.com]

The idea of using antibodies and antibodies linked to toxins as "magic bullets" to target specific disease-causing elements while sparing healthy cells and tissues was conceptualized over one hundred years ago by Nobel laureate Paul Ehrlich.[39] Clinical trials using mouse IgG antibodies began in the 1980s, and the first ADC to gain approval by the US Food and Drug Administration (FDA), gemtuzumab ozogamicin for acute myeloid leukemia, occurred in 2000. There are currently nine FDA-approved ADCs on the market, with more than half of those products having received approval within the last 2 years. Immunotoxin/ADC research has expanded beyond the realm of cancer therapeutics towards non-oncological applications such as antibody-antibiotic

conjugates for *Staphylococcus aureus* infection, ADCs for rheumatoid arthritis, and immunotoxin conditioning agents for HSCT.[40-43]

In the early 1990s, research efforts directed at developing lower-toxicity conditioning agents for HSCT that take advantage of antibody targeting led to the development of radioimmunotherapy (RIT) compounds by investigators at Fred Hutchinson Cancer Research Center.[44] RIT agents consist of monoclonal antibodies conjugated to radioisotopes, with the intent of delivering radiation specifically to malignant cells, enhancing anti-tumor efficacy while minimizing toxicity to normal tissues. Two RIT drugs are FDA-approved for use as part of HSCT conditioning regimens for B cell non-Hodgkin's lymphoma (Zevalin®: anti-CD20-^{90}Yttrium conjugate and Bexxar®: anti-CD20-^{131}Iodine conjugate). Many other RITs targeting antigens such as CD45, CD33 and CD66, and conjugated to various α-, β-, or γ-emitting radionuclides, have been tested in clinical studies for HSCT conditioning for both malignant and non-malignant disease.[45] However, these compounds as conditioning agents for HSCT are often used in combination with standard alkylating conditioning agents rather than as single agents.

Significant advances in the application of immunotoxins for HSCT conditioning have been made in the last five years. A seminal article was published in 2016 from researchers at Harvard University describing the use of a CD45-saporin immunotoxin to successfully condition immunocompetent mice for HSCT and achieve phenotypic correction in a sickle cell disease model.[46] Momentum in this space is progressively building. Back-to-back reports were published in 2019 from the research groups of Agnieszka Czechowicz and Derrick Rossi. One report describes the use of CD117-saporin immunotoxin to prepare immunocompetent mice for congenic

or syngeneic transplants while preserving immune function against viral and fungal pathogens, as well as the ability of anti-human CD117-saporin immunotoxin to deplete human HSCs from UCB in a xenograft experiment. The second report describes the use of CD117-saporin immunotoxin combined with a regimen for transient immune suppression consisting of antibodies against CD4, CD8 and CD40L, and rapamycin to accomplish full MHC-mismatched HSCT and donor-specific tolerance of skin allografts.[47] The same year Gao et al. reported a protocol that combined three saporin-based immunotoxins directed towards CD45.2, CD117 and CD8 to achieve engraftment of lentiviral vector (LV)-transduced HSPCs modified to express a platelet-specific factor VIII transgene in a murine hemophilia A model.[48] The following year two additional studies were reported employing immunotoxin conditioning for HSCT in different disease models. One publication describes the use of CD45-saporin with or without low-dose radiation in mouse models of severe combined immune deficiency and combined immune deficiency with immune dysregulation. Though optimal results were obtained combining CD45-saporin with 2 Gy TBI, immunotoxin treatment alone still resulted in 60% donor chimerism, full T and B cell reconstitution, and correction of humoral immune responses.[49] The second report describes the use of either CD45-saporin or CD117-saporin immunotoxins in a Fanconi anemia murine model; both agents were shown to facilitate multilineage engraftment of heterozygous donor cells as least as well as cyclophosphamide.[50] These findings build on previous work that showed the efficacy of naked CD117 (ACK2) antibody to enable HSCT in Fanconi mice.[51] In addition to these peer-reviewed studies, there has been an outpouring of preliminary reports (abstracts and preprints) describing the use of CD45 or CD117 immunotoxins for conditioning in HSCT mouse, non-human primate, and human xenograft models.[52-61]

The value and exciting potential for the development of non-genotoxic, antibody-based conditioning have been realized in the cell and gene therapy community. This is evidenced by the establishment of multiple private companies that are dedicated to the development and clinical translation of these novel therapies (Magenta Therapeutics, Inc. and Jasper Therapeutics, Inc.). Furthermore, multiple clinical trials are currently underway testing a human CD117 antibody (JSP191) for HSCT conditioning in patients with myelodysplastic syndrome or acute myeloid leukemia (ClinicalTrials.gov Identifier: NCT04429191), severe combined immune deficiency (ClinicalTrials.gov Identifier: NCT02963064), and Fanconi anemia (ClinicalTrials.gov Identifier: NCT04784052). Non-genotoxic antibody-based approaches are now being widely recognized as the future of HSCT conditioning.

1.1.5 Hematopoietic Stem Cell Gene Therapy

Inherited hematologic diseases, immune deficiencies, bone marrow failure disorders, and some inborn errors of metabolism can be cured with allogeneic HSCT. Despite this, allogeneic HSCT comes with its own risks. Outcomes are typically favorable when an HLA-identical sibling donor is available, however this is the case for only about 30% of patients in need of transplantation.[62] As HLA-matching between donor and recipient decreases, the risks of graft failure and GVHD increase. Both situations leave the patient vulnerable to severe complications and may cause transplant-related morbidity and mortality. In order to attenuate these risks, the recipient must undergo high levels of immune suppression before and after the procedure, which exacerbates morbidity risks. Moreover, chemotherapeutic drugs used for bone marrow ablation to prepare for donor cell engraftment are genotoxic and may cause malignancy. These risks therefore limit the

widespread application of allogeneic HSCT for the treatment of many non-malignant genetic diseases.[63]

An alternative approach involves the use of a patient's autologous HSCs that have been genetically modified to express a functional copy of the defective protein that is responsible for the phenotype of the underlying condition. The long-term repopulating HSC population in bone marrow is responsible for the continuous production of all blood cells. Genetic modification of these self-renewing, repopulating cells can guarantee life-long expression of the therapeutic gene product in these cells and impart permanent correction of the disease phenotype. This approach, referred to as hematopoietic stem cell gene therapy (HSC-GT), has evolved significantly over the last 30 years and the promise it holds for the treatment or cure of many rare and common genetic diseases cannot be overstated.

In HSC-GT, autologous bone marrow HSPCs are harvested from the patient, either directly from the posterior iliac crest or from mobilized peripheral blood by leukapheresis. The HSPC product is processed under sterile conditions and the $CD34^+$ HSPC population is enriched by immunomagnetic bead selection, cultured and activated in the presence of growth factors ex vivo. The cells are then transduced with a viral vector harboring the therapeutic transgene. The modified HSCs are harvested and transfused intravenously into the pre-conditioned recipient, after which the stem cells will home to vacant bone marrow niches to take up residence and reconstitute hematopoiesis with the new gene-corrected cells (**Figure 8**).

Figure 8

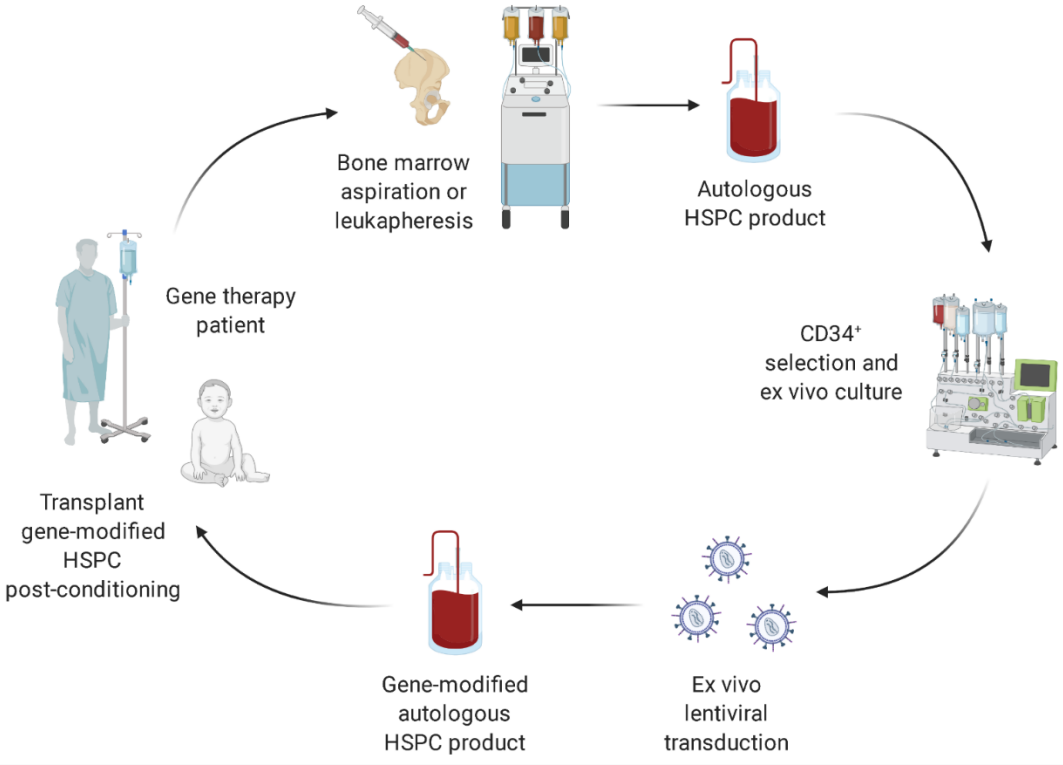


Figure 8. Ex vivo autologous hematopoietic stem cell gene therapy
Autologous bone marrow derived HSPCs are collected from the patient directly from the bone marrow or from the peripheral blood following mobilization. $CD34^+$ cells are enriched by immunomagnetic bead selection and activated in ex vivo culture. Cells are transduced with the gene therapy vector, after which they are harvested and infused back into the patient after the conditioning regimen. [Schematic created with Biorender.com]

Gene transfer methods for modification of HSCs using viral vectors began in the 1980s. Because of the frequency with which HSCs divide during the lifespan of an individual, permanent modification of the cellular DNA is the most efficient approach to ensure life-long expression of the therapeutic protein. Viral vectors based on retroviruses have been used most often for this purpose due to their DNA integrative capabilities. Initial vectors were based on the γ-retrovirus murine leukemia virus (MLV), which require breakdown of the nuclear envelope to gain nuclear entry for DNA modification, a challenge for HSC modification due to HSC quiescence and

infrequent entrance into the cell cycle. In 1992, the first HSC-GT clinical trial began for the treatment of adenosine deaminase severe combined immune deficiency (ADA-SCID) using MLV-based γ-retroviral vectors with great success.[63] Strimvelis™, an HSC-GT product that uses a γ-retroviral vector to introduce a functional ADA gene into autologous HSCs, became Europe's first market approved ex vivo HSC-GT for treatment of ADA-SCID in 2016.[64] However, subsequent trials using MLV-based γ-retroviral vectors for treatment of X-linked SCID encountered serious obstacles after 30% of patients (6 out of 20) developed acute lymphoblastic leukemia (ALL) due to integration of the vector upstream of proto-oncogenes and ectopic activation of these genes in T cells.[65]

Since then, lentiviral vectors (LVs) based on human immunodeficiency virus (HIV), developed by Luigi Naldini, Didier Trono and Inder Verma at the Salk Institute in 1996, have become the predominant gene therapy vector used for modification of HSCs.[66, 67] A distinct advantage of LVs is their ability to access the nuclear compartment in the absence of cell division and therefore, upon transduction, can more efficiently modify genomic DNA in quiescent cells. Improvements were progressively made to LV design to increase safety, inhibit viral replication, and prevent ectopic promoter and enhancer activity observed from γ-retroviral vectors. These self-inactivating (SIN) vectors have deletions in the U3 region of the 3' long terminal repeat (LTR), and all accessory genes from the wild-type virus that support HIV virulence and replication have been removed.[68] The current third-generation SIN LVs have become the vectors of choice for ex vivo HSC-GT applications (

Figure 9).

Figure 9

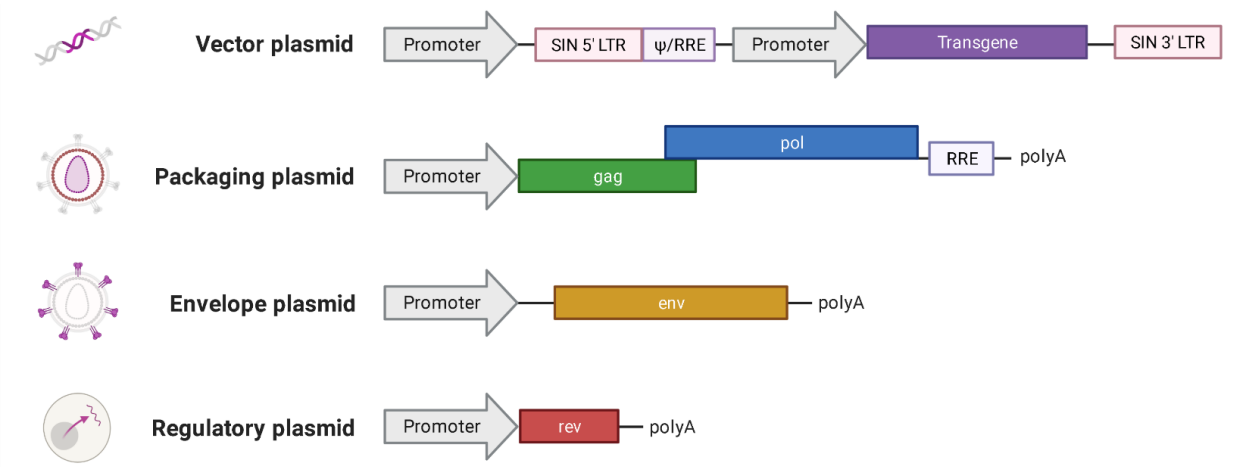


Figure 9. Third-generation lentiviral vector system
The third-generation system features separate plasmids harboring packaging, regulatory and envelope genes, as well as a vector plasmid harboring the transgene of interest and an internal promoter sequence. All other genes involved in viral propagation are deleted. Deletion in the 3' LTR U3 is transferred to the 5' end after reverse transcription and integration to create a replication incompetent, self-inactivating system. [Schematic created with Biorender.com]

The first HSC-GT clinical trials using LVs targeting primary immune deficiencies, X-linked SCID, Wiskott-Aldrich Syndrome, and the lysosomal storage disease metachromatic leukodystrophy (MLD) began more than ten years ago. Since then, HSC-GT clinical trials for over a dozen genetic diseases, primarily using LV modification, have been tested in clinical trials (**Table 3**). As of December 2020, three HSC-GTs have received market approval from the European Medicines Agency (**Table 4**) with many more in advanced stages in the United States.[69]

Table 3

Disease	Gene	ClinicalTrials.gov Identifiers
X-linked SCID (SCID X1)	IL2RG	NCT03311503 NCT03601286 NCT03217617 NCT01306019
Adenosine deaminase SCID (ADA-SCID)	ADA	NCT02999984 NCT02022696 NCT01852071 NCT03765632 NCT04140539 NCT03645460
Wiskott-Aldrich Syndrome (WAS)	WAS	NCT01347242 NCT01410825 NCT03837483 NCT01515462 NCT02333760
X-linked chronic granulomatous disease (X-CGD)	gp91phox	NCT01855685 NCT02757911 NCT02234934
Leukocyte adhesion deficiency (LAD)	CD18	NCT03812263 NCT03825783
ARTEMIS SCID	DCLRE1C	NCT03538899
Transfusion-dependent β-thalassemia (TDT)	HBB	NCT03207009 NCT02906202 NCT02453477 NCT03728322
Sickle cell disease (SCD)	HBB	NCT02140554 NCT03282656 NCT02151526 NCT02186418 NCT03964792 NCT02247843 NCT04091737
Fanconi anemia	FANCA	NCT03157804
Metachromatic leukodystrophy (MLD)	ARSA	NCT01560182 NCT03392987
X-adrenoleukodystrophy (X-ALD)	ABCD1	NCT01896102 NCT03852498
Mucopolysaccharidosis type I (MPS I)	IDUA	NCT03488394
Mucopolysaccharidosis type III (MPS III)	SGSH	NCT04201405
Fabry disease	GLA	NCT02800070 NCT03454893
Hemophilia A	F8	NCT04418414 NCT03818763 NCT03217032

Table 3. Genetic diseases treated in HSC-GT clinical trials registered on ClinicalTrials.gov as of March 2021. HSC-GT therapies have been tested for over a dozen diseases in clinical trials, most using LV for ex vivo genetic modification of autologous HSCs. Adapted from Staal et al.[70] and Tucci et al.[69]

Many exciting successes and promising outcomes have been achieved with regard to safety and efficacy in dozens of clinical trials employing LV for HSC-GT in a variety of disease settings. However, though the removal of strong LTR enhancers and the benign rather than oncogenic integration bias of LV as opposed to γ-retroviral vectors[71] have substantially improved the risks of insertional oncogenesis, the potential for genotoxicity caused by quasi-random integration of LV vectors within the genome has not been entirely eliminated. Indeed, two ongoing HSC-GT clinical trials sponsored by bluebird bio, Inc. were recently suspended due to reports of two patients who developed either acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) after receiving LentiGlobin LV gene therapy for sickle cell disease. In addition, Zynteglo™, which is manufactured using the same LV, was temporarily suspended from the market while these suspected unexpected serious adverse reactions were investigated.[72] After careful investigation, it was determined that the development of AML was unlikely to be associated with vector integration.[73] Still, these cases are a stern reminder that the development of improved vector designs that address these rare risks of adverse outcomes from LV insertion must continue to be pursued.[74]

Table 4

Product	Disease	Gene	Date of EMA Approval
Strimvelis™	Adenosine deaminase SCID (ADA-SCID)	ADA	May 26, 2016
Zynteglo™	Transfusion-dependent β-thalassemia (TDT)	HBB	May 29, 2019
Libmeldy™	Metachromatic leukodystrophy (MLD)	ARSA	December 17, 2020

Table 4. HSC-GTs that have received market approval in the European Union. Three therapeutic products based on the genetic modification and transplantation of autologous HSCs have been approved for use by the European Medicines Agency. Strimvelis uses a γ-retroviral vector, while Zynteglo and Libmeldy use LVs.

1.2 Hemophilia A

1.2.1 Hemophilia A Background

Hemophilia A (HA) is the most common bleeding disorder, affecting approximately 1:4000 male births. It is an X-linked recessive congenital disease, caused by mutation in the *F8* gene, leading to deficiency of the blood coagulation protein factor VIII (fVIII). HA is characterized by life-threatening protracted and excessive bleeding either spontaneously or secondary to trauma due to insufficient levels of functional fVIII activity in circulation.[75] This disease is classified according to clinical severity based on levels of residual fVIII activity. Normal fVIII concentration in plasma is defined as 1 IU/ml, which correlates to 100% normal fVIII activity. Patients with mild hemophilia have 0.05 – 0.40 IU/ml fVIII (5-40% normal levels) and typically bleed after significant trauma or surgery. Moderate hemophilia is classified as 0.01 – 0.05 IU/ml fVIII (1-5% normal levels) and may bleed after minimal trauma, injury, dental work, or surgery. Patients with severe hemophilia have less than 0.01 IU/ml fVIII (less than 1% normal levels). These patients experience spontaneous internal bleeding into muscles, soft tissues, organs and joints

(hemarthrosis) which leads to a progressive, debilitating arthropathy due to repeated joint bleeds. Patients with severe disease are also at risk for spontaneous, life-threatening bleeds into enclosed spaces, such as the cranial cavity. Intracranial hemorrhage is the most serious complication of severe HA, including during the neonatal period, resulting in high rates of disability and mortality.[76] Severe HA often manifests during the first few months of life, while moderate and mild cases may not present until later in childhood and adolescence. Bleeding from circumcision during the neonatal period, high incidence of bruising and contusions as children learn to ambulate, and musculocutaneous hemorrhage following intramuscular vaccination are characteristic presentations of severe HA in early childhood.[77]

1.2.2 Factor VIII and Its Role in Blood Coagulation

FVIII is a non-enzymatic plasma glycoprotein that is essential for normal blood coagulation. It is primarily synthesized in hepatic sinusoidal cells, although production in various cell types outside the liver has also been identified.[78] The *F8* gene is located at the distal end of the long arm of the X chromosome in band q28, spanning 186 kb. It consists of 26 exons and encodes a precursor polypeptide that is 2332 amino acids in length, plus a 19 amino acid signal peptide important for secretion. FVIII is synthesized as a 330 kDa single chain polypeptide containing three homologous A domains, two homologous C domains and one B domain, arranged as A1-A2-B-A3-C1-C2.[79] The N-terminal signal peptide is cleaved upon translocation into the endoplasmic reticulum and the single chain precursor molecule is cleaved in the B domain for secretion. The result is a heterodimer consisting of a 200 kDa heavy chain (A1-A2-B) and an 80 kDa light chain (A3-C1-C2) that are covalently linked.[78] Each domain contains binding sites for specific components of the coagulation cascade and therefore plays an important role in fVIII functionality.[79]

FVIII is a key component of secondary hemostasis (**Figure 10**). Hemostasis refers to the physiological process by which bleeding at the site of vascular injury is contained by the formation of a clot. Primary hemostasis involves the aggregation of platelets and formation of a platelet plug. Secondary hemostasis involves soluble proteins in plasma that generate a coagulation cascade, resulting in the formation of an insoluble fibrin clot. The two processes occur concomitantly and are tightly regulated, which allows the overall process of hemostasis to occur very rapidly upon injury and to ensure the process is confined to the site of injury.[80]

Upon secretion into the bloodstream, fVIII forms a stable noncovalent complex with von Willebrand factor (vWF). This association is critical for platelet adhesion and aggregation. vWF plays an important role in targeting fVIII to injured vasculature and concentrating it at exposed subendothelium. FVIII is activated (fVIIIa) by proteolytic cleavage of both the heavy and light chains by thrombin (fIIa), releasing the B domain. The fVIII heterodimer is converted to fVIIIa, a 165 kDa heterotrimer comprising A1, A2, and A3-C1-C2 domains. Cleavage in the light chain results in dissociation of vWF while exposing a phospholipid binding region necessary for cofactor activity in its activated form.[81]

FVIIIa serves as a procoagulant, glycoprotein cofactor of fIXa. Their association in the presence of Ca^{2+} ions and negatively charged phospholipids on the surface of activated platelets sets the stage for activation of the substrate fX in the tenase complex. Concurrently on the activated platelet surface, the prothrombinase complex can form between fXa and its glycoprotein cofactor fVa, with enzymatic activity towards prothrombin (fII), leading to activation and formation of thrombin

(fIIa). Finally, thrombin catalyzes the conversion of fibrinogen (fI) to fibrin (fIa), while also activating fXIII, leading to the formation and stabilization of a fibrin clot.[82]

Figure 10

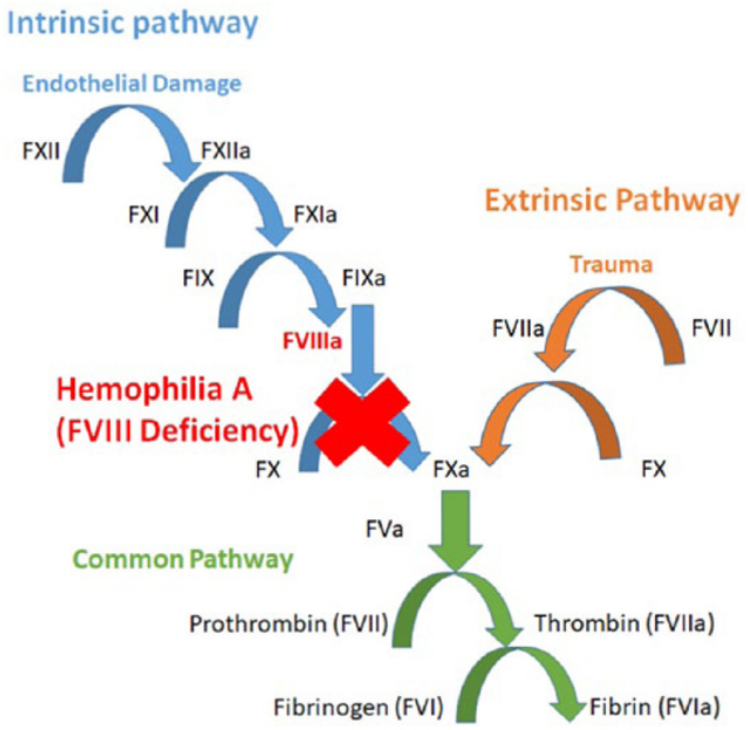


Figure 10. Intrinsic and extrinsic pathways of the coagulation cascade (secondary hemostasis) Hemophilia A leads to deficiency or absence of coagulation factor VIII, disrupting the ability to form a fibrin clot in response to tissue damage. [Schematic originally appeared in Moreira and Das.[83] Licensed under CC BY 4.0]

1.2.3 Current and Developing Therapies for Hemophilia A

Standard treatment for HA consists of management of acute bleeding and bleeding prophylaxis, both of which are treated with exogenous fVIII replacement products.[77] Prophylaxis can prevent episodes of hemathrosis and reduce risks of cerebral and soft tissue bleeds. It is considered a superior strategy over episodic management of bleeds and therefore represents the current standard

of care.[84] A major limitation of this strategy, however, is that fVIII has a short half-life of only about 12 hours, and therefore repeated intravenous infusions several times per week are necessary to maintain hemostasis. The implications of this include very high costs of factor replacement products, impact on patient quality of life and poor compliance, and ensuing problems with venous access.[84] Estimates of average annual direct medical costs for patients with mild to severe hemophilia exceed $200,000 per year, with 92% of costs attributed to factor replacement products.[85] Furthermore, in part due to high costs, approximately 80% of persons with hemophilia globally have no access to factor replacement products and remain untreated, particularly in the developing world.[86]

Recent efforts were made at developing newer recombinant version of fVIII with extended half-life (EHL), either by fusion of the protein with IgG1 Fc or albumin to encourage recycling through the neonatal Fc receptor (FcRn), or by slowing fVIII degradation and elimination through conjugation with chemicals such as polyethylene glycol (PEG). However, since the half-life of fVIII is inherently dependent on the half-life of its chaperone in blood vWF, these EHL modifications could only increase fVIII half-life less than two-fold, and therefore only decreases the burden of intravenous factor administrations from three injections per week to two.[84] Chhabra et al. have recently described a new class of fVIII replacement, termed BIVV001, that consists of B-domain deleted fVIII, the fVIII binding domain of vWF, Fcγ1 fragment, and longer half-life XTEN™ polypeptides. This new therapeutic chimera is independent from endogenous vWF, is hemostatically competent, and has an extended half-life of 25-31 hours in mice and 33-34 hours in monkeys.[87, 88]

Up to 40% of patients with severe HA will develop anti-fVIII neutralizing antibodies, referred to as inhibitors, after receiving fVIII replacement products.[89] The presence of inhibitors represents the most challenging complication to HA treatment, as bleeding episodes become much more difficult to manage and the costs of care increase significantly.[90] Immune tolerance induction (ITI) is the standard of care to eradicate inhibitors in patients who have developed them. However, ITI is only effective at eliminating inhibitors in about 70% of patients[91] and the long treatment period (several months to over a year), frequent infusions by central venous catheter, and extremely high costs are major drawbacks of the procedure.[92] Costs for ITI can exceed $1-4 million dollars per patient depending on prognosis.[85] Bypassing agents have been developed that restore hemostasis by circumventing fVIII in the coagulation cascade, and these have become the standard of care for prophylaxis and treatment of bleeding episodes in patients with inhibitors.[93] There are two currently available bypassing agents: recombinant fVIIa and activated prothrombin complex concentrate (aPCC). These agents have shown approximately 80% efficacy, but require increased frequency of infusions or large infusion volumes[90] and are very expensive.[94]

Ongoing efforts to design a therapeutic strategy that would not rely on factor replacement led to the development and market approval in 2017 of a bispecific monoclonal antibody, emicizumab, that mimics the cofactor function of fVIIIa in the tenase complex through high-affinity binding of fIXa and fX, promoting the generation of thrombin (**Figure 11**). Subcutaneous administration and longer half-life, allowing for 1–2-week dosing intervals, are distinct advantages offered by emicizumab. Despite its high cost, its licensing for use both in patients with or without inhibitors make it an important alternative to traditional and EHL factor replacement products. However, questions still remain regarding long-term comparisons of emicizumab and true fVIII products in

supporting joint health and wound healing.[84] Additionally, thrombotic complications have been observed when used in conjunction with aPCC.[95]

Figure 11

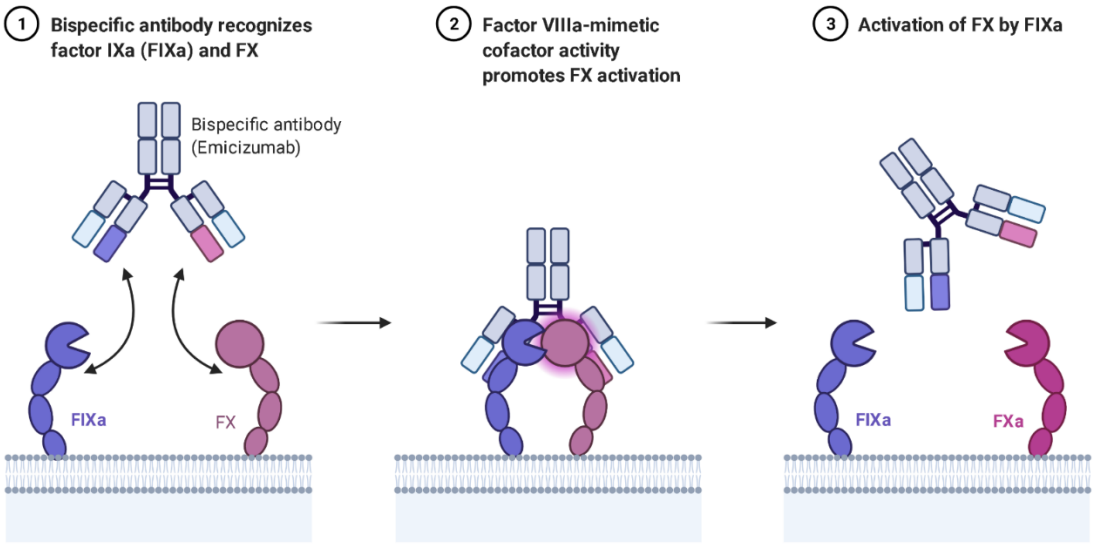


Figure 11. Mechanism of action of emicizumab
Emicizumab acts as a fVIIIa mimetic, binding fIXa and fX with high affinity, thereby promoting fX activation and downstream thrombin formation. [Schematic created with Biorender.com]

Other non-factor-based therapeutic approaches are in clinical or preclinical development. These agents have been designed to increase the formation of thrombin through suppression of coagulation inhibitors. Fitusiran is a short-interfering RNA (siRNA) targeting antithrombin messenger RNA in liver, thereby reducing antithrombin levels. Fitusiran is currently being tested in several phase III trials. Tissue factor pathway inhibitor (TFPI) is a protein that inhibits fXa, as well as the tissue factor/fVIIa complex of the extrinsic pathway. Several monoclonal antibodies that inhibit TFPI have been developed. One example, concizumab, is also currently in phase III trials.[96] Finally, activated protein C (APC) plays an important role in anticoagulation by

inactivating fVIIIa and fVa. Studies performed using a bioengineered protein KRK-α1AT in mice have demonstrated specific and irreversible inhibition of APC.[97]

1.2.4 Gene Therapy for Hemophilia A

Despite notable advances in factor- and non-factor-based therapies for HA, prohibitive costs, repeated injections, inhibitor development, and lack of universal efficacy remain significant hurdles to overcome and leave much room for improvement. The idea of using gene therapy to correct fVIII deficiency has been contemplated since the fVIII gene was cloned in the 1980s. Early research efforts developing gene therapy for hemophilia focused primarily on hemophilia B (HB) and correcting fIX gene expression, and results of the first human fIX clinical trials were published in 2011.[98] Gene therapy for HA has lagged behind HB until recently, in part due to its substantially larger cDNA size compared with fIX (9 kb versus 1.5 kb) which complicated packaging of the full-length transgene into standard gene therapy vectors. However, major strides have been made in the last several years and it is now possible that the first market approved hemophilia gene therapy product may be for HA.[99]

There are two primary transgene delivery approaches being pursued for HA gene therapy: adeno-associated viral (AAV) vectors and LVs. AAV vectors are the predominant strategy with the most long-term data available from clinical studies; it is likely that the first licensed product will be based on the AAV platform. The overarching goal of an AAV gene therapy for HA would be a single systemic vector administration which, due to tissue tropism of the specific AAV serotype, would deliver the therapeutic transgene to liver hepatocytes. Several hepatotropic varieties have been used for hemophilia gene therapy including AAV2, AAV5, AAV6, AAV8 and AAV10.[99]

One problem with this approach is the incidence of naturally occurring AAV neutralizing antibodies in the general population. Depending on ethnicity and geographical location, between 30-80% of individuals have antibodies to at least one of the AAV serotypes, and because of this pre-existing immunity approximately 50% of patients who would otherwise be eligible for an AAV-based gene therapy are considered ineligible.[99] Furthermore, due to immune responses to AAV capsid that occur after intravenous delivery of large vector doses, as well as cross-reactivity of anti-AAV antibodies across serotypes, AAV gene therapy could most likely only be administered one time. Therefore, the durability of the therapeutic effect after a single AAV administration is paramount. The most advanced gene therapy for HA in clinical studies is an AAV5-based investigational product developed by BioMarin Pharmaceutical, Inc. which is currently in phase I/II and phase III trials. Three years of data from the phase I/II study revealed substantial year after year declines in fVIII transgene expression following administration, prompting the FDA to issue a complete response letter denying market approval until further follow-up safety and efficacy data can be collected.[100] In addition to uncertain durability, differences observed between the phase I/II and phase III results raise further questions regarding the degree of variability and predictability of the therapeutic effect.[101]

AAV is considered a largely non-integrating vector, with the transgene persisting episomally in target cells (**Figure 12**), although genomic integrations have been observed, the significance of which has yet to be fully elucidated. The advantage of non-integration is an apparent decreased risk of insertional mutagenesis resulting from oncogenic genomic integration. Investigations of integration patterns of AAV sequences in preclinical and clinical studies have shown that these integrations occur in intergenic regions more than 90% of the time,[99] however a recent report of a

long-term HA AAV study in dogs showed substantial vector integration in liver biopsy samples and clonal expansion in 5 out of 6 dogs assessed.[102] Although none of the dogs showed evidence of neoplasm or altered liver function, 44% of integrations were found near cell growth genes, highlighting the importance of long-term follow-up to monitor potential genotoxic risks. An additional consideration regarding the episomal nature of AAV gene delivery is the impact of cell division on dilution of the transgene, which could have implications for the applicability of AAV approaches in children whose livers have yet to fully develop.

Figure 12

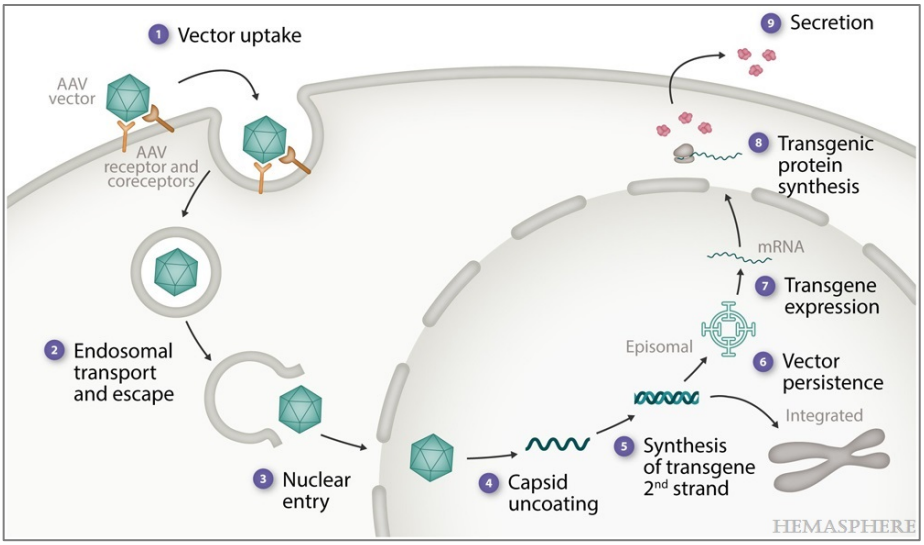


Figure 12. AAV vector gene delivery
AAV binds surface receptors that mediate endocytosis of the vector particle. AAV escapes the endosomal compartment, enters the nucleus, and capsid uncoating leads to release of the transgene. The single-stranded DNA template becomes double-stranded and persists in the nucleus indefinitely as an episome or may integrate into the genome at some frequency. Transgenic messenger RNA is transcribed and exported to the cytoplasm where protein synthesis takes place, followed by secretion of the transgene product. [Schematic originally appeared in Batty and Lillicrap.[99] Licensed under CC BY 4.0]

Finally, although acute adverse effects are rare, AAV vector delivery is known to cause transient liver toxicity in approximately 60% of subjects 4-12 weeks after administration.[99] Increases in liver enzymes are typically mild to moderate, however a negative effect on plasma factor levels is observed, secondary to hepatocyte death, though the pathogenic mechanisms are not yet understood. Recently, three deaths occurred among children enrolled in a high-dose AAV8 clinical trial for X-linked myotubular myopathy due to progressive liver dysfunction.[103, 104] Vector doses in this study were significantly higher than those used in human trials of hemophilia and the children that died had pre-existing intrahepatic cholestasis. Still, these unfortunate experiences underscore the critical need to better understand the short- and long-term effects of AAV transduction on liver cells so these devastating outcomes can be avoided.

The second approach to transgene delivery being pursued in HA clinical trials is ex vivo LV modification of autologous HSC. As discussed in depth in section 1.1.5, HSC-GT using LV has many advantages, including stable integration in the genome of long-term repopulating HSCs, ensuring permanent transgene expression from hematopoietic daughter cells for the lifetime of the individual. In the setting of HA, HSC-GT is an attractive and promising strategy towards durable expression of circulating fVIII from blood cells. In contrast to AAV, transgene dilution is not a concern and therefore the procedure would be effective in the pediatric population. Importantly, HSC-GT allows for the development of immunological tolerance to the highly immunogenic fVIII protein.[105] This is a distinct advantage of LV gene therapy approaches over AAV for HA. The ability of HSC-GT to induce tolerance to fVIII may also have important implications for treatment of HA patients with inhibitors.

After much anticipation, human trials testing HSC-GT using LV for HA are now being set in motion. There are currently three studies registered on ClinicalTrials.gov (**Table 3**). One study (ClinicalTrials.gov Identifier: NCT04418414) involves mobilized autologous peripheral blood $CD34^+$ HSPCs that will be modified ex vivo with recombinant LV harboring a high-expression bioengineered fVIII variant, termed ET3, driven by the myeloid-specific CD68 promoter to drive transgene expression in monocytes and macrophages. Patients will receive busulfan for bone marrow conditioning and anti-thymocyte globulin for transient immune suppression in the early post-transplantation period to prevent anti-fVIII immune responses while transgene-specific tolerance is being established. A second study (ClinicalTrials.gov Identifier: NCT03818763) will use mobilized autologous peripheral blood $CD34^+$ HSPCs modified ex vivo with LV harboring B-domain deleted human fVIII driven by a platelet-specific promoter with the goal of expressing and storing fVIII in platelets until activation and release at the site of tissue injury. Patients in this study will receive reduced intensity conditioning with melphalan and fludarabine in preparation for HSCT. This study will test safety and feasibility of this approach in HA patients with pre-existing high titer fVIII inhibitors. The third study (ClinicalTrials.gov Identifier: NCT03217032) employs a modified LV system, termed NHP/TYF,[106] to deliver a functional fVIII transgene to autologous HSCs and mesenchymal stem cells. No clinical data have yet been reported from these groundbreaking first-in-human studies.

1.3 Ataxia Telangiectasia

1.3.1 Ataxia Telangiectasia Background

Ataxia telangiectasia (AT) is a rare, autosomal recessive disorder caused by mutations in the ataxia telangiectasia mutated (ATM) gene. It is widely referred to as a genome or chromosomal instability

syndrome, DNA repair disorder, or DNA damage response syndrome. The disease affects approximately 1:40,000 – 1:100,000 live births with equal prevalence in males and females.[107] AT is a complex, pleiotropic disorder with multisystem involvement and highly variable disease presentations across individuals. Although phenotypic manifestations span a wide spectrum, classical AT disease is characterized by progressive neurodegeneration, cerebellar ataxia, oculocutaneous telangiectasia, immune deficiency, predisposition to malignancy, recurrent sinopulmonary infection, and radiation/radiomimetic sensitivity. Other common presentations include growth retardation, gonadal atrophy and sterility, premature aging, and insulin resistant diabetes.[108] Elevated α-fetoprotein levels in blood are also characteristic of AT and is a reliable clinical marker.[109] The disease typically manifests within the first five years of life with cerebellar ataxia as the first symptom, and progression of movement disorders leads to patients becoming wheelchair bound by their second decade. Death usually results from decline in pulmonary function secondary to recurrent respiratory infection or lymphoid cancer. Mild or atypical AT is associated with less severe disease manifestations, later onset, and longer survival. Certain ATM mutations, such as missense rather than truncating variants, allow higher levels of residual ATM protein kinase activity and are therefore associated with a milder, less debilitating clinical course.[110, 111]

Spinocerebellar neurodegeneration is the hallmark of AT. Progressive loss of Purkinje cells and granule neurons in the cerebellar cortex is the primary feature. The exact mechanisms leading to neurodegeneration are unknown, though the cause is likely multifactorial relating to defects in the DNA damage response, the cellular response to oxidative stress and inflammation, mitochondrial dysfunction, and defective cell cycle and epigenetic regulation.[107, 112] Immunodeficiency is

variable in AT, but usually involves lymphopenia, especially low T cells, and hypogammaglobulinemia, especially IgA, IgE, and IgG2, whereas IgM may be elevated. V(D)J recombination and class-switch recombination are critical functions for proper lymphocyte maturation and normal levels of immunoglobulin isotypes. Immune system abnormalities therefore result from the dependence of these functions on DNA double-strand break (DSB) repair processes that are defective in AT. Moreover, inability to appropriately repair programmed DNA DSBs in lymphocytes leads to increased risk of lymphoid malignancy.

1.3.2 ATM Kinase: Central Role in DNA Double-strand Break Repair and Other Diverse Functions

ATM is a serine/threonine protein kinase belonging to a family of phosphatidylinositol 3-kinase-related protein kinases (PIKKs) that is highly conserved from yeast to humans.[113] It is a large protein of 3056 residues and a molecular weight of approximately 350 kDa. ATM functions at the apex of the DNA DSB signaling cascade and coordinates the resulting cellular responses that lead to cell cycle arrest, DNA repair, or apoptosis.[110] Therefore, ATM serves a critical function in maintaining DNA and chromosomal integrity. The key role of ATM in the DNA DSB repair pathway is well characterized and may be considered its primary function, however an extensive map of ATM functional interactions, including over 1000 putative substrates, highlights the broad scope of ATM involvement in numerous and diverse cellular pathways and directly reflects the complex spectrum of phenotypic manifestations and multisystem involvement in AT (**Figure 13**).[114, 115] Many ATM substrates, such as p53 and BRCA1, are tumor suppressors that play essential roles in cell cycle regulation, underscoring the important relationship between ATM signaling, genomic integrity, and tumorigenesis.[110]

Figure 13

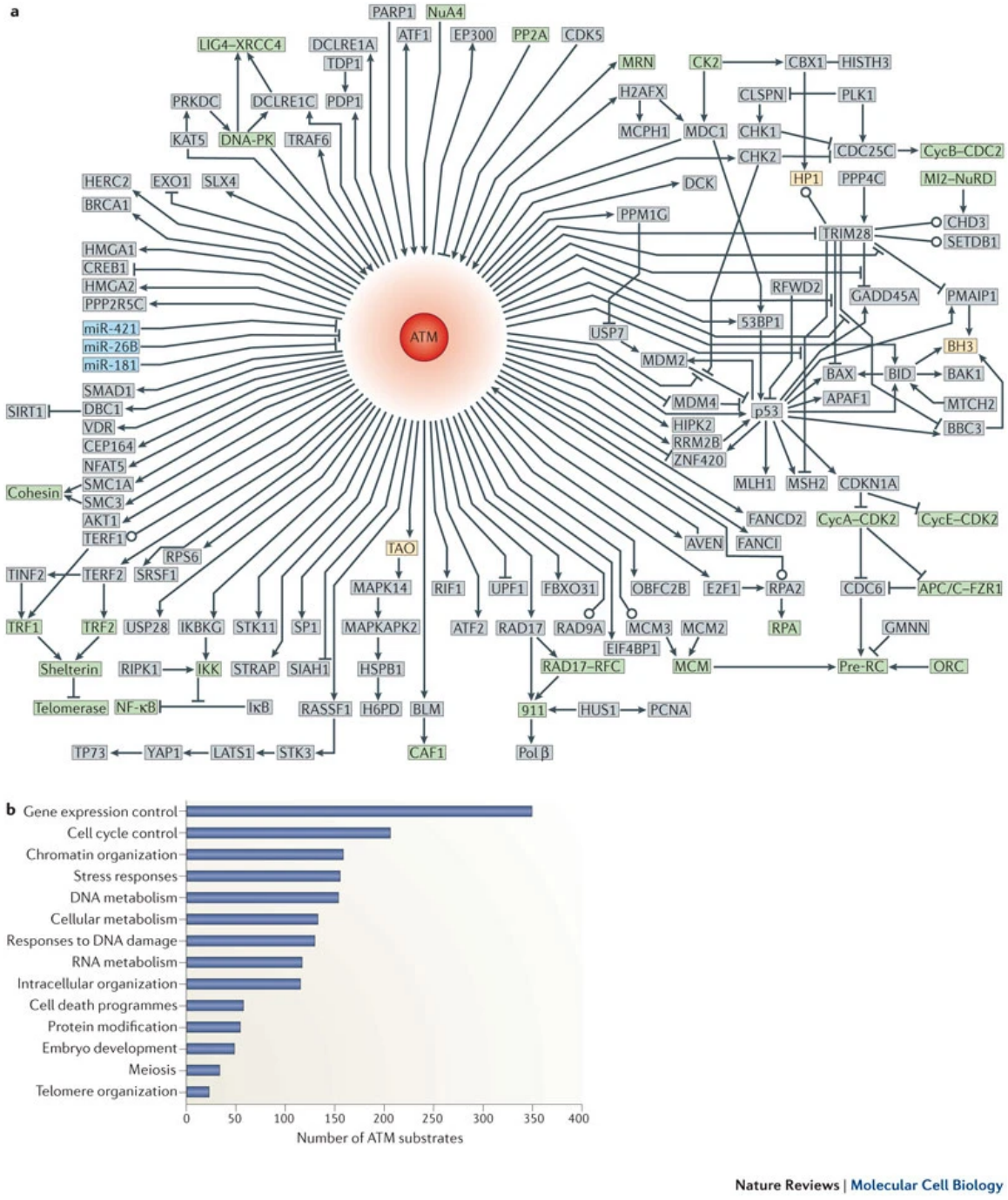


Figure 13. Broad scope of ATM functional interactions and substrates
(**A**) Map of ATM functional interactions, including individual proteins (gray), protein complexes (green), protein families (yellow), and microRNAs (blue). (**B**) Gene Ontology functional classifications of 1077 putative ATM substrates. [Reprinted with permission from Springer Nature: Shiloh Y & Ziv Y. The ATM protein kinase: regulating the cellular response to stress, and more. *Nat Rev Mol Cell Biol* **14,** 197-210 (2013). doi: 10.1038/nrm3546. License #5031560988957]

Inactive ATM protein exists as a multimer that, in response to DNA DSBs, autophosphorylates at serine 1981 and dissociates into active monomeric ATM kinase. Full activation of ATM activity in response to DNA damage depends first on activation of a heterotrimer protein complex consisting of MRE11, RAD50 and NBS1 (MRN complex). The MRN complex senses DNA DSBs and binds exposed DNA ends, leading to a conformational change in RAD50 that allows formation of a molecular tether between MRN complexes bound to each exposed end. ATM is recruited by tethered broken DNA and interacts with NBS1 through its C terminus.[110] Once activated, ATM then phosphorylates many downstream proteins to regulate the processes of cell cycle arrest, DNA repair or apoptosis (**Figure 14**). Mutations that disrupt the function of individual MRN complex components produce disorders with similar clinical and cellular phenotypes to AT. For example, MRE11 mutation causes AT-like disorder, also characterized by progressive cerebellar degeneration, whereas NBS1 mutation causes Nijmegen breakage syndrome, another chromosomal instability syndrome characterized by neurodevelopmental problems, growth defect, recurrent respiratory infections and increased cancer susceptibility.[116]

Figure 14

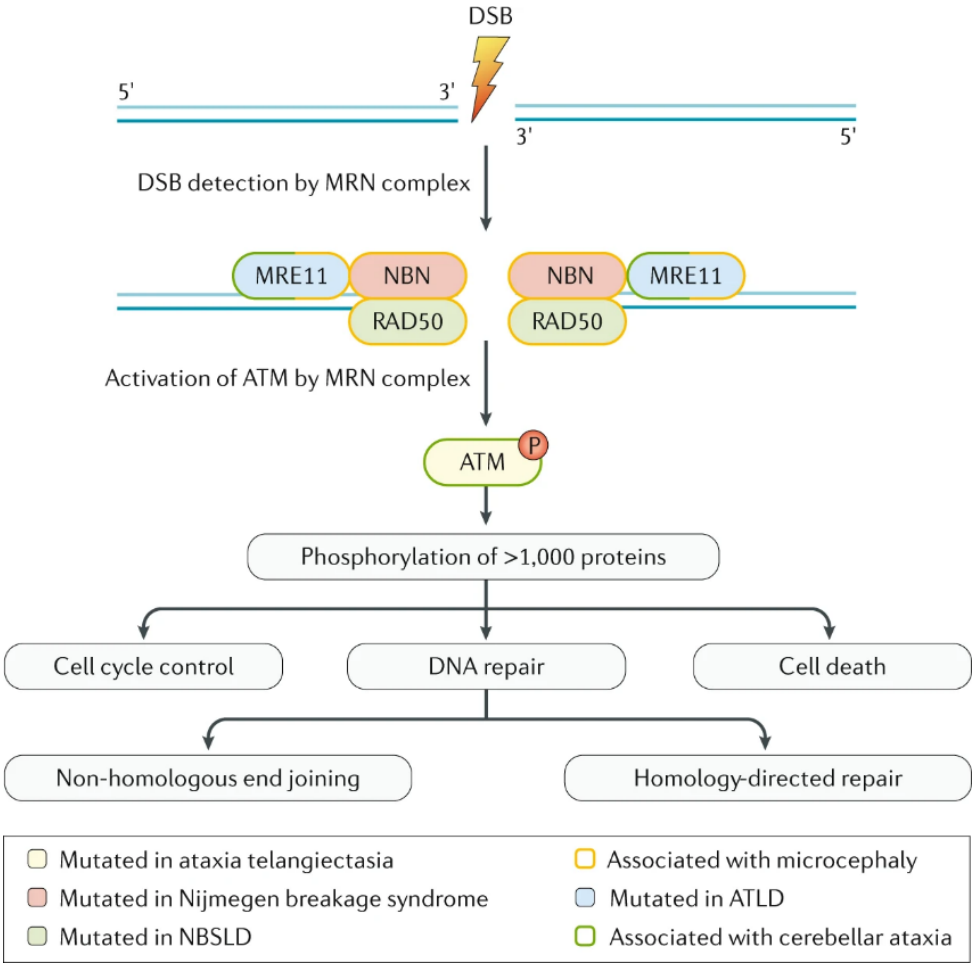


Figure 14. ATM and DNA double-strand break repair
MRE11, NBN/NBS1, and RAD50 (MRN complex) detect DSBs and bind exposed DNA ends, forming an intercomplex tether that recruits ATM. Activated ATM then phosphorylates thousands of downstream effectors that regulate the cell cycle, DNA repair, or apoptosis in response to DNA damage. [Reprinted with permission from Springer Nature: Taylor AMR, Rothblum-Oviatt C, Ellis NA, et al. Chromosome instability syndromes. *Nat Rev Dis Primers* **5,** 64, 64(2019). doi: 10.1038/s41572-019-0113-0. License #5031591424078]

Fueled largely by attempts to elucidate the molecular mechanisms driving neurodegeneration in AT, as well as other poorly understood AT disease manifestations such as premature aging and diabetes, many roles for ATM kinase outside of the DNA DSB pathway have been characterized. These discoveries have underlined the significant involvement of ATM in the maintenance of

cellular homeostasis (**Figure 15**). ATM is activated during times of cellular stress such as hypoxia, hyperthermia, hypotonic stress, and other triggers of replication stress.[114] ATM has been shown to play a role in the mitotic spindle checkpoint through phosphorylation and activation of the kinetochore protein BUB1.[117] It is present in the cytoplasm with demonstrated involvement in cytoplasmic signaling pathways that affect calcium and potassium ions levels.[118, 119] There is much evidence pointing to various roles of ATM in regulating oxidative stress. ATM can be activated by reactive oxygen species, indicating that ATM can be tuned to respond to different cellular stressors through distinct mechanisms of activation.[114] When reactive oxygen species are elevated, ATM downregulates mTOR (mammalian target of rapamycin) to promote autophagy,[120] and absence of ATM is associated with mitochondrial dysfunction and defects in mitophagy.[121] ATM has also been shown to influence production of the antioxidant cofactor NADPH in response to genotoxic stress through the pentose phosphate cycle, further supporting a role for ATM in maintaining redox balance.[122] Finally, involvement of ATM in insulin signaling has been demonstrated. In response to insulin, ATM phosphorylates a repressor of translation initiation factor eIF4E.[123] Loss of ATM leads to downregulation of protein synthesis, which may contribute to the growth defect observed in AT. Additionally, insulin signaling leads to ATM-dependent activation of protein kinase AKT, which enhances glucose uptake in some cells, and suggests a potential mechanism for development of insulin resistant diabetes in AT.[124]

Figure 15

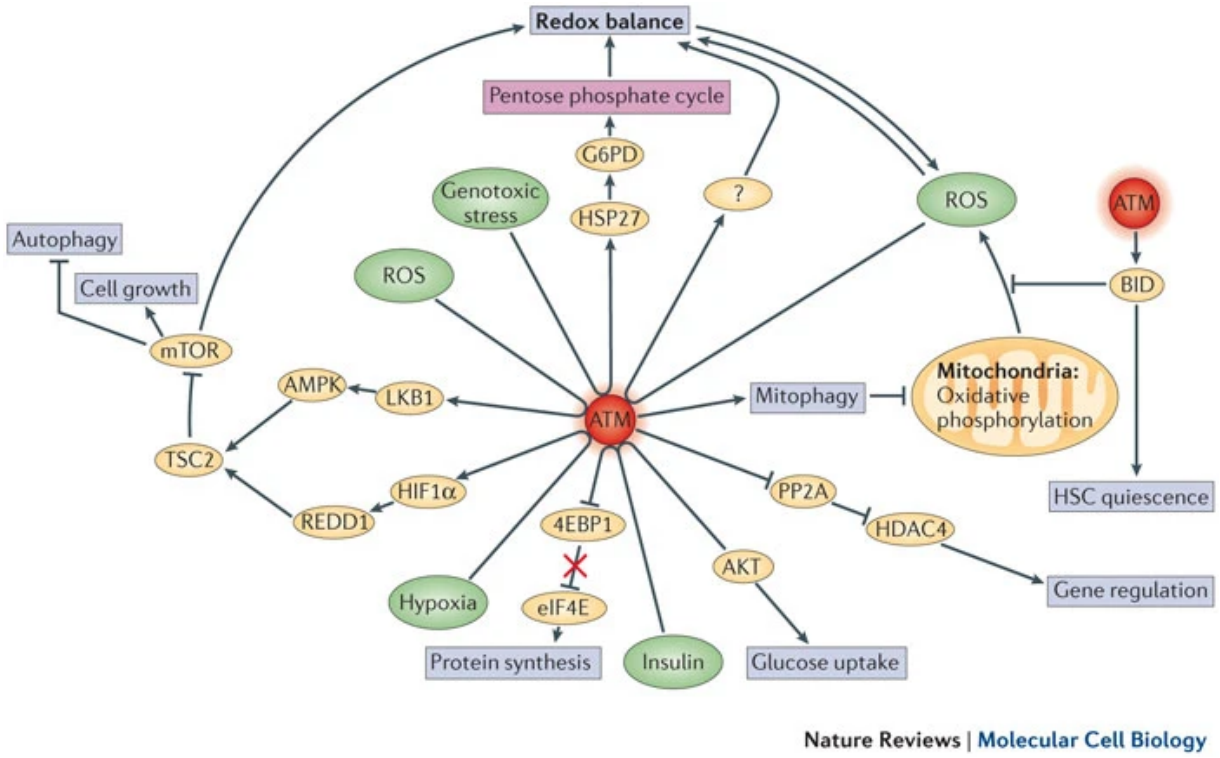


Figure 15. ATM and maintenance of cellular homeostasis
Evidence for roles of ATM in cellular pathways outside of DSB repair is accumulating. ATM is involved in diverse pathways including redox balance, insulin signaling, protein synthesis, cell growth, autophagy, and mitochondrial function. [Reprinted with permission from Springer Nature: Shiloh Y & Ziv Y. The ATM protein kinase: regulating the cellular response to stress, and more. Nat Rev Mol Cell Biol **14,** 197-210 (2013). doi: 10.1038/nrm3546. License #5031560988957]

1.3.3 Current and Developing Therapies for Ataxia Telangiectasia

There is no cure for AT. No specific treatment or drug is currently available that addresses all aspects of the disease.[125] Treatment of the disease is symptomatic and supportive. Management strategies are multidisciplinary and vary from patient to patient due to variable disease presentation, progression and complications. Physical therapy and exercise can be helpful for maintaining some function, muscle strength and overall well-being but does not improve ataxia or slow neurodegeneration.[126] Motor function from the basal ganglia can sometimes be improved

with L-dopa or dopamine agonists.[127] Anti-Parkinson drugs such as amantadine are commonly prescribed and have been shown to modestly improve parkinsonism, ataxia, speech and other motor symptoms.[107] Cerebellar tremors can also be treated with drugs such as gabapentin and propranolol.[128]

Steroid treatment has been shown to significantly improve ataxia symptoms in multiple trials. Short-term oral betamethasone effectively reduced ataxic symptoms in AT patients by approximately 30% as assessed by the International Cooperative Ataxia Rating Scale (ICARS).[129] These encouraging results led to the development of a novel therapeutic strategy that aimed to take advantage of the observed benefit of steroids while minimizing detrimental side effects from long-term steroid treatment. Dexamethasone was encapsulated into autologous erythrocytes (termed EryDex) before readministration to AT patients, allowing for controlled release of the drug for up to one month after treatment. Significant reductions in neurological symptoms by ICARS was observed without typical steroid side effects. Long-term follow up suggested that EryDex could potentially delay the natural neurological progression of AT.[130] Evidence has been reported that dexamethasone can induce a noncanonical splicing event, leading to an *ATM* transcript with an alternative open reading frame that preserves the kinase domain.[131] However, subsequent studies failed to demonstrate induction of alternative, functional *ATM* transcripts in AT lymphoblasts, fibroblasts or an *ATM*-knockout HeLa cell line by dexamethasone.[132] Therefore the mechanism of action of amelioration of symptoms clinically observed in AT patients from steroid treatment remains unclear.

Approximately one-third of AT patients die from chronic lung disease, a consequence of recurrent sinopulmonary infections exacerbated by dysphagia and immune deficiency. Although multiple immune deficiencies such as lymphopenia and hypogammaglobulinemia are prevalent in AT, systemic or opportunistic infections are uncommon. Management of respiratory tract infection and pulmonary complications can be achieved with vaccination, prophylactic antibiotics and immunoglobulin replacement therapy.[133] Immunization against common respiratory pathogens can boost immunity even in patients with low immunoglobulin levels[107] and the practice of prophylactic antibiotic administration has prolonged life expectancy in AT patients.[134]

An additional one-third of AT patients die from malignancy. Lymphoid malignancies are most common in children, while adults are susceptible to hematologic malignancy and various solid tumors, including breast, liver and gastric.[107] Heterozygous carriers also have a significantly increased (>25%) lifetime risk of breast cancer.[135] The underlying DNA DSB repair defect precludes therapeutic ionizing radiation in these patients and DNA-damaging chemotherapy imparts substantially increased risk of secondary malignancy and other toxicities. There are no consensus treatment protocols for AT patients with cancer, and the exact strategy pursued depends on patient-specific factors.[136] In general, chemotherapy is given using dose-adjusted protocols with radiomimetic alkylating drugs given at reduced doses or avoided altogether.[127]

In addition to EryDex, which is currently in phase III trials (ClinicalTrials.gov Identifier NCT03563053), several other investigational therapies for AT are currently being tested in humans. An active phase II study testing N-acetyl-L-leucine, a modified amino-acid ester that has been used in France since 1957 for the treatment of vertigo, is assessing whether this drug can

improve various neurological and quality of life measures in AT (ClinicalTrials.gov Identifier NCT03759678). An n-of-1 expanded access IND study is currently being conducted at Boston Children's Hospital testing an individualized antisense oligonucleotide (ASO) gene therapy for a two-year-old child with AT.[137] The goal of ASO therapy is to target aberrant splice sites in the mRNA transcript such that normal *ATM* splicing is restored, and full-length functional ATM protein can be translated.[138] The patient has received multiple intrathecal injections of the ASO product, with hopes that cerebellar cells will receive enough of the drug to restore functional ATM protein.[139] If successful, the results could pave the way for further development of novel, individualized ASO-based therapies for AT and related diseases.

Traditional gene therapy strategies using viral vectors are challenging for AT because the *ATM* gene is large. The genomic DNA harbors 66 exons[140] and full-length *ATM* cDNA spans approximately 9.1 kb,[141] which is the approximate maximum packaging capacity of LVs. Several reports have described successful delivery and expression of an *ATM* transgene in vitro and in vivo; however these studies relied on either herpes simplex virus 1 (HSV-1)[142, 143] or HSV/AAV hybrid amplicon vectors,[144] which are unlikely to be clinically translatable.[141, 145] Carranza et al. recently reported successful transduction of human AT fibroblasts with *ATM* cDNA using an LV.[141] Transduced cells expressed ATM protein, could repair DNA DSBs, and exhibited less radiosensitivity and improved cell cycle function. However, vector titer and transduction efficiency of $ATM^{-/-}$ cells was low. Whether this approach will be effective and technically feasible in an in vivo setting remains to be determined.

Non-viral gene therapy strategies are also being explored. Dixon and colleagues at the University of Nottingham are developing glycosaminoglycan-binding enhanced transduction (GET) peptides[146] paired with a Sleeping Beauty transposon system to deliver and integrate a full-length therapeutic *ATM* transgene. Preliminary results demonstrating efficacy and feasibility of this approach in an AT model have yet to be reported.

1.3.4 Allogeneic Hematopoietic Stem Cell Transplantation for Ataxia Telangiectasia

Allogeneic HSCT is a potentially curative treatment modality for immune deficiencies and at least two-thirds of AT patients have both cellular and humoral immune defects.[147, 148] Allogeneic HSCT has the potential to correct immune deficiency and prevent lymphoid malignancy in AT by reconstitution of the immune system with ATM-competent cells. Although successful HSCT would provide clear benefits, it has long been considered a controversial option for AT due to the extreme sensitivity of these patients to standard DNA damaging conditioning agents. Only a few published case reports are available, and compared with other DNA DSB disorders, outcomes of HSCT for AT are worse (≈25% overall survival). Allogeneic HSCT has therefore not been widely recommended as a standard treatment option for AT.[149] A fatal outcome was reported in 2012 after HSCT was performed on a 22-month-old AT patient for a misdiagnosed immune deficiency. AT diagnosis was made post-mortem. The patient was conditioned with a fully myeloablative regimen of treosulfan, fludarabine, and anti-thymocyte globulin (ATG), and despite full donor lymphohematopoietic reconstitution, the patient died from liver failure after 10 months. The cause of toxicity was not determined, but the conditioning regimen could not be ruled out as a contributing factor.

Transplants that followed were usually performed to treat leukemia or lymphoma with a known AT diagnosis, or pre-emptively in an effort to prevent the development of malignancy. Reduced intensity regimens must be used, as a survey of data from 38 transplant centers worldwide found that myeloablative conditioning in AT led to GVHD, multiorgan failure, viral activation, post-transplantation lymphoproliferative disorder, and death.[149] In 2013, successful allogeneic HSCT was reported in a 3-year-old patient with AT and acute lymphocytic leukemia (ALL).[150] The patient received a dose-adjusted chemotherapy protocol for ALL, and then a modified Fanconi anemia conditioning regimen consisting of busulfan, fludarabine and ATG prior to HSCT. Mild chemotherapy-induced toxicities were reported, including leukopenia, agranulocytosis, mucositis, and multiple viral infections that were easily managed. Despite achieving only mixed chimerism (33% donor neutrophils, eventually increasing and stabilizing at 60-80%) the patient experienced low hematological toxicity and remained in leukemia remission at least 8 years post-transplantation with normal blood count values and without the need for intravenous immunoglobulin.[151] The same group reported HSCT in two additional AT patients: a 12-month-old patient referred for HSCT diagnosed with undefined, radiosensitive, severe combined immunodeficiency, and a 23-month-old patient with known AT who was transplanted pre-emptively after the death of an affected older brother. All patients received modified Fanconi anemia conditioning regimens, and all survived HSCT without significant adverse effects. All patients experienced recovery of T cells and immunoglobulin production, and none of the patients developed symptomatic infections, including pulmonary infections.[151] These clinical observations are encouraging and suggest that some AT patients may tolerate reduced intensity regimens and benefit from allogeneic HSCT. Indeed, another 2018 report described a 4-year-old AT patient who was transplanted pre-emptively under reduced intensity conditioning and experienced correction

of T cell lymphopenia, serum immunoglobulins, vaccine responsiveness, and delayed progression of neurological impairment.[152] The patient retained full restoration of immunity 7 years post-transplantation, described in a later report.[148] The same report also described a second AT patient receiving pre-emptive HSCT at 5 years of age; reduced intensity conditioning was well-tolerated, and rapid engraftment and immune reconstitution was achieved in the absence of GVHD or organ toxicity up to 1.5 years post-transplantation.[148]

In contrast, a 2016 report described a 13-year-old AT patient with Epstein-Barr virus (EBV)-positive B cell non-Hodgkin lymphoma.[153] The patient initially received rituximab followed by chemotherapy for his malignancy, which were tolerated well, and complete remission was achieved. HSCT was still pursued due to the underlying immune defect and current EBV-associated lymphoproliferation. Although this patient also received a reduced intensity Fanconi anemia conditioning regimen, he developed remarkable, life-threatening toxicities, including severe mucosal toxicity and gastrointestinal bleeding, hemorrhagic cystitis, severe veno-occlusive disease with hepatomegaly, and respiratory distress syndrome requiring mechanical ventilation. This case exemplifies the variability in patient responses and outcomes following allogeneic HSCT and genotoxic conditioning in the setting of AT. The extent to which age, nature of malignancy, characteristics of the specific mutation, or other factors contribute to these variable outcomes remains to be determined. However, these published case reports highlight the significant benefits that are attainable from immune reconstitution with ATM-competent cells, including prevention of malignancy and prevention of infection. They also underscore the need for safer, non-genotoxic agents that can be used to effectively condition the bone marrow in AT patients to accept healthy

donor cells while minimizing widespread toxicities caused by agents that damage DNA in the setting of underlying DNA repair defect.

1.4 Hypothesis

HSCT is a powerful therapeutic modality that can cure many hematologic and immune disorders, yet it is underutilized for non-malignant diseases due to the inherent toxicities, short- and long-term adverse effects associated with DNA-damaging, genotoxic conditioning agents.[46, 154] In non-malignant disease settings, adequate clearance of endogenous HSCs in bone marrow niche spaces must be attained to make space for engraftment of healthy allogeneic donor cells or ex vivo gene-modified autologous cells. As LV-HSC-GTs for non-malignant diseases gain traction, it is imperative that non-genotoxic methods of bone marrow conditioning and, if necessary, immune suppression are developed to minimize adverse effects and risk of malignancy in order to increase uptake of these novel curative therapies. Similarly, non-genotoxic approaches to bone marrow conditioning would significantly increase the number of patients eligible for allogeneic HSCT who might otherwise be poor candidates due to certain vulnerabilities, such as extreme sensitivity to DNA damage or advanced age. We hypothesize that antibody-based conditioning regimens, namely, a stem cell-targeting CD117-saporin immunotoxin for bone marrow conditioning and naked T and/or B cell targeting monoclonal antibodies for immune suppression, can enable engraftment of ex vivo gene modified autologous HSCs or healthy allogeneic HSCs in the absence of genotoxic insult and regimen-related toxicities typically associated with traditional conditioning agents. This dissertation first describes the successful implementation of a CD117-saporin immunotoxin in combination with a T cell targeting monoclonal antibody regimen to achieve therapeutic expression of circulating transgenic fVIII protein after ex vivo LV-HSC-GT, curing

hemophilia A in a murine model in the absence of genotoxicity or loss of fertility. Second, the use of CD117-saporin immunotoxin in an allogeneic HSCT murine model of ataxia telangiectasia is demonstrated. Though immune suppression was not required for engraftment of donor cells in this setting, a monoclonal antibody regimen of T and B cell depleting antibodies was included to deplete endogenous *Atm*-deficient lymphocytes in order to attenuate the risk of host-derived lymphoid malignancy. Cellular and humoral immune functions were restored in HSCT recipients after non-genotoxic conditioning. Finally, this dissertation will conclude with a discussion of the future of HSCT conditioning.

Chapter 2

Non-genotoxic conditioning facilitates hematopoietic stem cell gene therapy for hemophilia A using bioengineered factor VIII

This manuscript was published in *Molecular Therapy: Methods & Clinical Development*

Russell AL, Prince C, Lundgren TS, Knight KA, Denning G, Alexander JS, Zoine JT, Spencer HT, Chandrakasan S, Doering CB

Non-genotoxic conditioning facilitates hematopoietic stem cell gene therapy for hemophilia A using bioengineered factor VIII

Molecular Therapy: Methods & Clinical Development. 2021 May 5;21:710-727.
doi: 10.1016/j.omtm.2021.04.016

Athena Russell designed and performed experiments, wrote and edited the manuscript.

Chengyu Prince, Taran Lundgren, Kristopher Knight, Gabriela Denning, and Jaquelyn Zoine performed experiments.

Jordan S. Alexander developed experimental techniques.

Christopher Doering, Shanmuganathan Chandrakasan, and Trent Spencer conceived experiments and reviewed the manuscript.

2.1 Abstract

Hematopoietic stem and progenitor cell lentiviral gene therapy is a promising strategy towards a lifelong cure for hemophilia A. The primary risks associated with this approach center on the requirement for pre-transplantation conditioning necessary to make space for, and provide immune suppression against, stem cells and blood coagulation factor VIII, respectively. Traditional conditioning agents utilize genotoxic mechanisms of action, such as DNA alkylation, that increase risk of sterility, infection and developing secondary malignancies. In the current study, we describe a non-genotoxic conditioning protocol using an immunotoxin targeting CD117 (c-kit) to achieve endogenous hematopoietic stem cell depletion and a cocktail of monoclonal antibodies to provide transient immune suppression against the transgene product in a murine hemophilia A gene therapy model. This strategy provides high-level engraftment of hematopoietic stem cells genetically modified ex vivo using recombinant lentiviral vector encoding a bioengineered high-expression factor VIII variant, termed ET3. Factor VIII procoagulant activity levels were durably elevated into the normal range and phenotypic correction achieved. Furthermore, no immunological rejection or development of anti-ET3 immunity was observed. These preclinical data support clinical translation of non-genotoxic antibody-based conditioning in hematopoietic stem and progenitor cell lentiviral vector gene therapy for hemophilia A.

2.2 Introduction

Hemophilia A (HA) is the most common severe congenital bleeding disorder caused by a deficiency in blood coagulation factor VIII (fVIII) due to mutations in the *F8* gene. The disease affects approximately 1:4000 male births and is associated with an elevated risk of bleeding-related mortality. In severe cases (less than 1% fVIII activity), persons with HA suffer spontaneous

bleeding into joints leading to progressive, debilitating arthropathy, as well as life-threatening bleeds into closed spaces such as intracranial or retroperitoneal cavities.[155] Standard of care involves either prophylactic fVIII replacement or fVIII mimetic therapy. Although largely effective, these existing therapies are economically challenging, if not completely prohibitive, for the global majority. Furthermore, even with optimal therapy, bleeding and joint damage/pain still occur and unmet clinical need exists.[84, 156]

HA has a longstanding history with experimental gene therapies.[157, 158] It is likely that sustained, steady state production and circulation of fVIII would overcome most of the limitations of existing replacement, mimetic or bypassing therapies. It has been established in animal models and clinical trials that modest increases in circulating fVIII activity can lead to clinically significant reduction in bleeding challenge assays and annualized bleed rate, respectively. Furthermore, due to the lack of cell type-specific post-translational modifications, e.g., gamma carboxylation, virtually any cell type can biosynthesize and secrete functional fVIII into the circulation. Gene transfer strategies employing adeno-associated viral (AAV) vectors to target liver hepatocytes or lentiviral vectors (LV) targeting hematopoietic stem and progenitor cells (HSPCs) ex vivo have advanced into clinical testing. Multiple AAV-fVIII gene therapies have already progressed into phase 3 clinical studies, although concerns remain regarding long-term safety and durability. In contrast, HSPC LV-fVIII gene therapies are just beginning phase 1/2 testing and no clinical data have been released. Additionally, systemic in vivo LV delivery targeting hepatocytes for HA gene therapy is in preclinical development. This approach has potential advantages over in vivo AAV delivery, e.g., vector integration allows stable transgene expression, even during substantial organ growth that occurs in children, and the low prevalence of antibodies against LV in the general population.

However, many questions remain to be answered to ensure the safety and feasibility of this approach. Although vesicular stomatitis virus surface glycoprotein (VSV-G), the most common LV pseudotype, allows for considerable tropism to the liver,[159] VSV-G-LV have extremely broad tropism[160] and since genotoxicity remains an inherent risk with integrating vectors, extensive biodistribution studies will be needed. Challenges associated with vector quantity and quality will also need to be overcome before LVs can be administered systemically in humans.[159]

Ex vivo LV modification of autologous HSPCs followed by transplantation (HSCT) is a promising approach for gene therapy of HA[161, 162] and other monogenic blood and immune cell diseases.[163, 164] Since HSPCs are responsible for producing a life-long supply of blood cells, gene replacement strategies that target these long-term repopulating cells offer the potential for continuous production of fVIII from daughter cells and correction of disease phenotypes for the lifetime of the individual. Our laboratory has created a bioengineered high-expression fVIII variant, termed ET3, that has been optimized for recombinant LV delivery to HSPCs.[165-170] Expression of the codon-optimized ET3 transgene is driven by the CD68 promoter, enabling high-level monocyte-lineage restricted expression of ET3 upon HSPC differentiation.[171] ET3 is secreted by gene-modified monocyte and macrophage populations into the bloodstream and sustained plasma ET3 levels thereby confer lifelong hemostatic correction.

HSCT is a powerful and potentially curative therapy used for a broad range of malignant and non-malignant diseases,[15] with its therapeutic potential further amplified when combined with gene therapy. However, the clinical impact of HSCT gene therapy is primarily limited by the cytotoxic and genotoxic effects of conventional conditioning agents such as total body irradiation (TBI) and

alkylating chemotherapeutics, which are DNA damaging.[172] Significant acute and long-term toxicities and treatment-related mortality are associated with non-targeted, genotoxic conditioning regimens, including organ toxicity, infertility, and risk of secondary malignancy.[173-176] Myeloablative conditioning regimens cause cytopenias in the early post-transplant period before engraftment of leukocyte and platelet populations, leaving the patient vulnerable to infection and bleeding.[15, 17, 177] Thrombocytopenia and attendant increased bleeding risk caused by genotoxic conditioning is particularly undesirable in the setting of HA. Therefore, the development of alternative, non-genotoxic conditioning agents and regimens is a high priority translational objective for the fields of HSCT and HSPC-directed gene therapy.

2.3 Materials and Methods

Mice. Exon 16-disrupted hemophilia A mice[178] backcrossed to C57BL/6 background were used as transplantation recipients in this study. Congenic donor mice were CD45.1^{+} (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ; JAX stock #002014). Mouse strains were bred and maintained at an Emory University Division of Animal Resources facility. Male and female mice 7 – 10 weeks of age were used in this study. Transplant recipients within each experiment were age-matched and randomized to experimental groups. Animal studies were approved by Emory University Institutional Animal Care and Use Committee.

Immunotoxin preparation and conditioning. Biotinylated anti-CD117 (clone 2B8, Biolegend, San Diego, CA) was combined at a 1:1 molar ratio with either streptavidin-ZAP (ATS, San Diego, CA) or an in-house developed streptavidin-saporin product.[179] CD117-saporin immunotoxin was delivered by retro-orbital injection at a dose of 0.5 mg/kg on day -5 before HSCT. Rabbit anti-

mouse ATG (Cedarlane, Burlington, NC) was administered by intraperitoneal injection at a dose of 30 mg/kg on day -5. Anti-CD4 (clone GK1.5, Bio X Cell, Lebanon, NH) and anti-CD8 (clone YTS 169.4, Bio X Cell, Lebanon, NH) were administered in 100 μg doses by intraperitoneal injection on day -2 and day 0. Anti-CD40L (clone MR-1, Bio X Cell, Lebanon, NH) was administered in 500 μg doses by intraperitoneal injection on day 0. Irradiation controls were exposed to either 11 Gy cesium-137 or 9 Gy x-ray radiation in split doses on day 0. Further reagent details are provided in Table S1.

Bone marrow depletion and flow cytometric analysis. Mice were conditioned with CD117-sap immunotoxin and sacrificed after 5 days. Femurs were dissected and crushed using a mortar and pestle. Bone marrow was strained through a 40 μm cell strainer and RBCs lysed using Hybri-Max RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). Bone marrow leukocytes were stained for flow cytometric detection of HSPCs using the following LSK-SLAM markers: lineage cocktail, CD117 (c-Kit), Sca-1, CD48, and CD150. Data was acquired using a BD™ LSR II (San Jose, CA) or Cytek® Aurora (Fremont, CA) flow cytometer. Flow cytometric analysis was performed using FlowJo v10.7.1 (BD, Ashland, OR). Further reagent details are provided in Table S1.

Histology. Fresh tissues were isolated and fixed in 10% neutral buffered formalin for 24-48 hours, and then transferred to 70% ethanol before paraffin embedding. For acute toxicity studies, HA mice were conditioned with CD117-sap immunotoxin and sacrificed after 5 days. Paraffin-embedded sections were stained with H&E and tissue morphology evaluated for signs of toxicity or pathological lesions by a veterinary pathologist. For long-term morphological analysis, tissues were collected from HA mice that had received immunotoxin conditioning and gene therapy with

sustained ET3 expression after 34 weeks. H&E-stained paraffin-embedded sections were evaluated for normal tissue morphology after long-term engraftment by a veterinary pathologist. Histology services were provided by the Histology and Molecular Pathology Laboratory at Yerkes National Primate Research Center (Atlanta, GA).

Sca-1$^+$ cell isolation, ex vivo expansion, transduction, and transplantation. Sca-1$^+$ isolation from murine bone marrow was performed by immunomagnetic bead selection as previously described.[180] Briefly, bone marrow from CD45.1$^+$ donor mice was harvested and incubated with a biotinylated anti-Sca-1 antibody (Biolegend, San Diego, CA) followed by incubation with anti-biotin microbeads (Miltenyi, Gaithersburg, MD). Labeled cells were then passed through an LS column loaded on a MidiMACS™ Separator (Miltenyi). Sca-1$^+$ cells were harvested and cultured in serum-free StemSpan™ medium (Stem Cell Technologies, Vancouver, BC) for three days in the presence of mouse SCF (100 ng/ml), mouse IL-3 (20 ng/ml), human IL-11 (100 ng/ml), and human Flt-3 ligand (100 ng/ml). All cytokines were purchased from R&D Systems (Minneapolis, MN). Sca-1$^+$ cells were transduced on day -1 and day 0 with half volume CD68-ECO-ET3-LV(LV production described in detail by Lytle et al.[161]) at a density of 2.0 x 10^6 Sca-1$^+$ cells/ml and multiplicity of infection (MOI) of 13 – 63. Transduced CD45.1$^+$ Sca-1$^+$ HSPCs were harvested, washed and resuspended in PBS, and then transplanted by retro-orbital injection into preconditioned HA mice.

Peripheral blood collection and analysis. Peripheral blood was periodically collected from the retro-orbital sinus in 3.8% w/v sodium citrate anticoagulant. CBC analyses were performed using a HemaTrue Veterinary Hematology Analyzer (Heska, Loveland, CO). Remaining whole blood

was centrifuged at 2000 x *g* for 15 minutes at 4 °C. Plasma was removed and frozen at -80 °C until used for downstream analyses. Cell pellets were lysed using Hybri-Max RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) and peripheral blood leukocytes were stained for flow cytometric detection of donor- and host-derived cells using monoclonal antibodies against the following surface markers: CD45.1, CD45.2, CD3, B220 (CD45R) and Gr-1 (Ly-6G/Ly-6C). Data was acquired using a BD™ LSR II (San Jose, CA) or Cytek® Aurora (Fremont, CA) flow cytometer. Flow cytometric analysis was performed using FlowJo v10.7.1 (BD, Ashland, OR). Further reagent details are provided in Table S1.

ET3 activity measurement. ET3 activity was measured in plasma samples using the Chromogenix Coatest® SP Factor VIII chromogenic assay kit (Diapharma, West Chester Township, OH) according to manufacturer's instructions. Standard curves were generated using Factor Assay Control Plasma (George King Bio-Medical, Overland Park, KS). Absorbance at 405 nm was measured kinetically using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA).

Vector copy number measurement. Vector copy number in DNA samples isolated from peripheral blood, spleen and bone marrow was determined by quantitative real-time polymerase chain reaction (qPCR) using primers specific for RRE sequences present in the CD68-ECO-ET3-LV construct as previously described.[171] Briefly, genomic DNA (gDNA) was isolated, and 50 or 100 ng DNA was analyzed in triplicate by generating a 1X Power SYBR™ Green Master Mix (Thermo-Fisher, Waltham, MA) containing 250 nM primers. Standard curve was generated by analyzing serial dilutions of RRE plasmid DNA suspended in 50 ng HEK-293T/27 gDNA as

background. qPCR measurements performed on an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA).

Anti-ET3 ELISA assay. Mouse plasma was analyzed for the presence of anti-ET3 IgG by ELISA. Microtiter plates were coated with 1.5 μg/ml ET3 antigen in coating buffer (20 mM bicine, 2 mM $CaCl_2$, pH 9.0) overnight at 4 °C. Plates were washed twice with wash buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, 0.05% sodium azide, pH 7.4) and then incubated in blocking buffer (wash buffer plus 2.0% bovine serum albumin) overnight at 4 °C. Plates were washed twice with wash buffer immediately before the assay. Mouse plasma samples were diluted 1:20 in blocking buffer and 25 μl plasma dilution was added to ET3-coated wells. Plates were incubated at room temperature for 1 hour, and then washed twice with wash buffer. Goat anti-mouse IgG-alkaline phosphatase (AP) was diluted 1:1000 and 25 μl added to each well. Plates were incubated at room temperature for 1 hour, and then washed twice with wash buffer. AP substrate was reconstituted according to manufacturer's instructions (AP Substrate Kit, Cat #1721063, Bio-Rad, Hercules, CA) and 40 μl was added to each well. Plates were incubated room temperature for 20 minutes, and reaction was then quenched by adding 0.4 M NaOH. Absorbance at 405 nm was measured using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA).

Modified Bethesda assay. Plasma was tested for inhibitory activity against ET3 using a modification of the Bethesda assay[181] as previously described.[171] Briefly, fVIII-deficient plasma was reconstituted with ET3, to a final concentration of 0.8 – 1.2 IU/mL and kept on ice until use. Equal volumes of reconstituted plasma and test plasma were incubated for 2 hours at 37 °C, followed by measurement of residual ET3 activity using the one-stage coagulation assay. Residual

ET3 activity was calculated by dividing the ET3 activity in the test sample by that of the reconstituted plasma sample. Dilutions were identified that produced at least two values in the range between 40% and 60% residual activities. One Bethesda unit (BU) per milliliter is defined as the dilution of inhibitor that produces 50% inhibition of fVIII activity using the published reference curve for interpolation.[181]

Phenotypic correction tail snip assay. Phenotypic correction of the hemophilia A bleeding phenotype was assessed using a quantitative tail snip assay as previously described.[182] Briefly, 15 ml conical tubes containing 13 ml PBS were weighed before the procedure and placed in a 37 °C water bath. Mice were anesthetized with isoflurane and a transection made 4 mm from the distal tip of the tail. Snipped tails were immediately placed in pre-weighed PBS tubes at 37 °C and bleeding into the tube was allowed for 40 minutes. Tubes were capped and weighed at the conclusion of the assay and the amount of blood lost from each mouse was calculated. Mice were euthanized upon completion of the procedure.

ATG binding studies. HA mice were injected intraperitoneally with either 30 mg/kg rabbit anti-mouse ATG (Cedarlane, Burlington, NC) or PBS. After four hours, mice were sacrificed and peripheral blood, bone marrow and spleen cells were isolated. Single cell suspensions from each tissue were incubated with mouse Fc block, followed by mouse anti-rabbit IgG secondary antibody to detect the presence of surface bound ATG antibodies. Cells were then washed and stained with an LSK-SLAM flow cytometry panel for bone marrow cells, or a mature lineage marker panel for blood and spleen cells. Data was acquired using a Cytek® Aurora (Fremont, CA) flow cytometer.

Flow cytometric analysis was performed using FlowJo v10.7.1 (BD, Ashland, OR). Further reagent details are provided in Table S1.

Statistical analysis. Shapiro-Wilk test was used for normality testing of continuous variables. When comparing two groups, Student's t-test or Mann-Whitney U test was used depending on normality of data. When comparing more than two groups, one-way ANOVA with Tukey's multiple comparison tests was performed for normally distributed data, and Kruskal-Wallis test with Dunn's multiple comparison test was performed for nonparametric analyses. Data are reported as mean ± sample standard deviation (SD). Statistical analysis was performed using GraphPad Prism v9.0.0 (San Diego, CA).

2.4 Results

Generation of saporin-based anti-mouse CD117 immunotoxin (CD117-sap). For this study, we generated an immunotoxin using a monoclonal antibody (mAb) specific for the stem cell factor (SCF) receptor CD117 (clone 2B8) present on mouse HSPCs as the targeting moiety, coupled to the type I ribosome-inactivating protein toxin saporin. Commercially available biotinylated CD117 mAb was linked to a streptavidin-saporin conjugate that was either purchased commercially (see Supplemental Materials) or generated in-house (**Figure S1A**). Commercial saporin is isolated directly from seeds of the *Saponaria officinalis* plant, whereas our in-house product is a recombinant version.[179] Our laboratory has optimized the production and purification of recombinant saporin, as well as the bioconjugation of saporin to streptavidin for immunotoxin generation. Figure S1B shows a representative Coomassie-stained polyacrylamide gel of

biotinylated CD117 mAb, commercial saporin, in-house generated recombinant saporin, and their associated CD117-sap products after conjugation.

CD117-sap selectively depletes HSCs and spares non-hematopoietic tissues in HA mice. We first evaluated the ability of CD117-sap immunotoxin to deplete HSCs in HA mice, thereby creating space in bone marrow niches for engraftment of ET3 gene-modified donor HSPCs. HA mice were conditioned with 0.5 mg/kg CD117-sap and bone marrow was evaluated for depletion of HSPC subsets by flow cytometry. Although CD117-expressing hematopoietic progenitor cell (HPC) populations, such as Lineage (Lin)$^-$ Sca-1$^+$ CD117$^+$ (LSK) and Lin$^-$ Sca-1$^+$ CD117$^+$ CD48$^+$ CD150$^-$ (MPP) compartments, were minimally affected five days after CD117-sap treatment, we observed robust and specific depletion of the short-term (ST-HSC) and long-term repopulating hematopoietic stem cell (LT-HSC) populations (Lin$^-$ Sca-1$^+$ CD117$^+$ CD48$^-$ CD150$^-$ and Lin$^-$ Sca-1$^+$ CD117$^+$ CD48$^-$ CD150$^+$, respectively) compared with control mice that received no immunotoxin (**Figure 16A and Figure 16B**). C57BL/6 mice were conditioned with 0.5 mg/kg CD117-sap at various time points and transplanted with 5 x 10^6 congenic whole bone marrow cells to determine optimal timing of immunotoxin conditioning relative to transplantation. CD117-sap conditioning 5 days before transplantation led to 90.6 ± 5.9% donor myeloid chimerism at 4 weeks post-transplantation that increased to 94.5 ± 3.3% by 16 weeks (**Figure S2**). Based on these observations, a five-day conditioning period was selected for all future studies.

Although expression of CD117 is found predominantly in the HSPC compartment, certain non-hematopoietic cells and tissues express some level of CD117, such as Cajal cells of the gastrointestinal tract, germ cells, and some neurons.[183] Histological analysis of various CD117$^+$

tissues in conditioned mice and untreated controls was performed five days after conditioning to investigate whether CD117-sap immunotoxin elicited toxicity in healthy $CD117^+$ tissues outside the hematopoietic compartment. No evidence of gross toxicity or pathological lesions in any tissues was observed by necropsy or histology following CD117-sap treatment (**Figure 16C**). Collectively, these data illustrate that CD117-sap immunotoxin conditioning potently depletes ST- and LT-HSC populations in HA mice without causing overt toxicity in non-target tissues.

Figure 16

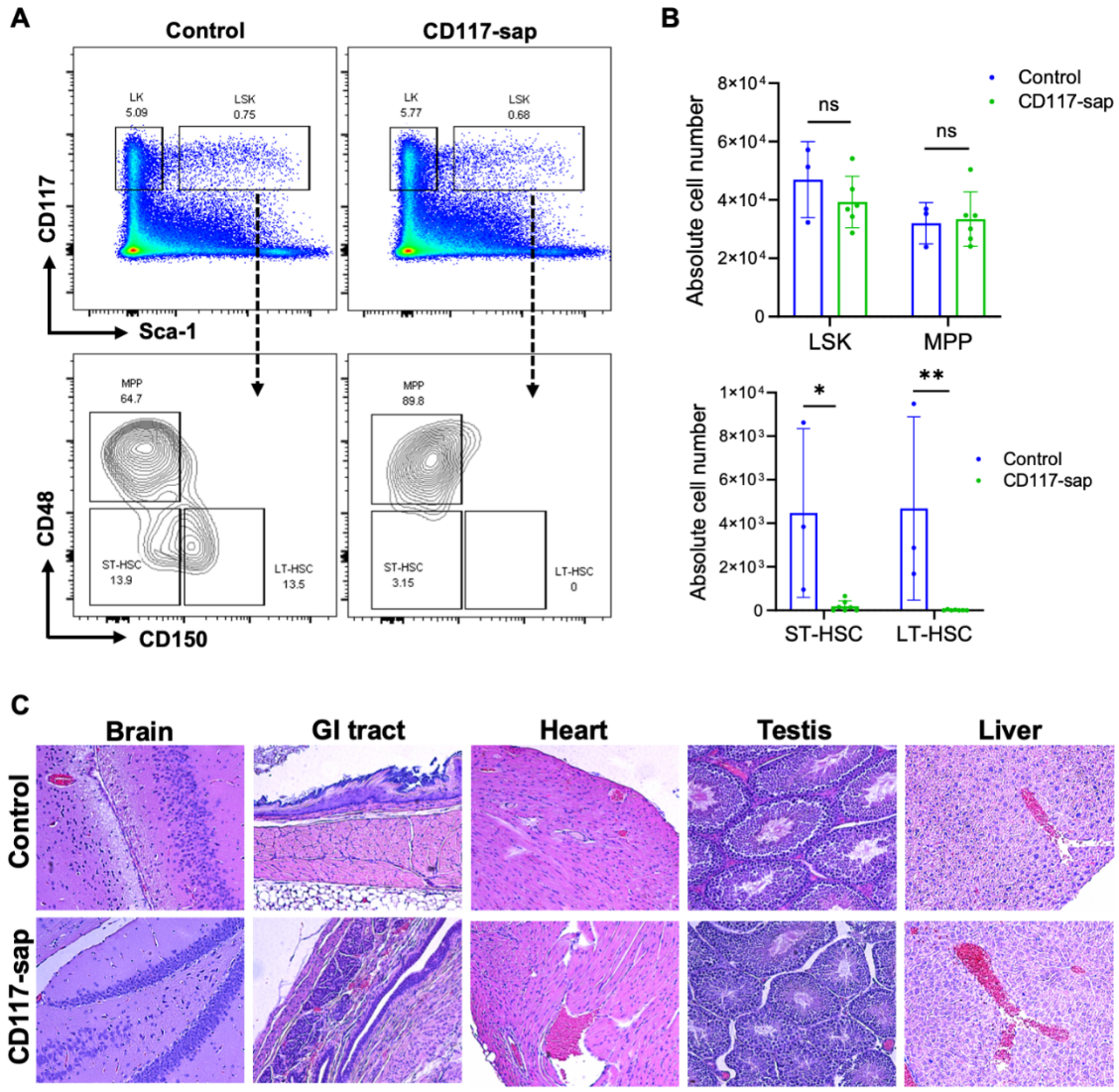


Figure 16. CD117-saporin immunotoxin selectively depletes HSCs and spares non-hematopoietic tissues in hemophilia A mice

(**A**) Representative flow cytometry plots of bone marrow demonstrate selective and robust depletion of short-term (LSK CD48$^-$ CD150$^-$) and long-term (LSK CD48$^-$ CD150$^+$) HSC compartments 5 days post-conditioning with CD117-sap. (**B**) Quantification of HSPC depletion after treatment with CD117-sap immunotoxin (n = 6) compared with no immunotoxin (n = 3) illustrates selective HSC depletion. Data represent mean ± sample SD. Statistical comparison performed using Mann-Whitney U test. *p < 0.05, **p < 0.01, ns = not significant (**C**) H&E staining of non-hematopoietic tissues known to express CD117 revealed no evidence of toxicity or histopathological lesions.

CD117-sap plus anti-thymocyte globulin enables engraftment of ET3 modified LT-HSCs in a subset of mice. We next evaluated whether CD117-sap immunotoxin could effectively condition the bone marrow of HA mice to engraft congenic donor HSPCs modified using an LV harboring our expression codon-optimized (ECO) ET3 transgene driven by the CD68 promoter (CD68-ECO-ET3-LV). We have previously shown that host immune suppression using T cell costimulatory blocking agents or anti-thymocyte serum was necessary to achieve high donor chimerism of gene-modified cells and sustained fVIII activity in the absence of inhibitor development in LV-HSCT studies using various non-myeloablative conditioning regimens and a B-domain deleted porcine fVIII transgene.[155] These observations informed our hypothesis that transient immune suppression would likely be necessary to enable long term engraftment of ET3 gene-modified HSPCs and durable fVIII expression in the context of non-genotoxic immunotoxin conditioning.

CD45.2$^+$ HA mice were conditioned with 0.5 mg/kg CD117-sap with or without 30 mg/kg mATG, or mATG alone, and then transplanted 5 days later with 1 x 10^6 CD68-ECO-ET3-LV transduced CD45.1$^+$ Sca-1$^+$ HSPCs. Mice conditioned with TBI were included as positive controls to ensure viability and engraftment potential of the HSPCs following ex vivo culture and transduction. Depletion of ST-HSC and LT-HSC compartments in mice conditioned with CD117-sap + mATG were analyzed and findings were consistent with patterns observed in mice conditioned with CD117-sap alone (**Figure S3A**). Peripheral blood and plasma were collected every two weeks and complete blood counts (CBCs), multilineage donor cell chimerism and ET3 activity were measured (**Figure 17A**). Conditioning with CD117-sap + mATG permitted normal white blood cell (WBC) and lymphocyte counts within 2-4 weeks after transplantation and did not produce prolonged leukopenia. Platelet counts were also preserved in CD117-sap + mATG conditioned

mice and monocyte counts were not negatively impacted by the conditioning regimen (**Figure 17B and S3B**). In a separate experiment, leukocyte and lymphocyte subpopulations following mATG administration were tracked for 21 days post-conditioning to examine kinetics of depletion and recovery (**Figure S4**). Two weeks after transplantation, 1 of 7 mice (across two independent experimental cohorts) conditioned with CD117-sap + mATG failed to engraft ET3 gene-modified HSPCs (**Figure S5A, orange diamond**), while the remaining six mice achieved early myeloid chimerism of 72.5 ± 33.3% (mean ± sample SD). Mice receiving CD117-sap alone or mATG alone universally failed to engraft gene-modified cells (**Figure 17C**). By four weeks post-transplantation, myeloid chimerism levels decreased from 95.6% to 0.22% and 86.4% to 8.94% in two additional mice conditioned with CD117-sap + mATG (**Figure S5A, red square and brown square**). The remaining four mice in this group maintained high levels of donor myeloid chimerism up to 24 weeks post-transplantation. T and B cell chimerism continued to rise in these mice for the duration of the study (**Figure 17C and S5**). Upon sacrifice at 30 weeks post-transplantation, engraftment of donor-derived LT-HSCs in chimeric HA mice conditioned with CD117-sap + mATG was 80.2 ± 16.1% (**Figure 17D and S6A**). For comparison of the CD117-sap + mATG regimen with another non-myeloablative regimen, in a separate set of experiments HA mice were conditioned with three doses of 15 mg/kg busulfan and two doses of 20 mg/kg mATG before transplantation with 1 x 10^6 CD68-ECO-ET3-LV transduced CD45.1$^+$ Sca-1$^+$ HSPCs. TBI conditioned mice were included as controls for comparison of donor chimerism and fVIII activity. Eight weeks post-transplantation, mice conditioned using CD117-sap + mATG with sustained engraftment achieved higher mean donor leukocyte chimerism than mice conditioned with the busulfan + mATG regimen (73.2 ± 13.9% vs. 60.1 ± 11.0%, respectively) (**Figure S6B**).

Bone marrow from one primary recipient conditioned with CD117-sap + mATG (95.2% myeloid chimerism, 88.2% LT-HSC chimerism) and one conditioned with TBI (98.8% myeloid chimerism, 97.4% LT-HSC chimerism) was harvested and transplanted into lethally irradiated secondary HA recipients (n = 5 secondary recipients). Early CD45.1$^+$ myeloid chimerism in secondary recipients reached 99.0 ± 0.7% four weeks after serial transplantation and remained at this level for 24 weeks. B cell CD45.1$^+$ chimerism reached 95.4 ± 2.5% by eight weeks and was sustained, while T cell CD45.1$^+$ continued to increase throughout the study (**Figure 17E**). Results were similar in secondary recipients of TBI donor marrow (**Figure S7A**). CD45.1$^+$ LT-HSC engraftment was 83.3 ± 22.4% in secondary recipients receiving CD117-sap + mATG-conditioned bone marrow and 99.9 ± 0.06% for secondary recipients receiving TBI-conditioned bone marrow (**Figure 17D and S6A**), which were consistent with levels measured in the primary recipients.

Taken together, these data demonstrate that CD117-sap + mATG enabled high-level engraftment of CD68-ECO-ET3-LV modified donor LT-HSCs in the absence of prolonged lymphopenia in a majority of HSCT recipients. However, 43% (3 out of 7) of mice conditioned with CD117-sap + mATG did not maintain long-term donor engraftment. Despite this variability, immune suppression with mATG was required for successful engraftment of ET3 gene-modified cells in HA mice.

Figure 17

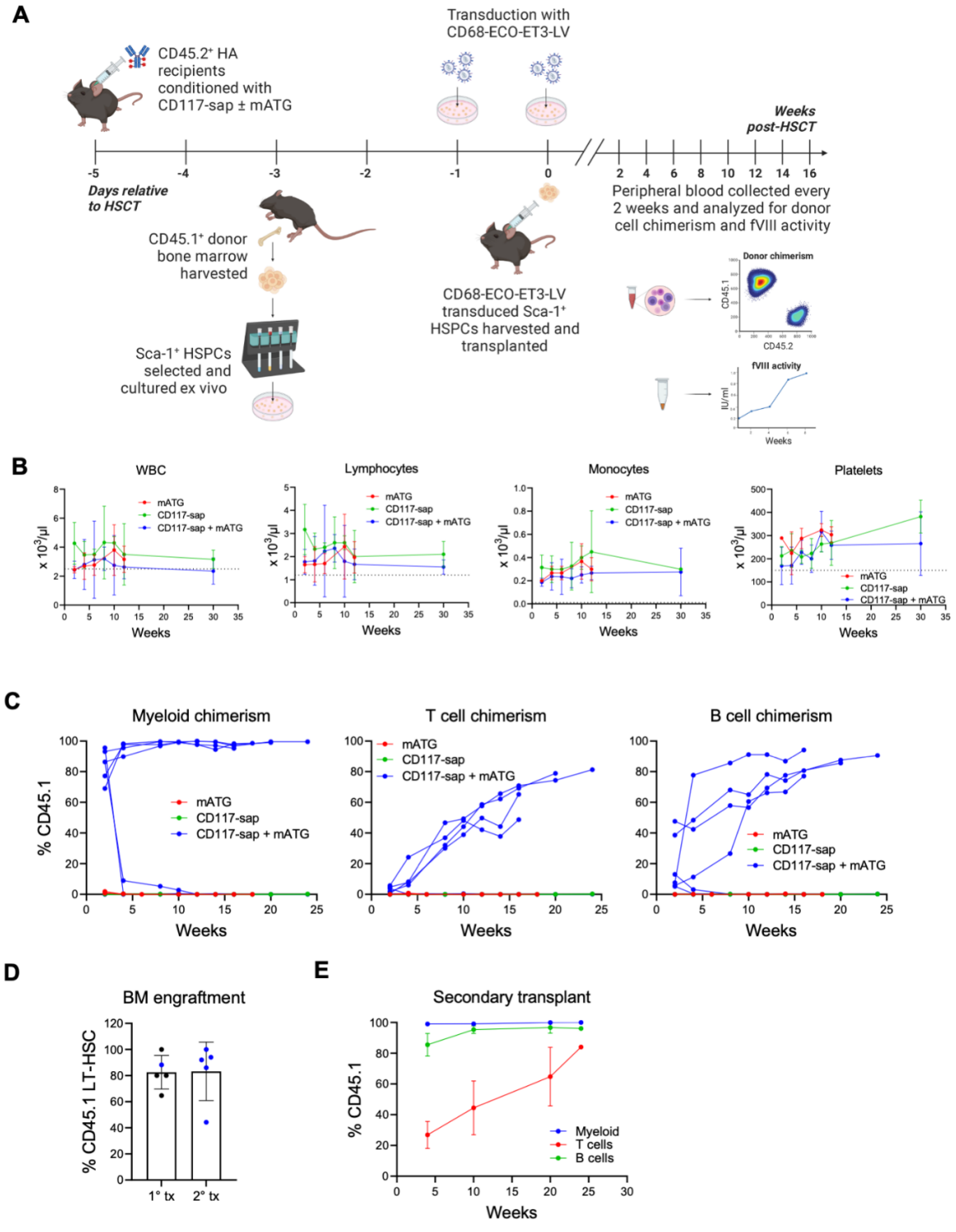


Figure 17. CD117-sap + mATG conditioning enables engraftment of CD68-ECO-ET3-LV transduced LT-HSCs and multilineage chimerism in a majority of recipients

(**A**) Schematic of experimental design illustrates timeline of CD117-sap + mATG conditioning followed by transplantation of ET3 gene-modified Sca-1$^+$ cells in HA mice. (**B**) CD117-sap + mATG conditioning (n = 7) did not produce prolonged leukopenia or lymphopenia in the early post-transplantation period. No detrimental effects to monocyte populations were observed. Platelet counts were preserved in the early post-transplantation period. Dotted line represents lower limit of reference range for each population. Data represent mean ± sample SD. See also Figure S3B and S4. (**C**) High-level early myeloid chimerism was observed in 6 out of 7 mice conditioned with CD117-sap + mATG two weeks post-transplantation. Multilineage hematopoietic chimerism of donor-derived cells was sustained for up to 30 weeks in 4 out of 7 of HA recipients conditioned with CD117-sap + mATG. Conditioning with CD117-sap or mATG alone did not permit engraftment. See also Figure S5. (**D**) Donor-derived LT-HSCs engrafted in bone marrow of mice conditioned with CD117-sap + mATG (n = 5) for primary transplant (1° tx) and secondary transplant (2° tx) recipients (n = 5). Blue dots represent primary recipient that acted as donor for respective secondary recipients. Data represent mean ± sample SD. See also Figure S6A. (**E**) Secondary HA recipients (n = 5) conditioned with 9 Gy total body irradiation were serially transplanted with whole bone marrow from a primary HA recipient conditioned with CD117-sap + mATG and followed for 24 weeks. Results demonstrate that immunotoxin conditioning enabled engraftment of donor-derived long-term repopulating HSCs in primary recipients. Data represent mean ± sample SD. See also Figure S7A.

CD117-sap + mATG conditioning promotes therapeutic and durable ET3 expression. Next, we examined whether high-level engraftment of ET3 gene-modified HSPCs could produce sustained therapeutic levels of ET3 hemostatic activity in HA recipients after CD117-sap + mATG conditioning. We then compared these results with those obtained using the non-myeloablative busulfan + mATG regimen or TBI. Plasma was collected and tested by chromogenic assay. ET3 activity in mice conditioned with CD117-sap + mATG that sustained long-term donor chimerism measured 0.21 ± 0.15 IU/ml (21% the normal human level) and 0.13 ± 0.18 IU/ml (13% of normal) in busulfan + mATG mice as early as two weeks post-HSCT. By six weeks, curative levels of 0.88 ± 0.35 IU/ml (88% of normal) were achieved in the busulfan + mATG group, and 0.62 ± 0.40 IU/ml (62% of normal) using CD117-sap + mATG conditioning, with ET3 activity continuing to increase for the duration of the study in the latter group. Mice conditioned with CD117-sap + mATG that did not maintain donor chimerism also lost fVIII activity (**Figure 18A**).

Vector copy number (VCN) of CD68-ECO-ET3-LV was measured in DNA isolated from peripheral blood of mATG, CD117-sap, or CD117-sap + mATG treated mice 10 weeks post-transplantation. Peripheral blood VCN in mice conditioned with CD117-sap + mATG that maintained the graft was 0.19 ± 0.05 copies per genome (**Figure 18B and S7B**). ELISA assays were performed to detect the presence of anti-ET3 IgG antibodies to determine whether mice conditioned with mATG, CD117-sap alone, or CD117-sap + mATG that failed to durably engraft gene-modified HSPCs also mounted a humoral response against the ET3 transgenic protein. Although none of the six mice conditioned with CD117-sap alone without mATG immune suppression successfully engrafted ET3 gene-modified HSPCs at any point during the study, only two developed measurable levels of anti-ET3 antibodies (**Figure 18C**). Similarly, of the three mice conditioned with CD117-sap + mATG that failed to maintain engraftment during the study, only one developed antibodies against ET3. Bethesda assays were performed to quantify titers in mice that developed ET3 neutralizing inhibitors (**Figure S7C**). The immunological mechanism responsible for rejection of ET3 gene-modified cells in mice that did not produce ET3 inhibitors was not identified.

ET3 activity in secondary HA recipients was measured by chromogenic assay for 24 weeks after serial transplantation. Levels of ET3 activity reached 6.7 ± 1.3 IU/ml (660% of normal) four weeks after secondary HSCT with CD117-sap + mATG donor marrow and were sustained for the duration of the study (**Figure 18D**). These findings illustrate that CD68-ECO-ET3-LV HSPC-directed gene therapy using non-genotoxic CD117-sap + mATG conditioning enables durable therapeutic levels of ET3 activity in HA mice following engraftment of gene-modified cells.

However, in approximately one-third of mice that did not maintain long-term engraftment under CD117-sap or CD117-sap + mATG conditioning, an immune response to ET3 was observed.

Figure 18

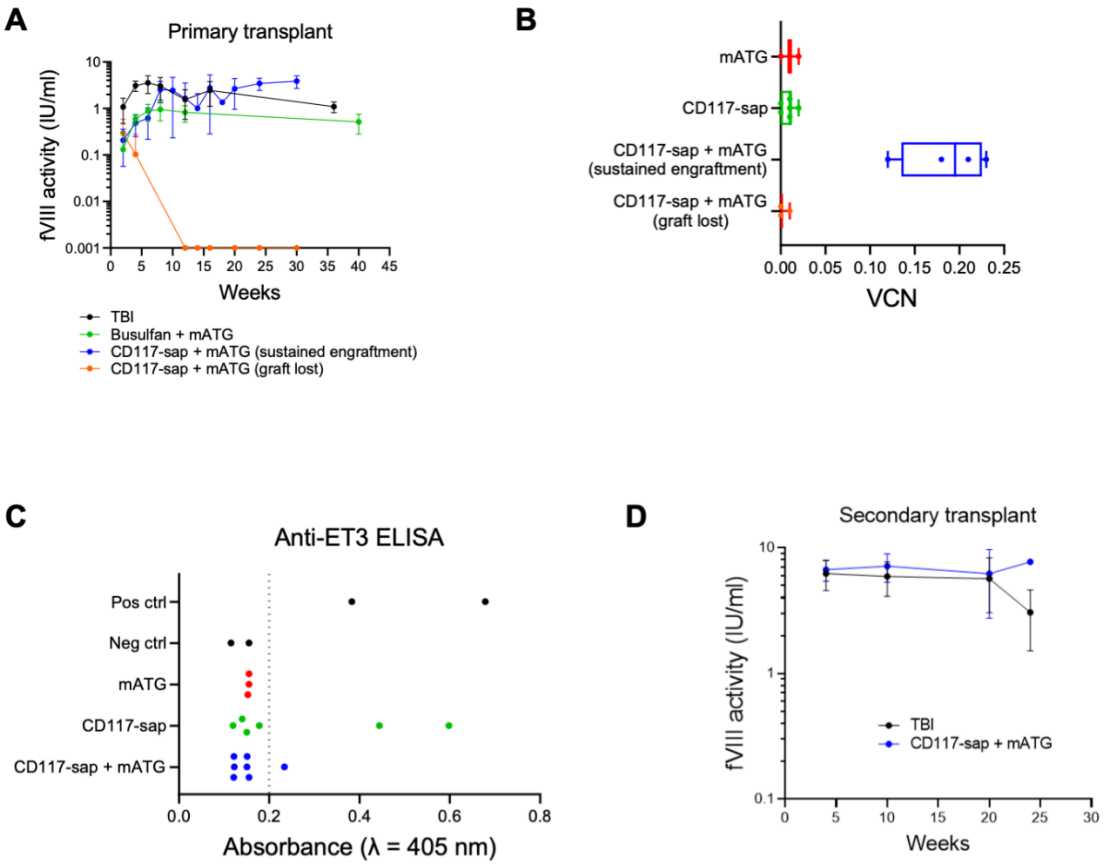


Figure 18. HA mice engrafted with gene-modified HSCs following CD117-sap + mATG conditioning exhibited therapeutic levels of plasma ET3 activity while some non-engrafted mice developed ET3 inhibitors

(A) ET3 activity was determined by chromogenic assay in HA mice that maintained long-term engraftment of ET3 gene-modified HSCs. (TBI, n = 9; Busulfan + mATG, n = 5; CD117-sap + mATG, n = 4; CD117-sap + mATG (graft lost), n = 3) Data represent mean ± sample SD. (B) Vector copy number was measured by real-time PCR of proviral DNA in peripheral blood of primary recipients 10 weeks post-transplantation. (mATG, n = 3; CD117-sap, n = 6; CD117-sap + mATG, n = 4; CD117-sap + mATG (graft lost), n = 3) See also Figure S7B. (C) Anti-ET3 antibodies were detected by ELISA in three mice 14 weeks post-transplantation. Dotted line represents threshold for positive signal. Inhibitors were detected in two mice that failed to engraft gene-modified HSCs following conditioning with CD117-sap alone. One mouse conditioned with CD117-sap + mATG did not have sustained engraftment and developed ET3 inhibitors.

Bethesda titers shown in Figure S7C. (D) Sustained high levels of ET3 activity measured in lethally irradiated HA secondary recipients following serial transplantation indicate engraftment of gene-modified, LT-HSCs after TBI (n = 5 secondary recipients) or CD117-sap + mATG (n = 5 secondary recipients) conditioning in primary recipients. Data represent mean ± sample SD.

mATG exhibits a broad-ranging binding profile that includes mature and primitive hematopoietic cells. In an effort to identify potential causes for why certain mice conditioned with CD117-sap + mATG failed to engraft or maintain engraftment of ET3 gene-modified cells, we turned our attention to mATG. The mATG used in our study was produced by immunizing rabbits with mouse thymocytes and collecting the IgG fraction. Human ATG products are known to contain a multitude of antibodies with varying specificities, with new target antigens being continually identified that can be found on a variety of cell types and involved in diverse cellular pathways.[184, 185] With concerns over whether residual mATG in circulation at the time of HSCT could potentially bind donor HSCs and interfere with engraftment, we aimed to characterize the binding profile of the mATG reagent.

HA mice were injected intraperitoneally (IP) with 30 mg/kg mATG and sacrificed after 4 hours (**Figure 19A**). Blood, spleen, and bone marrow cells were harvested and stained with an anti-rabbit IgG secondary antibody to detect cells bound by mATG, and then stained for LSK-SLAM markers in bone marrow, or mature lineage markers in spleen and blood. Flow cytometric analysis revealed substantial mATG binding to every cell population examined in all three tissues (**Figure 19B**). As expected, mATG antibodies were extensively bound to T cell subsets, yet mATG was also detected on B cells and Gr-1^{+} myeloid cells. Furthermore, considerable binding of mATG antibodies was detected on all lineage-negative bone marrow subsets evaluated, including ST- and LT-HSC

populations. Median fluorescence intensity (MFI) of mATG staining (as measured by secondary antibody signal) was greatest in the peripheral blood $CD8^+$ T cell compartment, while MFIs of the various bone marrow HSPC compartments were at least an order of magnitude lower than those measured from peripheral blood T cells (**Figure 19C**). Nevertheless, clear signals indicating mATG binding to HPC and HSC populations were observed (**Figure 19D and S8**). Similar results were obtained from binding studies using peripheral blood, bone marrow and spleen cells from HA mice incubated with mATG in vitro and analyzed by flow cytometry (**Figure S9A and S9B**). Additionally, in transplantation studies, when mATG was administered the day before and the day of transplant, engraftment of transplanted HSPCs was abrogated (**Figure S9C, red line**). These results suggest off-target effects of mATG, potentially through direct interaction with the transplanted gene-modified HSPCs, likely confounded the previous studies and contributed to the variability in outcomes observed.

Figure 19

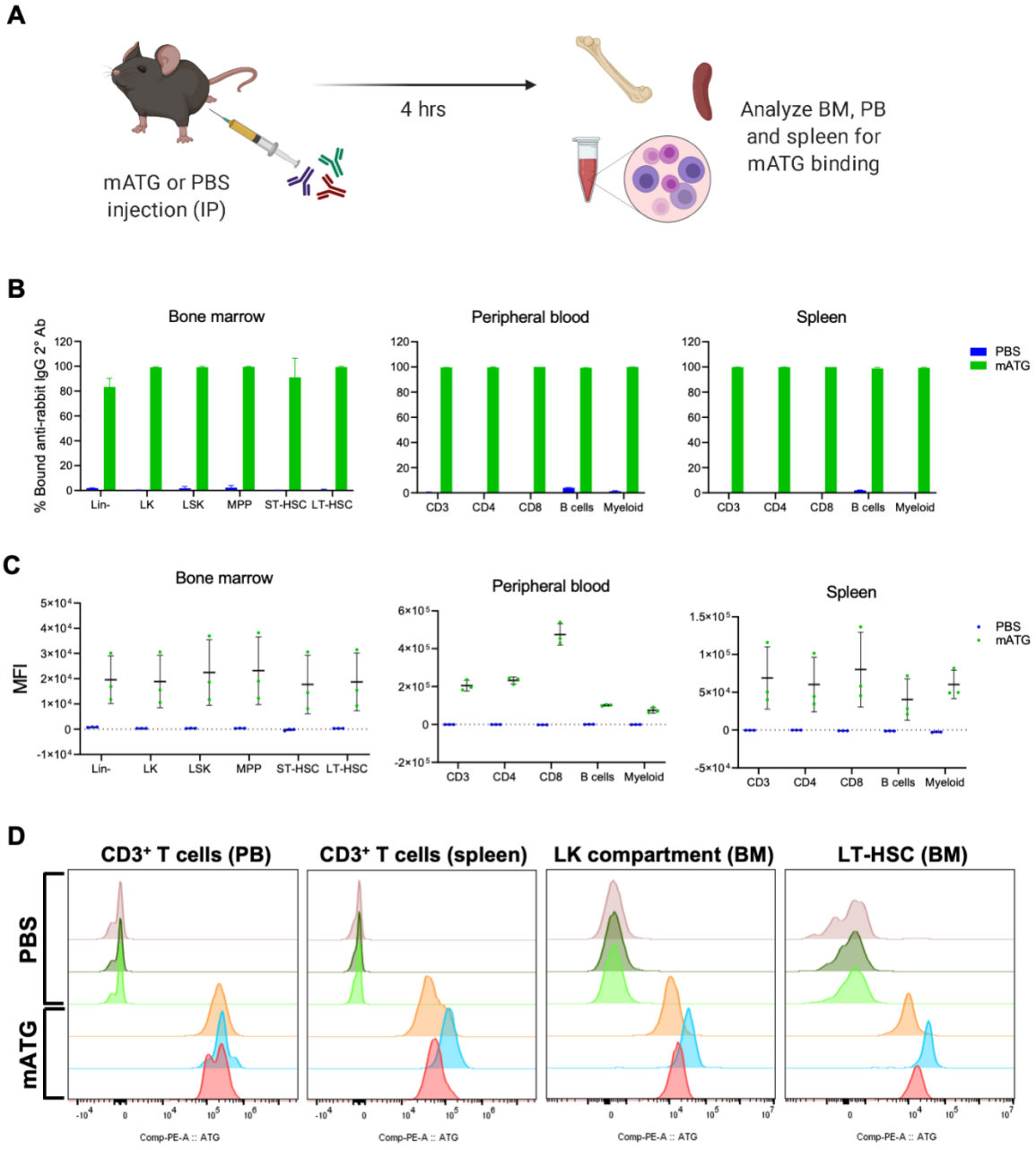


Figure 19. Rabbit anti-mouse mATG binds mature and primitive hematopoietic cells in peripheral blood, spleen and bone marrow

(**A**) Schematic illustrates experimental design of in vivo mATG binding study. (**B**) mATG binding to various cell populations in each tissue was detected by flow cytometry using an anti-rabbit IgG secondary antibody. Data represent mean ± sample SD. (n = 3 in each group) See also Figure S9A. (**C**) Median fluorescence intensity (MFI) of mATG staining reveals greatest intensity of binding on $CD8^+$ peripheral blood T cell subset. MFI measured in bone marrow subsets were 10- to 20-fold lower than in peripheral blood subsets. See also Figure S9B. (**D**) Flow cytometry histograms exemplify mATG binding on spleen

and peripheral blood (PB) T cells, Lin^- $CD117^+$ (LK) HSPCs and LT-HSCs in bone marrow (BM). See also Figure S8.

CD117-sap plus T cell-targeted immune suppression invariably enables engraftment of ET3-modified LT-HSCs. Immune suppression is a requirement for engraftment of CD68-ECO-ET3-LV gene-modified cells. Although there are important aspects of mATG immune suppression that enabled engraftment and ET3 expression in a majority of recipients, there appeared to be additional aspects that could also be interfering. We aimed to develop a more targeted approach to immune suppression in our gene therapy model in an attempt to replicate the important aspects of mATG mechanism of action on immune suppression while excluding potential confounding effects relating to the polyvalent and non-thymocyte specific nature of mouse-specific ATG. Since the immune response to fVIII is T cell-dependent,[186, 187] we designed a naked monoclonal antibody regimen specifically targeting T cells in order to control host T cell responses directed at ET3-modified cells. HA mice were conditioned with CD117-sap followed by antibody cocktails directed at CD4, CD8 and CD40L antigens (collectively referred to as 'T cell mAbs'), and then transplanted with 2.5 x 10^6 CD68-ECO-ET3-LV transduced $CD45.1^+$ $Sca\text{-}1^+$ HSPCs (**Figure 20A**). CD4 (clone GK1.5) and CD8 (clone YTS169.4) mAbs produce in vivo depletion of respective T cell subsets, whereas CD40L mAb (clone MR-1) blocks CD40/CD40L signaling. CD40L is primarily expressed on activated $CD4^+$ T cells, and the interaction of CD40L with CD40 expressed by antigen-activated B cells is critical for humoral immune responses against T cell-dependent antigens.[188]

This regimen permitted normal total lymphocyte counts by two weeks post-transplantation and normal monocyte and platelet counts throughout the study (**Figure 20B and S10A**). In a separate

experiment, leukocyte and lymphocyte subpopulations following T cell mAbs administration were tracked for 21 days post-conditioning to examine kinetics of depletion and recovery (**Figure S4**). Of 13 mice across two independent experimental cohorts, mice conditioned with CD117-sap + T cell mAbs uniformly maintained multilineage donor chimerism throughout the study up to 30 weeks post-transplantation with no incidence of immune rejection (**Figure 20C**). Mean myeloid chimerism at 30 weeks was 64.9 ± 23.2% (range: 37.5 – 99.9%), T cell chimerism was 88.0 ± 7.5% (range: 77.1 – 98.0%) and B cell chimerism was 82.2 ± 14.2% (range: 63.6 – 96.2%). A subgroup of mice that received CD117-sap + T cell mAbs conditioning and mock transduced Sca-1^+ HSPCs achieved only slightly higher levels of donor chimerism in each cellular compartment (**Figure S10B**). Upon sacrifice, engraftment of donor-derived CD68-ECO-ET3-LV transduced LT-HSCs in chimeric HA mice conditioned with CD117-sap + T cell mAbs was 73.6 ± 23.5% (range: 23.0 – 95.2%) (**Figure 20D and S11A**).

Bone marrow from two primary recipients that received CD117-sap + T cell mAbs conditioning and ET3-modified HSPCs (myeloid chimerism 97.9% and 76.9% and LT-HSC chimerism 94.1% and 46.2% when sacrificed at 16 weeks post-transplantation) was harvested and transplanted into lethally irradiated secondary HA recipients (n = 6 secondary recipients per donor). CD45.1^+ myeloid chimerism in secondary recipients reached 98.8 ± 0.66% from donor 1 and 80.1 ± 18.3% from donor 2 after 16 weeks (**Figure 20E, blue and red circles**), consistent with levels measured in respective primary recipients at the same time point. Secondary recipients were sacrificed after 30 weeks and CD45.1^+ LT-HSC chimerism measured 99.3 ± 1.5% in recipients of donor 1 and 41.8 ± 29.1% in recipients of donor 2 (**Figure 20D and S11A**). These values were also consistent with LT-HSC chimerism measured in respective primary recipients.

Taken together, these data demonstrate that, although both regimens facilitate high multilineage engraftment in durably engrafted mice, CD117-sap + T cell mAbs conditioning, in contrast to the CD117-sap + mATG regimen, enabled reliable engraftment of CD68-ECO-ET3-LV modified donor LT-HSCs in the absence of prolonged lymphopenia and with no incidence of immunological rejection in 100% of gene therapy recipients.

Figure 20

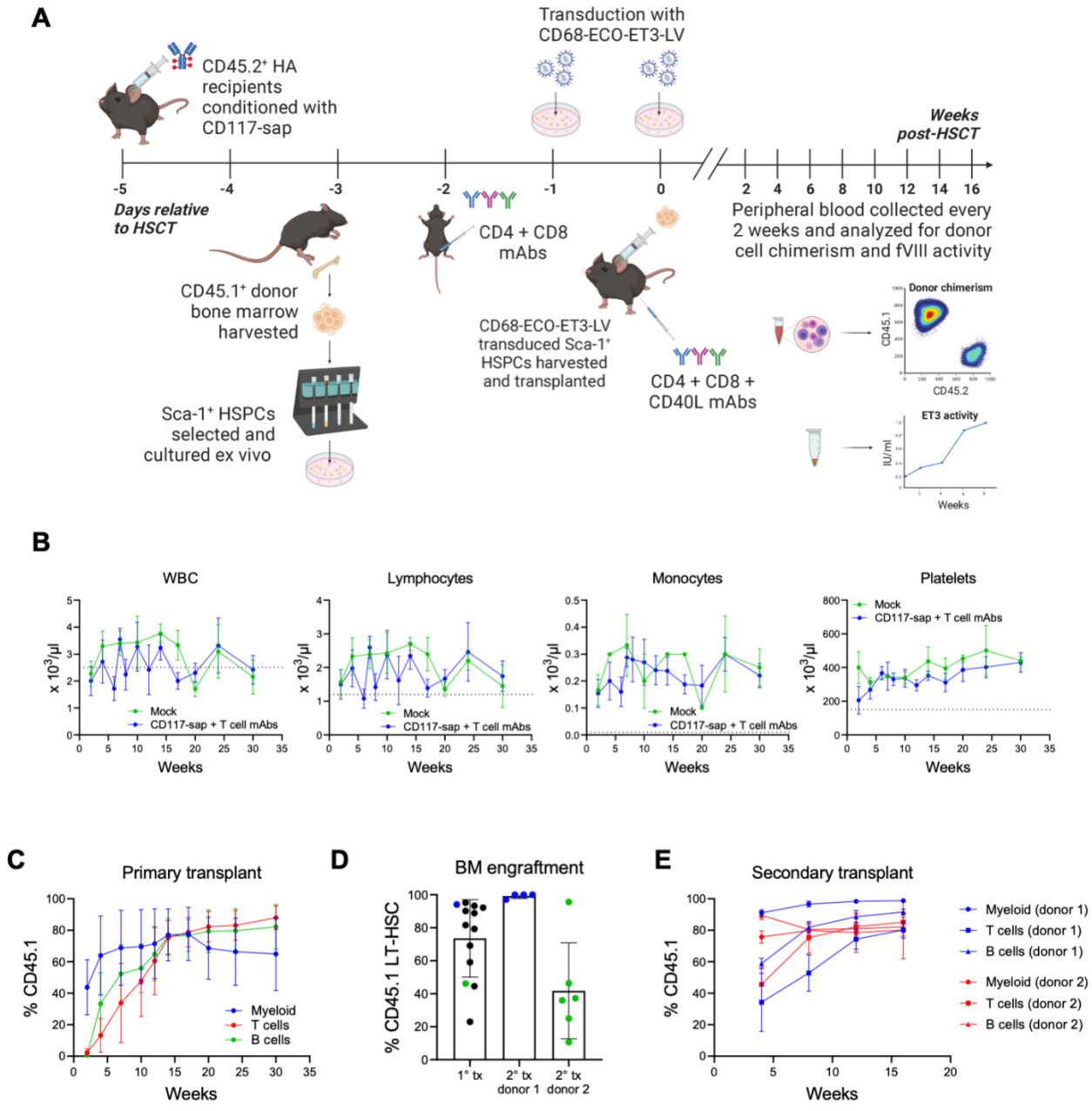


Figure 20. CD117-sap + T cell mAbs conditioning enables engraftment of CD68-ECO-ET3-LV gene-modified LT-HSCs and multilineage hematopoietic chimerism in all transplant recipients

(**A**) Schematic of experimental design illustrates timeline of CD117-sap + T cell mAbs conditioning followed by transplantation of ET3 gene-modified Sca-1^{+} cells in HA mice. (**B**) CD117-sap + T cell mAbs (ET3-transduced cell transplant, n = 13; mock transduced cell transplant, n = 3) conditioning did not produce prolonged leukopenia or lymphopenia in the early post-transplant period. No detrimental effects to monocyte populations were observed. Platelet counts were preserved in the early post-transplantation period in mice conditioned with CD117-sap + T cell mAbs. Dotted line represents lower limit of reference range for each population. Data represent mean ± sample SD. See also Figure S10A and S4. (**C**)

Multilineage hematopoietic chimerism of donor-derived cells was sustained for up to 30 weeks in 100% of HA recipients conditioned with CD117-sap + T cell mAbs (n = 13). Data represent mean ± sample SD. See also Figure S10B. (**D**) Donor-derived LT-HSCs engrafted in bone marrow of mice conditioned with CD117-sap + T cell mAbs (n = 13) for the primary transplant (1° tx) and secondary transplant (2° tx) recipients from two donors (n = 6 per donor). Data represent mean ± sample SD. Blue and green dots represent donors for respective secondary transplant recipients. See also Figure S11A. (**E**) Secondary HA recipients (n = 6 in each group) conditioned with 9 Gy total body irradiation were serially transplanted with whole bone marrow from two primary HA recipients conditioned with CD117-sap + T cell mAbs and followed for 16 weeks. Results demonstrate that immunotoxin conditioning enabled engraftment of donor-derived long-term repopulating HSCs in primary recipients. Data represent mean ± sample SD.

Sustained therapeutic levels of ET3 activity uniformly achieved using CD117-sap + T cell mAbs conditioning. Plasma samples were tested by chromogenic assay to ensure ET3 gene-modified HSCs engrafted under CD117-sap + T cell mAbs conditioning could enable long-term therapeutic expression of circulating plasma ET3. Four weeks post-transplantation using the new targeted regimen, curative ET3 levels of 0.49 ± 0.31 IU/ml were achieved, steadily increasing to 1.76 ± 1.0 IU/ml by 30 weeks (**Figure 21A and S11B**). At the time of sacrifice, copies of CD68-ECO-ET3-LV proviral DNA were measured from bone marrow and spleen. In mice receiving the CD117-sap + T cell mAbs regimen, VCN measured 2.5 ± 1.8 copies per genome in bone marrow and 1.9 ± 0.68 in spleen (**Figure 21B and S11C**).

ET3 activity in secondary HA recipients was measured by chromogenic assay for 16 weeks after serial transplantation. At the time of secondary transplantation, ET3 activity measured 2.60 IU/ml in donor 1 and 1.04 IU/ml in donor 2. Measured ET3 activity in secondary recipients were consistent with those measured in the donors; ET3 activity in secondary recipients of donor 1 bone marrow reached 2.70 ± 0.24 IU/ml, while activity in secondary recipients of donor 2 marrow reached 1.35 ± 0.59 IU/ml (**Figure 21C and S11B**). VCN of secondary recipients was quantified

from bone marrow and spleen at the time of sacrifice 30 weeks post-HSCT. Copies of CD68-ECO-ET3-LV proviral DNA in bone marrow measured 1.3 ± 0.33 (donor 1) and 0.71 ± 0.21 (donor 2) and spleen measured 1.47 ± 0.20 (donor 1) and 0.72 ± 0.29 (donor 2) (**Figure 21B and S11C**).

We performed a tail snip bleeding assay to assess correction of the characteristic bleeding phenotype of HA mice to demonstrate the functional efficacy of circulating plasma ET3 activity achieved from HSCT gene therapy under CD117-sap + T cell mAbs conditioning. After 40 minutes of bleeding from a 4 mm excision from the tip of the tail, untreated HA control mice lost 22.9 ± 3.9 mg of blood per gram body weight. By contrast, wild-type fVIII-competent controls and mice treated with ET3 gene therapy bled significantly less, losing only 0.67 ± 2.2 and 1.1 ± 0.37 mg blood per gram body weight, respectively (**Figure 21D**). Together these data demonstrate that CD68-ECO-ET3-LV HSPC-directed gene therapy using non-genotoxic CD117-sap + T cell mAbs conditioning enables long-term expression and functional activity of ET3, restoring hemostasis and correcting the disease phenotype in HA mice.

Figure 21

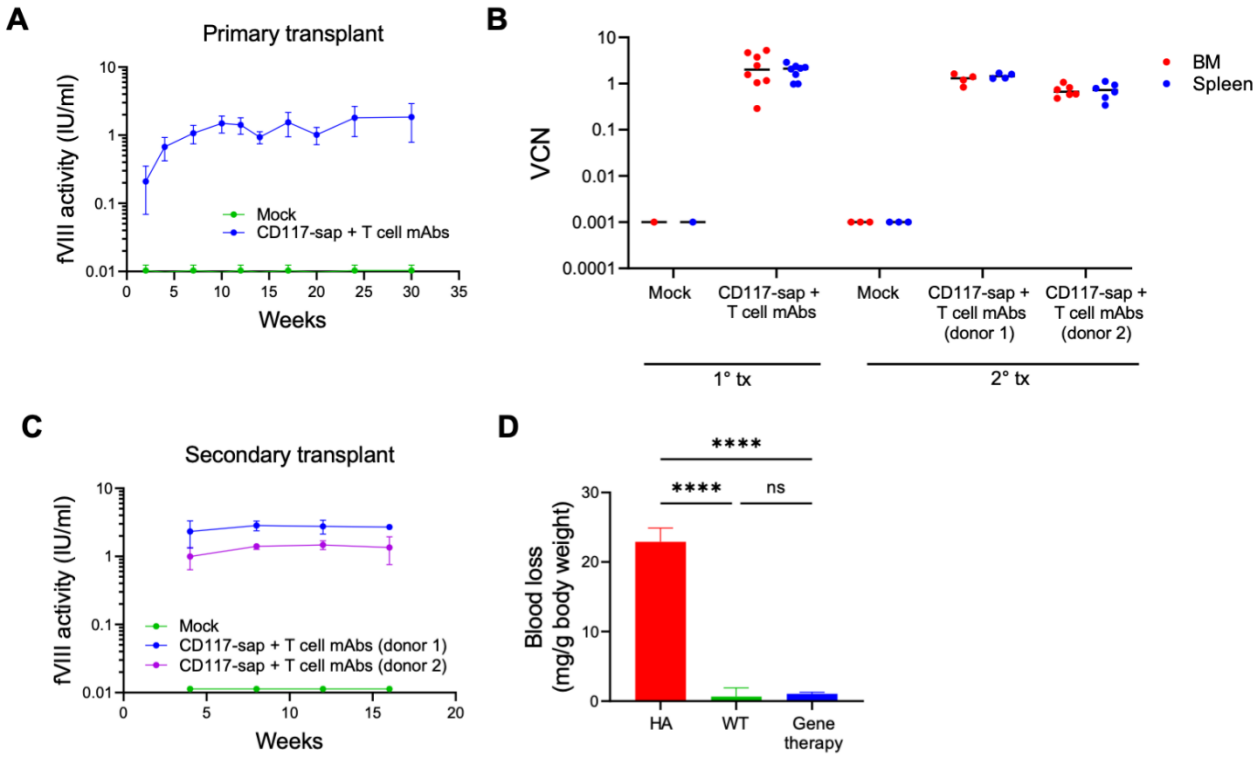


Figure 21. HA mice conditioned with CD117-sap + T cell mAbs and engrafted with CD68-ECO-ET3-LV gene-modified HSCs exhibited therapeutic levels of plasma ET3 activity and phenotypic correction

(**A**) ET3 activity was determined by chromogenic assay of plasma from HA mice engrafted with ET3 gene-modified HSCs following CD117-sap + T cell mAbs conditioning (n = 13) and mice that received CD117-sap + T cell mAbs conditioning and mock transduced HSCT (n = 3). Data represent mean ± sample SD. See also Figure S11B. (**B**) Vector copy number in primary (n = 8) and secondary (n = 4-6) recipients was measured by real-time PCR of proviral DNA from bone marrow and spleen. See also Figure S11C. (**C**) Sustained high levels of ET3 activity measured in lethally irradiated HA secondary recipients following serial transplantation indicate engraftment of ET3 gene-modified, LT-HSCs in primary recipients. (Mock, n = 3; CD117-sap + T cell mAbs, n = 6 in each group) Data represent mean ± sample SD. See also Figure S11B. (**D**) Correction of HA bleeding phenotype was observed using tail snip bleeding assay in gene therapy mice conditioned with CD117-sap + T cell mAbs (n = 3 in each group). Data represent mean ± sample SD. Statistical comparison performed using one-way ANOVA with Tukey's multiple comparisons test. ****$p < 0.0001$, ns = not significant

Normal tissue morphology and fertility after engraftment of gene-modified HSCs and ET3 expression. Histological analysis of H&E-stained sections of various organs 34 weeks post-

transplantation was performed to ensure the absence of organ toxicity following long-term engraftment of ET3-modified cells and CD68 promoter-driven ET3 expression from tissue-based macrophages. No evidence of toxicity, pathological lesions or inflammatory processes were observed in liver, spleen, gonad, bone marrow, or brain due to ET3 gene therapy under CD117-sap + T cell mAb conditioning (**Figure 22A**). Six weeks after CD117-sap + T cell mAbs conditioning and engraftment of ET3-modified HSCs, chimeric HA males were mated to untreated HA females to assess fertility and reproductive function. Chimeric males mated successfully and produced indistinguishable litter sizes compared to control HA breeder males (**Figure 22B**). Mating of chimeric HA males was followed up to 20 weeks post-engraftment and no reproductive dysfunction was observed during this observation period.

Figure 22

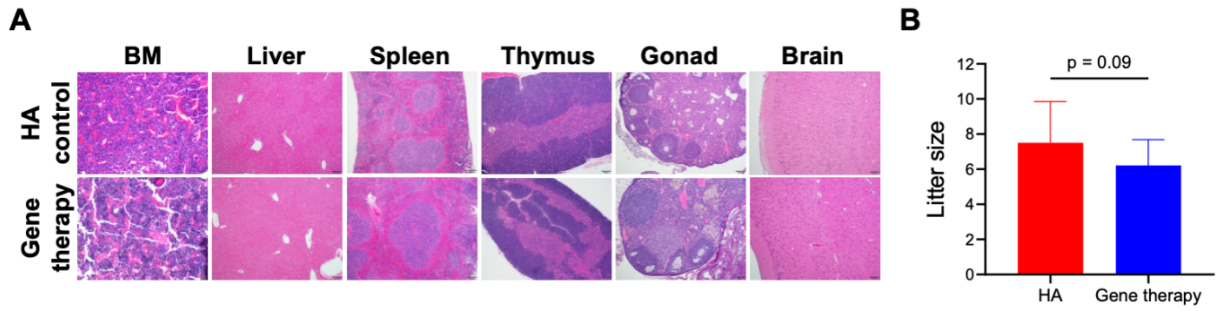


Figure 22. HA mice engrafted with ET3-modified cells after CD117-sap + T cell mAbs conditioning exhibit healthy tissue morphology and normal litter sizes
(**A**) H&E staining of various tissues revealed no evidence of toxicity or inflammatory processes following long-term engraftment of ET3 gene-modified cells. (**B**) Average litter size was not significantly different between control HA breeder males (n = 12 litters) and HA males engrafted with ET3 gene-modified HSCs following CD117-sap + T cell mAbs conditioning (n = 19 litters) indicating normal reproductive capacity following ET3 gene therapy with non-genotoxic conditioning. Statistical comparison performed using Student's t-test. Data represent mean ± sample SD.

2.5 Discussion

Autologous HSC-directed gene therapy represents a promising strategy for providing a lifelong cure for hemophilia A. However, risks associated with DNA damaging, genotoxic conditioning agents traditionally required for successful HSCT remain a concern. The preclinical findings described herein suggest that these risks can be mitigated. We demonstrate that endogenous HSCs can be specifically depleted in HA mice using a non-genotoxic immunotoxin targeting CD117 and, coupled with a non-genotoxic antibody cocktail targeting host T cells for immune suppression, HSCs modified to contain a functional copy of the fVIII variant ET3 can be successfully and durably engrafted with no signs of immunological rejection. Upon monocyte differentiation and tissue-based macrophage and dendritic cell maturation, sustained therapeutic levels of ET3 are secreted into plasma, restoring hemostasis and correcting the HA phenotype in the absence of humoral immunity against the transgenic protein.

Anti-CD117 mAb conjugated to the ribosome-inactivating protein toxin saporin (sap) was utilized to create a potent HSC-targeted immunotoxin to condition bone marrow in preparation for HSCT gene therapy. Other studies have demonstrated the effectiveness of this antibody-toxin combination[47, 50, 189] or CD45-sap immunotoxin[46, 49, 50] for non-genotoxic conditioning in traditional HSCT models. We have found CD117-sap to be more effective than CD45-sap in enabling specific HSC depletion and donor cell engraftment in pilot studies with HA and C57BL/6 mice (**Figure S12**). The more restricted expression of CD117 compared with CD45 in the hematopoietic compartment enabled the use of a 6-fold lower dose (0.5 mg/kg versus 3.0 mg/kg) of CD117-sap to achieve robust HSC depletion in the absence of prolonged lymphopenia, making it more cost-effective, as well as limiting exposure of the recipient to the drug-toxin conjugate. Due to its lack of a lectin B-chain, and therefore significantly diminished ability to enter cells

unilaterally, saporin has a high safety profile.[190] However, since vascular leak syndrome has been previously identified as a dose-limiting toxicity in immunotoxin clinical trials,[191] administration of a minimal dose is favorable.

Published reports describing the application of non-genotoxic antibody-based conditioning for HSPC-directed gene therapy are limited. In a recent study, a combination of CD117-, CD45- and CD8-sap immunotoxins was necessary to achieve engraftment of fVIII-modified HSCs and sustained platelet-confined fVIII expression.[48] To our knowledge, only one report has described the use of a single agent CD117-targeting immunotoxin to facilitate engraftment of LV-modified HSPCs.[58] Autologous $CD34^+$ HSPCs from rhesus macaque were modified with a β-globin construct and successfully engrafted under CD117-amanitin conditioning. In contrast to our study, these studies did not involve production of a secreted, foreign therapeutic gene product that is known to be highly immunogenic (i.e., fVIII in hemophilia A), a unique challenge that requires the induction of sufficient immune tolerance to support lifelong expression of a new circulating protein. Nevertheless, observations from this preliminary nonhuman primate study are encouraging evidence that CD117-based immunotoxin conditioning may be safely and effectively translated to human gene therapy trials in the near future.

In the current study, host immune suppression was found to be a requirement for achieving engraftment of ET3 gene-modified HSPCs using CD117-sap conditioning. We initially sought to incorporate mATG into our non-genotoxic regimen. Human ATG products (e.g., Thymoglobulin®, ATGAM®) are FDA-approved hyperimmune therapeutics commonly used in the prevention of acute rejection of solid organ allografts[192] and graft-versus-host disease (GVHD)

in allogeneic HSCT.[193] ATG is known to elicit rapid T cell depletion, although other potential mechanisms for immune suppression have been posited and the full spectrum of its multifaceted mechanisms of action have yet to be completely elucidated.[194] Nevertheless, ATG is an effective immune suppressive agent in humans, and its use is incorporated into a current clinical trial of HSPC-directed LV gene therapy for hemophilia A (ClinicalTrials.gov Identifier: NCT04418414). We found that the addition of mATG enabled long-term engraftment of ET3 gene-modified donor cells in the majority of HA mice conditioned with CD117-sap. Still, over 40% of recipients receiving CD117-sap + mATG did not exhibit sustained engraftment. ATG contains a wide variety of polyvalent antibodies with a broad range of binding specificities. Known target antigens include markers expressed on T and B lymphocytes, but also on monocytes, dendritic cells, and endothelium.[185] Some target antigens, such as CD45 and CD11b are found on many different leukocyte populations, while HLA class I & II and β2 microglobulin expression is widespread even outside the hematopoietic compartment. Antibodies targeting CXCR4, an antigen present on HSCs, are also found in ATG.[184] Although clinical grade ATG products for human use are better characterized, the heterogeneous nature, incomplete characterization, and lot-to-lot variability of the mouse-specific ATG reagent may contribute to inconsistent outcomes in the setting of murine HSCT. Despite its promiscuous binding profile (**Figure 19, S8 and S9**), we found that mATG alone does not effectively deplete endogenous HSCs (**Figure S3A**) and therefore does not condition the bone marrow in a way that permits appreciable levels of gene-modified donor cell chimerism (**Figure 17C and S5B**). However, if residual mATG remains in circulation at the time of transplantation, it may bind receptors such as CXCR4 in the donor HSC graft. This could lead to either downregulation of surface expression (i.e., modulation, a known functional mechanism of ATG action[194]) or physical inhibition of chemotactic interactions by blocking the CXCR4/SDF-

1 axis and disrupting donor HSC homing to bone marrow niches. We explored adjustments to the timing and frequency of mATG administration in HSCT studies and found that when mATG was administered on day -1 and on the day of transplantation, engraftment of donor HSPCs was significantly impaired (**Figure S9C**). Further, when ATG was administered 5 days before HSCT, with an additional dose given 3 days after HSCT, chimerism declined dramatically within the first 8 weeks. In corroboration of these findings, Jin and colleagues recently reported that rabbit anti-human ATG also binds human bone marrow cells and purified $CD34^+$ human fetal liver cells in vitro. Moreover, human hematopoietic reconstitution in the periphery, as well as human cell engraftment in spleen and bone marrow, were completely abrogated when human ATG was administered to NSG mice 1 day after $CD34^+$ cell transplantation. In contrast, graft failure did not occur in controls that received ATG 21 days after transplantation or PBS, providing further evidence that circulating ATG at the time of HSCT can interfere with HSC engraftment.[195] Also in concordance with our observations (**Figures 16B and S3A**), Jin et al. found that although ATG can bind human HSPCs and impede engraftment, HSCs residing in bone marrow niches, though coated in ATG after administration, are resistant to depletion. In spite of these observations, to our knowledge there are no reports that show ATG interferes with HSPC engraftment in the setting of clinical human HSCT and it is used widely and successfully in this context. Therefore, we believe the current findings underscore the critical importance of optimal timing of ATG administration. Indeed, in another recent study, Samelson-Jones et al. reported that appropriate timing of ATG administration was a critical factor in the prevention of humoral immunity against factor IX (fIX) in a nonhuman primate AAV2 gene therapy model.[196] As a non-genotoxic conditioning regimen for HSCT gene therapy for HA is moved towards clinical translation, timing and dosage of human ATG will be thoroughly explored for its utility as part of this regimen in order to take advantage

of its important immune suppressive actions while minimizing its ability to interfere with HSC engraftment.

Although human ATG remains a strong candidate as part of a clinically translatable non-genotoxic regimen, inconsistent outcomes in murine studies paired with a demonstrated, unequivocal requirement for immune suppression, led us to refine our approach in the murine model to more precisely target the T cell-specific antigens CD4, CD8 and CD40L, as T cells are required for the development of anti-fVIII immunity. Chabra et al. took a similar approach, using a regimen of four naked antibodies aimed at depleting host HSCs (CD117 [clone ACK2] and CD47 mAbs) and host T cells (CD4 and CD8 mAbs) to accomplish donor HSC engraftment in a murine model of minor MHC-mismatched allogeneic HSCT.[34] This approach was successful at accomplishing approximately 20% donor HSC engraftment, but required at least 11 consecutive antibody injections and 3 donor HSPC transplants. A more recent study combined 1.5 mg/kg CD117-sap with CD4, CD8, CD40L mAbs and rapamycin to achieve full MHC-mismatched HSCT using whole bone marrow.[47] Though long-term donor LT-HSC chimerism reached only 3.4%, this was enough to establish donor-specific skin allograft tolerance. In our gene therapy model, we were able to accomplish an average of 75.1% bone marrow chimerism of donor LT-HSCs, with 100% of transplant recipients maintaining long-term engraftment, using a 3-fold lower dose of CD117-sap, enabling therapeutic transgene expression without immune rejection by administering only 3 doses of depleting agents (a single immunotoxin administration plus two injections of mAb cocktails) and one HSPC transplant. This protocol limits the immunotoxin dose, invasiveness of the procedure, and minimizes potential risks associated with multiple injections – an important consideration for patients with HA.

For each component of this protocol, reagents with human activity will need to be identified to advance into clinical testing. There are many research grade anti-human CD117 recombinant, humanized mAbs, scFv or Fab fragments that are commercially available with known inhibitory activity of CD117 signaling (e.g., clones 19, Ab1, Hum10) that can be tested for in vivo human $CD34^+$ cell depletion. Several anti-human CD117 mAbs (e.g., LMJ729, CK6, and SR-1) have been tested in preclinical or clinical studies of HSCT conditioning or for cytotoxicity toward CD117-expressing cancers. A clinical trial testing an LMJ729-based antibody-drug conjugate (LOP628) for acute myelogenous leukemia (ClinicalTrials.gov Identifier: NCT02221505) was discontinued in 2015 during phase 1 when three participants developed hypersensitivity reactions attributed to co-engagement of FcγR and CD117 present on mast cells.[197] Promising preclinical results were later obtained by Shizuru and colleagues using SR-1 to deplete both normal and myelodysplastic syndrome (MDS) human HSCs in murine xenograft models,[198] as well as endogenous HSPCs in cynomolgus macaques using the humanized version AMG191.[199] A current phase 1 dose escalation trial sponsored by Jasper Therapeutics testing JSP191 (formerly AMG191) as a conditioning agent in patients undergoing HSCT for severe combined immune deficiency (SCID) has shown encouraging early results for this indication, achieving up to 7% donor granulocyte chimerism 24 weeks post-transplantation (ClinicalTrials.gov Identifier: NCT02963064).[200] However, similar to the anti-mouse CD117 mAb clone ACK2, SR-1/JSP191 are CD117 antagonists, accomplishing HSC depletion by blocking SCF signaling. Previous studies in mice have shown that while naked antagonistic CD117 mAbs are successful at achieving bone marrow conditioning and donor HSC engraftment in the setting of immune deficiency,[31] appreciable donor engraftment in immunocompetent hosts using this approach required the addition of low dose

radiation[33] or additional agents for CD47 blockade.[34] Although varying levels of $CD34^+$ HSPC depletion were demonstrated in immunocompetent nonhuman primates using JSP191, successful engraftment has not yet been reported. Endogenous HSPC depletion and subsequent HSPC engraftment using JSP191 could potentially be enhanced as a saporin-based immunotoxin for gene therapy applications in immunocompetent hosts. Another potential candidate for clinical development of an anti-human CD117 mAb is clone 104D2. Czechowicz et al. demonstrated the ability of a 104D2-saporin conjugate to eliminate the development of human myeloid cells in peripheral blood of mice xenografted with human umbilical cord blood HSCs up to 120 days post-conditioning,[189] implying that this immunotoxin was successful at clearing human HSCs in vivo. Alternatively, clone 17F11 was shown to elicit robust downregulation of surface CD117 on MO7e cells in a similar fashion as its cognate ligand SCF,[201] suggesting induction of receptor internalization, an important characteristic of antibodies that create potent immunotoxins. Clone 17F11 could also be explored for its efficacy as a human HSC-targeted immunotoxin.

Aside from human ATG, there are multiple avenues that could be explored for translation of the more targeted immune suppressive component of our protocol. Alemtuzumab targets CD52 present on T and B cells, as well as monocytes, macrophages, NK cells and some granulocytes. It is used clinically for the treatment of chronic lymphocytic leukemia and multiple sclerosis, and has also been used as a T cell-depleting agent for GVHD prophylaxis or as an alternative to ATG in allogeneic HSCT settings.[202-205] Muromonab-CD3, an FDA-approved mouse mAb used to treat acute rejection of solid organ grafts, has also been used in bone marrow transplant settings.[206-208] Similar to the mAb cocktail used in the current study, these drugs clear host T cells and may be effective in our HSCT gene therapy protocol. Alternatively, investigational mAbs that specifically

target CD4 (e.g., MAX.16H5[209, 210] or IT1208[211]) or CD3[212] (e.g., teplizumab, otelixizumab, visilizumab or foralumab) might be considered. A similar approach to that taken in a recent phase 1/2 clinical trial of HSC-directed LV gene therapy for the primary immunodeficiency Wiskott-Aldrich syndrome, in which fludarabine and rituximab (CD20 mAb) were used to achieve immune cell depletion prior to transplantation, could be pursued.[213, 214]

The addition of T cell costimulatory blockade to our regimen using CD40L mAb ensured that if any residual T cells remained following CD4/CD8 mAb depletion and became activated through an encounter with ET3 protein, CD40L on these cells would be prevented from interacting with CD40 on B cells, dendritic cells and other antigen presenting cells, inhibiting humoral and cell-mediated immune responses to ET3. In a study of MHC-mismatched murine HSCT, Langford-Smith and colleagues found that blockade of both signal 1 (CD4/CD8 block) and signal 2 (CD40L block) was essential for long-term donor engraftment using non-myeloablative conditioning (low dose busulfan); signal 1 or signal 2 blockade alone was insufficient to prevent rejection.[215] In a xenograft model of T cell-mediated rejection of human $CD34^+$ cells, Oh et al. demonstrated that costimulatory blockade using CTLA4-IgG1 (abatacept) in the early post-transplantation period prevented antigen-specific T cell alloreactivity and $CD34^+$ rejection.[216] CTLA4-Ig fusion proteins target a different costimulatory pathway, working as competitive inhibitors of CD28/B7 signaling, thereby promoting anergy in activated T cells. Two CTLA4-Ig drugs on the market, abatacept and belatacept, are clinically approved for treatment of rheumatoid arthritis and prophylaxis of renal transplant rejection, respectively. Abatacept in particular has been studied for its potential application as GVHD prophylaxis in various allogeneic HSCT settings,[217-219] as well as for its ability to prevent anti-vector immune responses and allow repeated AAV8 administration in fIX

gene therapy.[220] Since thromboembolic complications in a phase 1 trial of human CD40L mAb therapy suspended clinical development of a CD40L-targeting agent for human use,[221] newer mAbs are being developed and tested in clinical trials that disrupt CD40/CD40L signaling by instead targeting CD40 (e.g., bleselumab).[222-224] As an alternative to costimulatory blocking agents, basiliximab, an interleukin-2 receptor antagonist that prevents T cell replication and T cell-dependent B cell activation, has been used in combination with ATG as GVHD prophylaxis[225] and may be effective at suppressing anti-fVIII immune responses in our regimen.

In summary, we report successful implementation of a completely non-genotoxic antibody-based conditioning regimen to facilitate HSC-directed gene therapy in a preclinical murine model of HA. We developed a method for achieving high-level, long-term engraftment of ET3-modified donor HSPCs, sustained curative levels of plasma procoagulant activity, phenotypic correction, and elimination of immunological rejection. The studies described herein demonstrated an explicit requirement for immune suppression to enable engraftment of ET3 gene-modified cells. Further studies will focus on the development of a human-specific non-genotoxic regimen that combines CD117 immunotoxin with non-genotoxic, clinically available immune suppressive agents, such as ATG, alemtuzumab and/or abatacept. Nevertheless, these findings establish strong proof-of-concept towards translation of a non-genotoxic approach to HSCT conditioning for HA gene therapy to the clinic in the future.

2.6 Acknowledgements

This work was supported by funding from the National Institutes of Health (NIH)/National Heart, Lung and Blood Institute (NHLBI) (C.B.D., H.T.S., and G.D.) and Hemophilia of Georgia (C.B.D.

and H.T.S.). Research reported in this publication was supported in part by the Pediatrics/Winship Flow Cytometry Core of Winship Cancer Institute of Emory University, Children's Healthcare of Atlanta and NIH/NCI under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Original schematic figures created with Biorender.com.

2.7 Supplemental Information

Figure S1

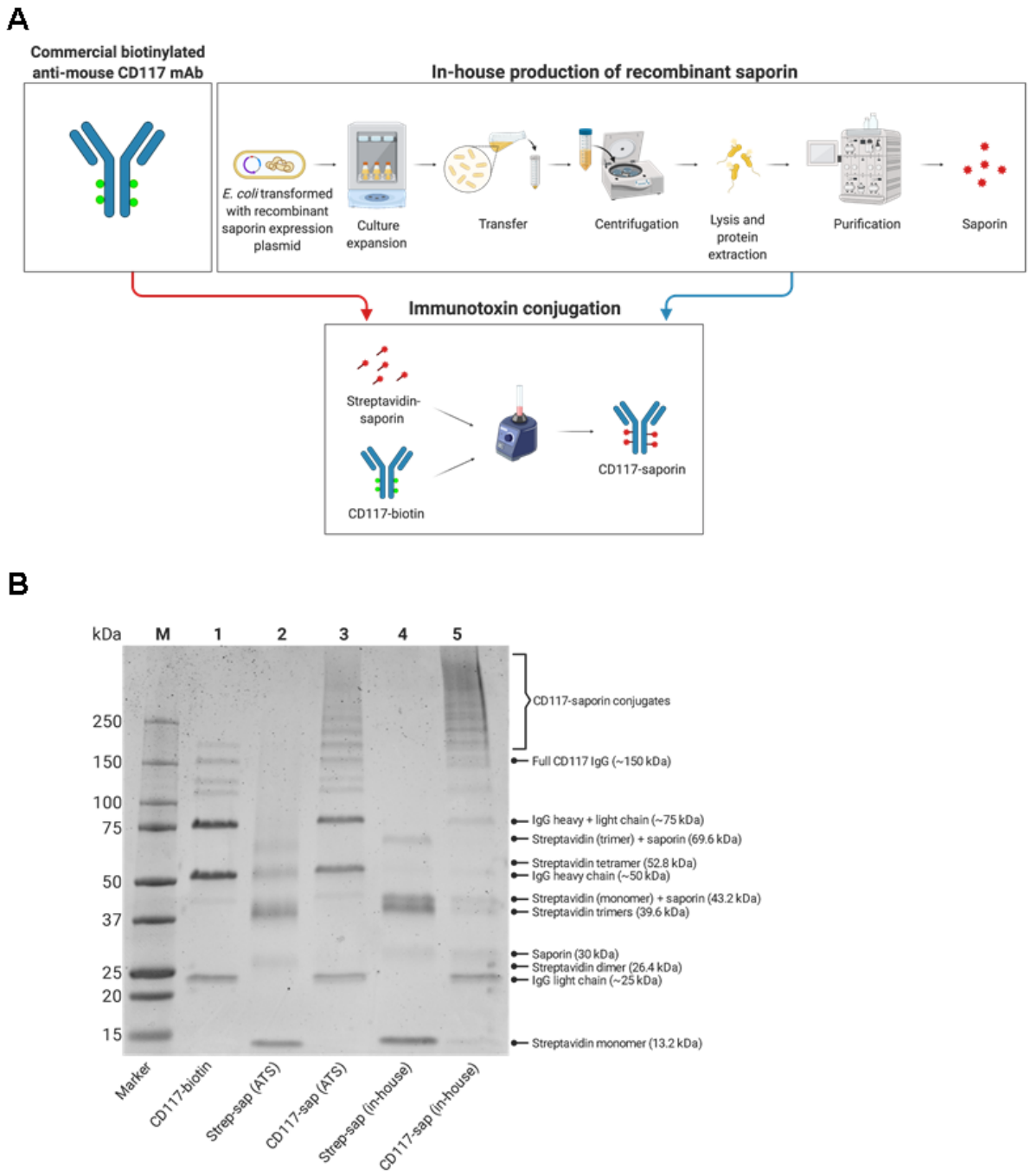


Figure S1. (**A**) Recombinant saporin was generated and bioconjugated to streptavidin in-house. Streptavidin-saporin (strep-sap) was then linked to biotinylated CD117 mAb to produce the final CD117-saporin immunotoxin product. (**B**) Coomassie-stained SDS-PAGE shows CD117 (2B8) mAb, commercial strep-sap, in-house recombinant strep-sap, and their associated CD117-sap conjugates.

Figure S2

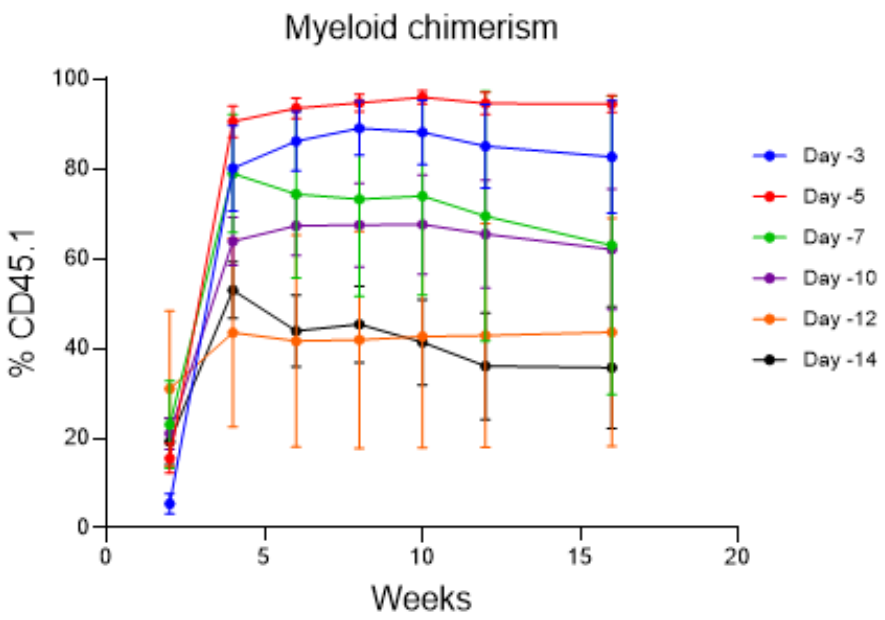


Figure S2. C57BL/6 mice were conditioned with 0.5 mg/kg CD117-sap at various time points, and then transplanted with 5 x 10^6 $CD45.1^+$ whole bone marrow cells. Optimal engraftment kinetics in the myeloid compartment were attained when immunotoxin was administered 5 days prior to transplantation. Data represent mean ± sample SD.

Figure S3

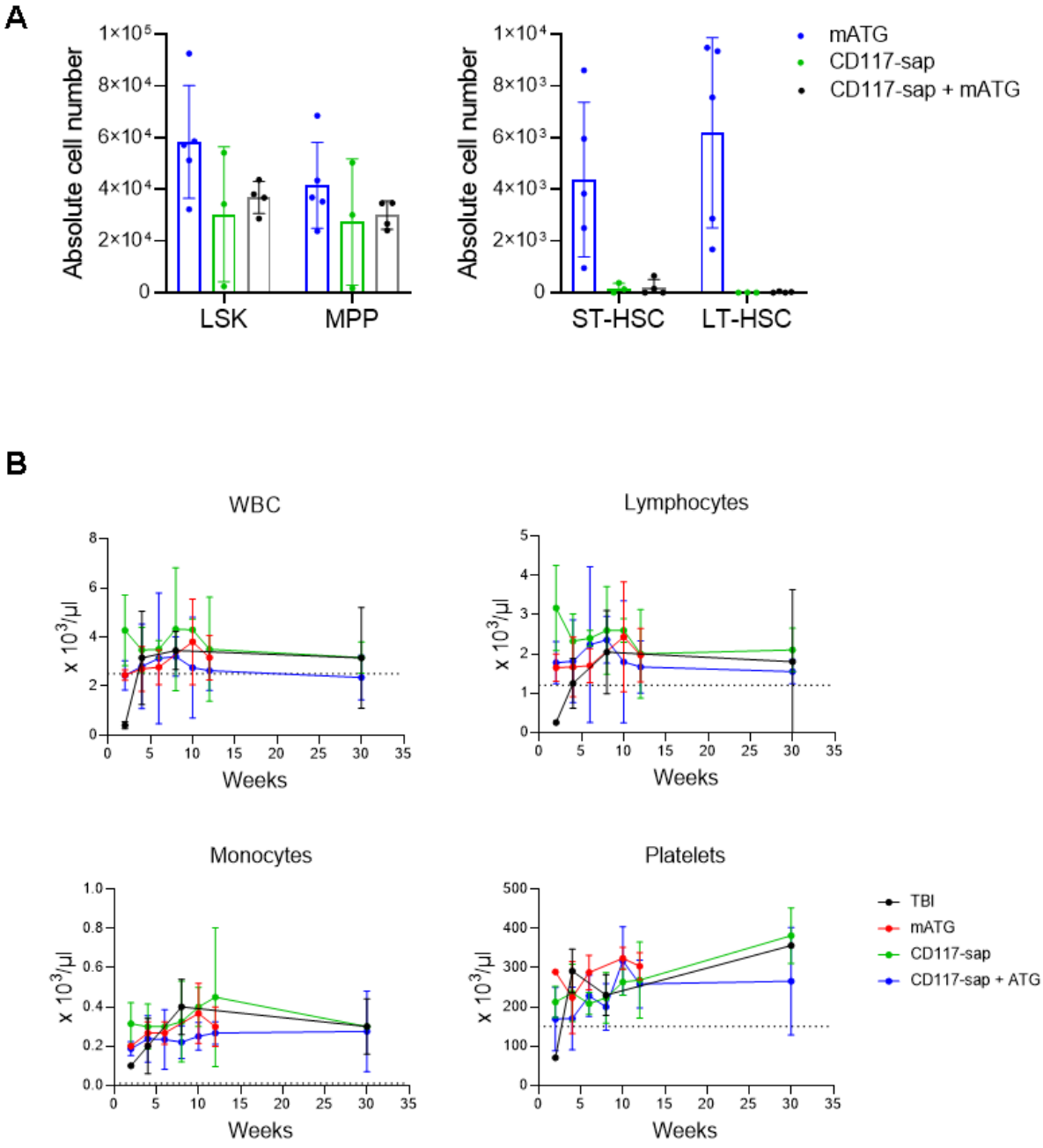


Figure S3. (**A**) ST-HSC and LT-HSC compartments are selectively and robustly depleted after CD117-sap or CD117-sap + mATG conditioning. mATG alone did not produce depletion of HSPC or HSC compartments. (**B**) Treatment with mATG (n = 3), CD117-sap (n = 7) or CD117-sap + mATG (n = 7) did not produce prolonged cytopenias. Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. TBI mice exhibited leukopenia and thrombocytopenia until 4 weeks post-transplantation. See also Figure 14B.

Figure S4

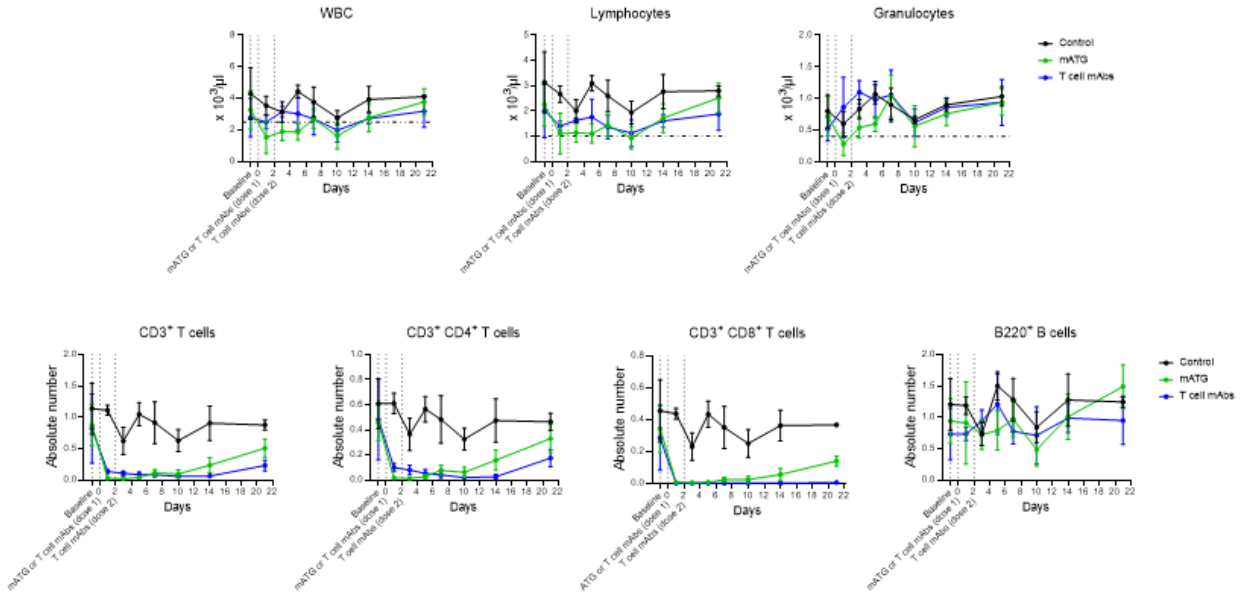


Figure S4. HA mice were injected intraperitoneally with either 30 mg/kg mATG or the T cell mAbs regimen (dosing and schedule as described in Materials and Methods). Peripheral blood was collected periodically for 21 days to follow the kinetics of depletion and recovery in WBC and lymphocyte compartments resulting from antibody immune suppression. Horizontal dotted line represents lower limit of reference range for respective populations. See also Figure 14B and 17B.

Figure S5

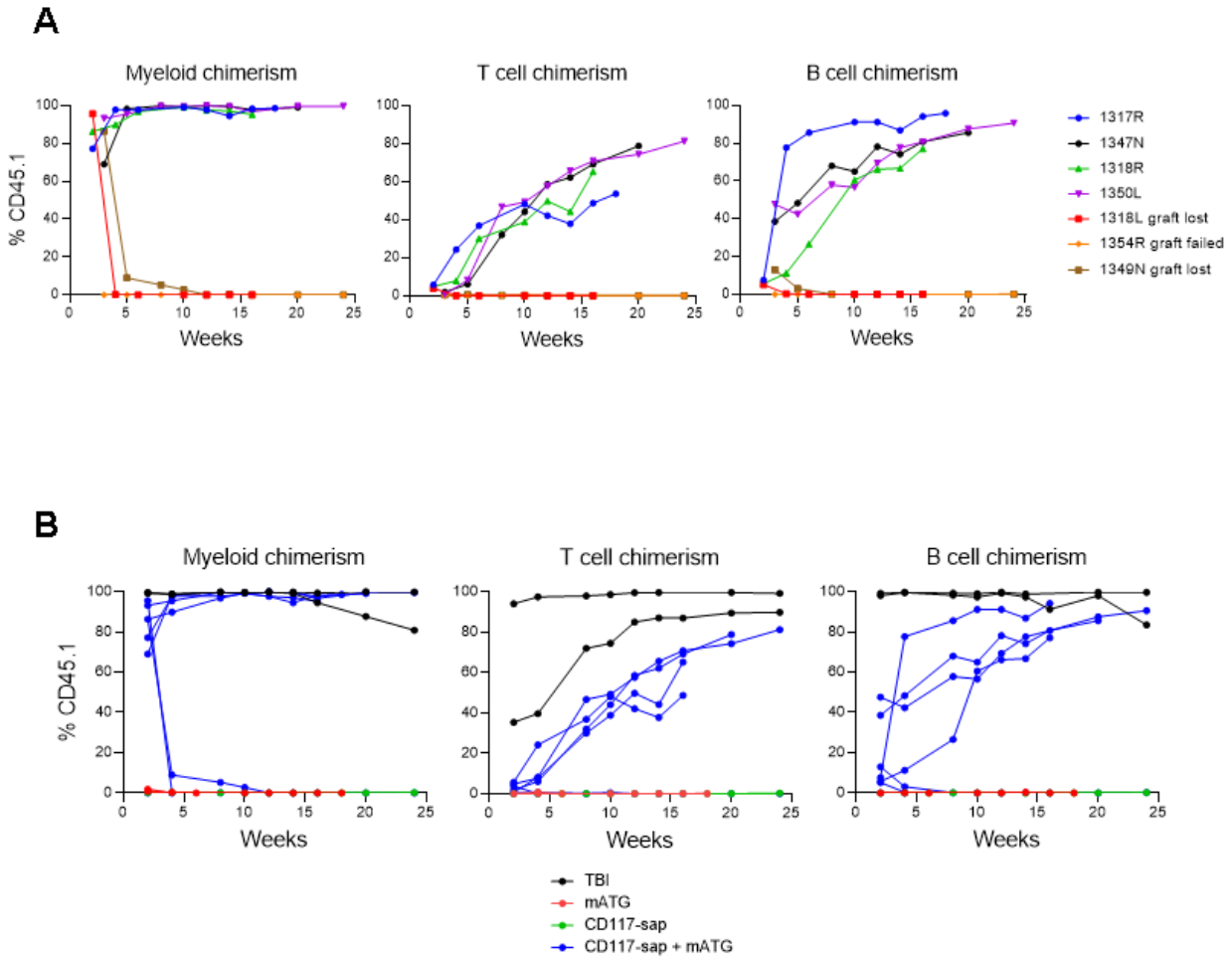


Figure S5. (**A**) Two weeks after transplantation, 1 of 7 mice (across two independent experimental cohorts) conditioned with CD117-sap + mATG failed to engraft ET3 gene-modified HSPCs (orange diamond), while the remaining six mice achieved early myeloid chimerism of 72.5 ± 33.3% (mean ± sample SD). By four weeks post-transplantation, myeloid chimerism levels decreased from 95.6% to 0.22% and 86.4% to 8.94% in two additional mice conditioned with CD117-sap + mATG (red square and brown square). (**B**) Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. See also Figure 14C.

Figure S6

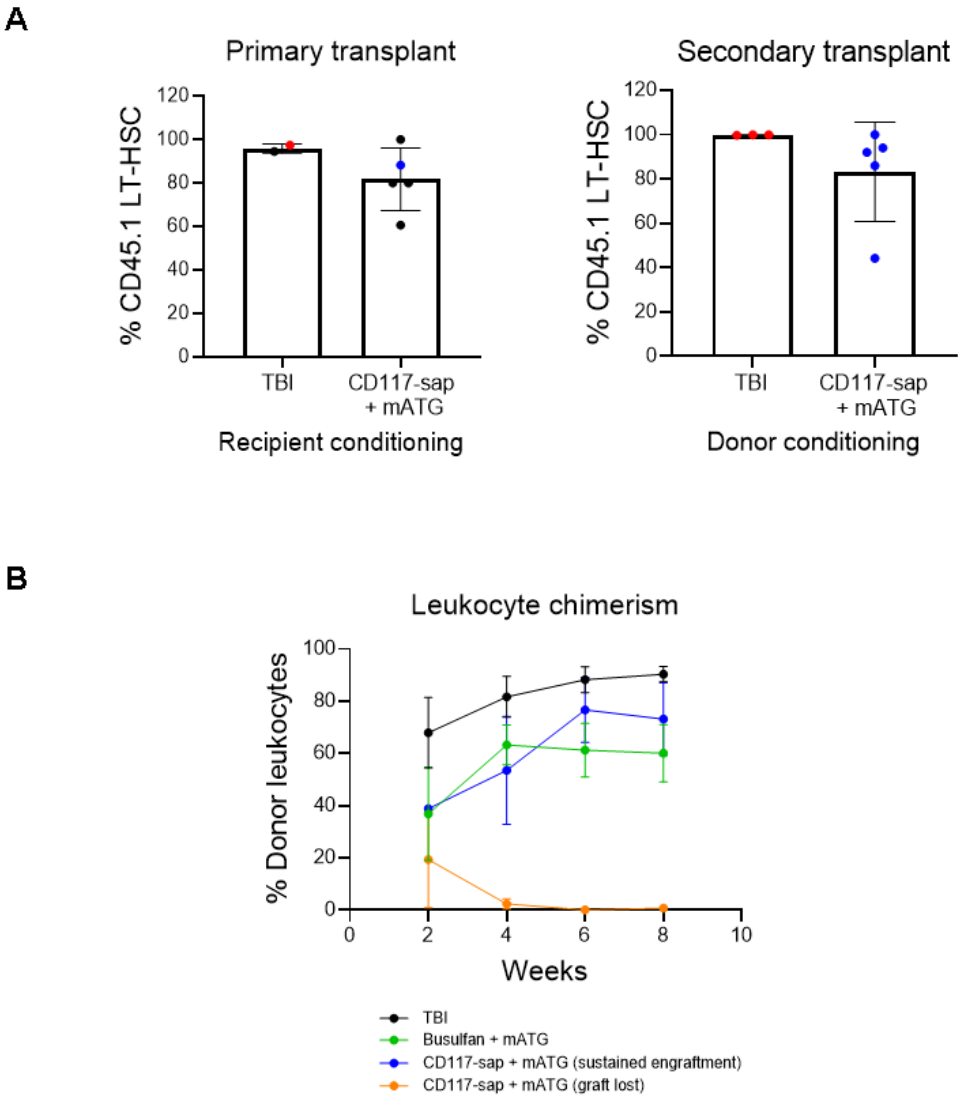
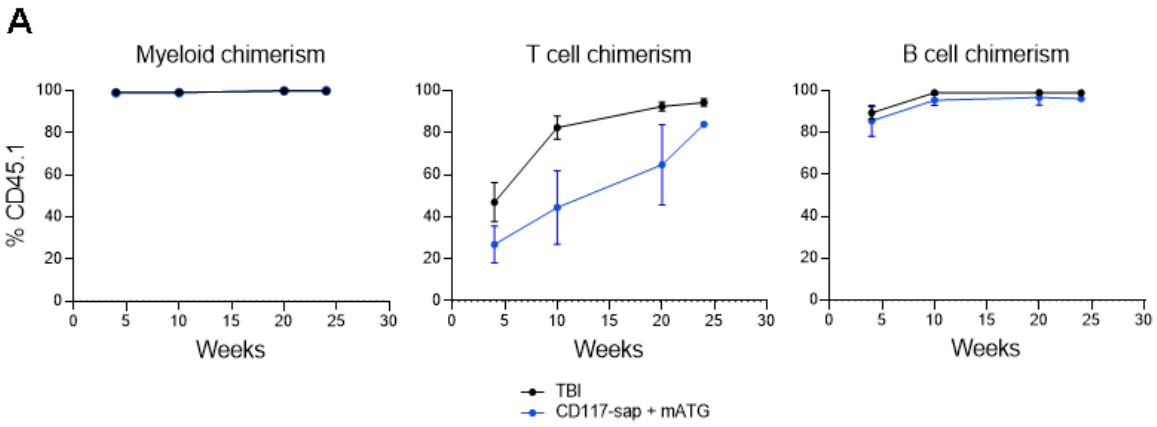
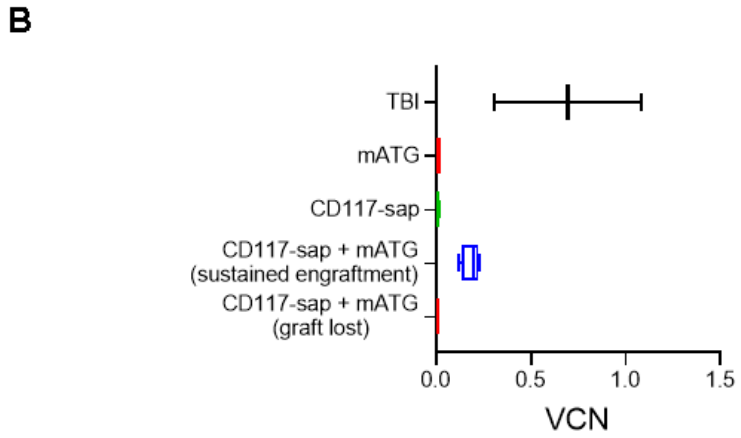


Figure S6. (**A**) Engraftment of donor-derived LT-HSCs in chimeric HA mice conditioned with CD117-sap + mATG was 80.2 ± 16.1% (primary transplant). Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. Donor-derived LT-HSCs in TBI mice was 95.9 ± 2.1%. Bone marrow from one primary recipient conditioned with CD117-sap + mATG (blue dot, left panel) and one conditioned with TBI (red dot, left panel) were harvested and transplanted into lethally irradiated secondary HA recipients (n = 5 secondary recipients per donor). $CD45.1^{+}$ LT-HSC engraftment was 87.2 ± 19.6% in secondary recipients receiving CD117-sap + mATG-conditioned bone marrow (blue dots, right panel) and 99.9 ± 0.06% for secondary recipients receiving TBI-conditioned bone marrow (red dots, right panel). See also Figure 14D. (**B**) High donor leukocyte chimerism after CD68-ECO-ET3-LV HSCT is achievable using TBI (n = 9) or non-myeloablative conditioning (busulfan + mATG or CD117-sap + mATG) (n = 4-5). Three mice conditioned with CD117-sap + mATG did not maintain long-term engraftment of ET3 gene-modified cells (orange). Data represent mean ± sample SD.

Figure S7





C

Bethesda Titers in Mice That Developed ET3 Inhibitors

Conditioning	Outcome	Titer (BU/ml)
CD117-sap (mouse 1)	No engraftment	50
CD117-sap (mouse 2)	No engraftment	44
CD117-sap + mATG	86.4% myeloid engraftment, followed by rejection beginning 4 weeks post-transplantation	37

Figure S7. (A) High-level multilineage chimerism in secondary recipients after TBI or CD117-sap + mATG conditioning indicates engraftment of donor LT-HSCs in primary recipients. (n = 5 in each group) Data represent mean ± sample SD. See also Figure 14E. **(B)** Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. Peripheral blood VCN in mice conditioned with CD117-sap + mATG was 0.19 ± 0.05 copies per genome and 0.70 ± 0.54 copies per genome in mice conditioned with TBI. Proviral DNA was undetectable in mice treated with mATG alone, CD117-sap alone, or mice that lost engraftment under CD117-sap + mATG conditioning. See also Figure 15B. **(C)** Modified Bethesda assay was used to measure inhibitor titers of mice from Figure 15C at 24 weeks post-transplantation.

Figure S8

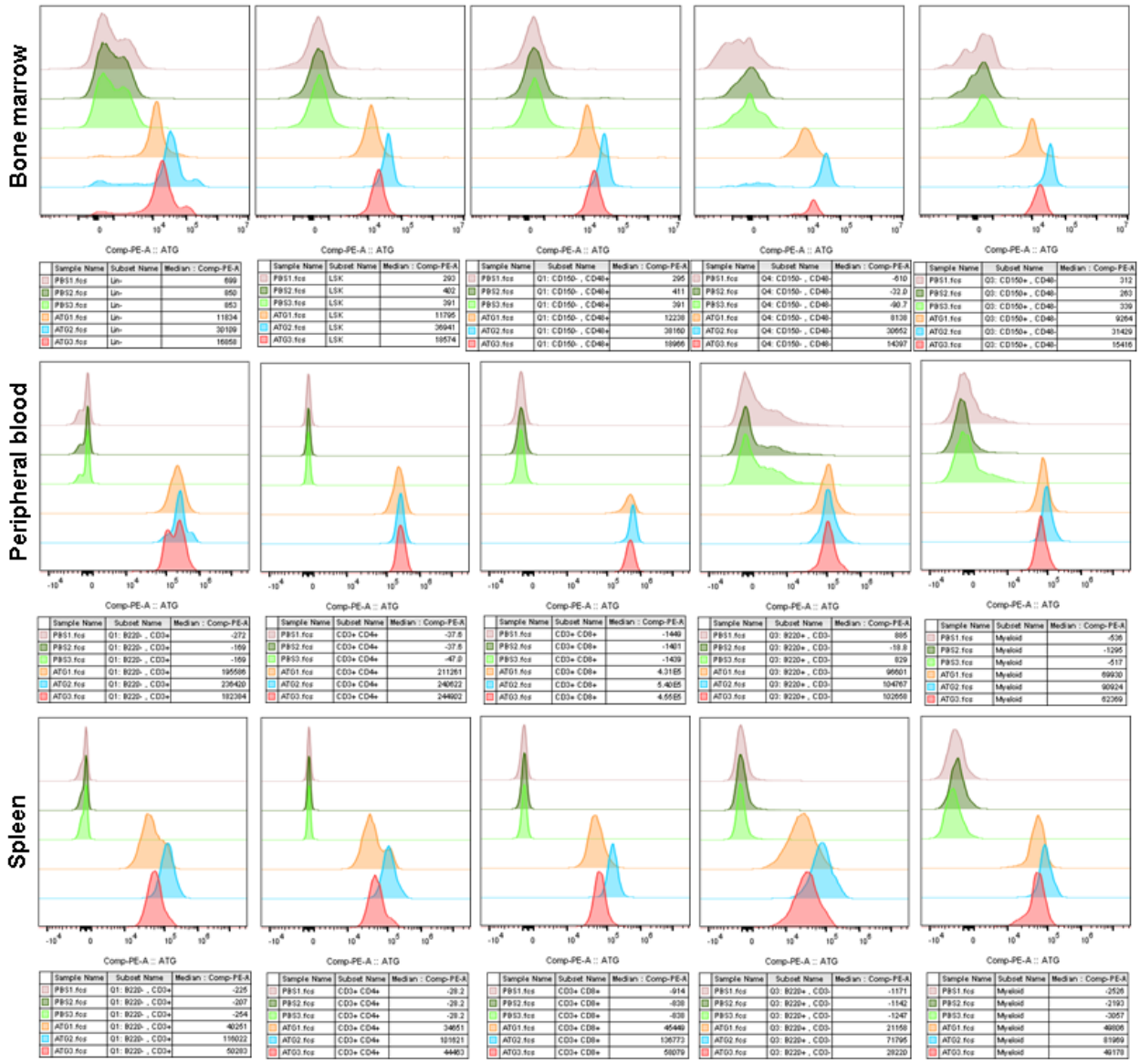


Figure S8. Flow cytometric analysis revealed measurable mATG binding in vivo to all primitive and mature hematopoietic cell populations examined. (n = 3 in each group) See also Figure 16.

Figure S9

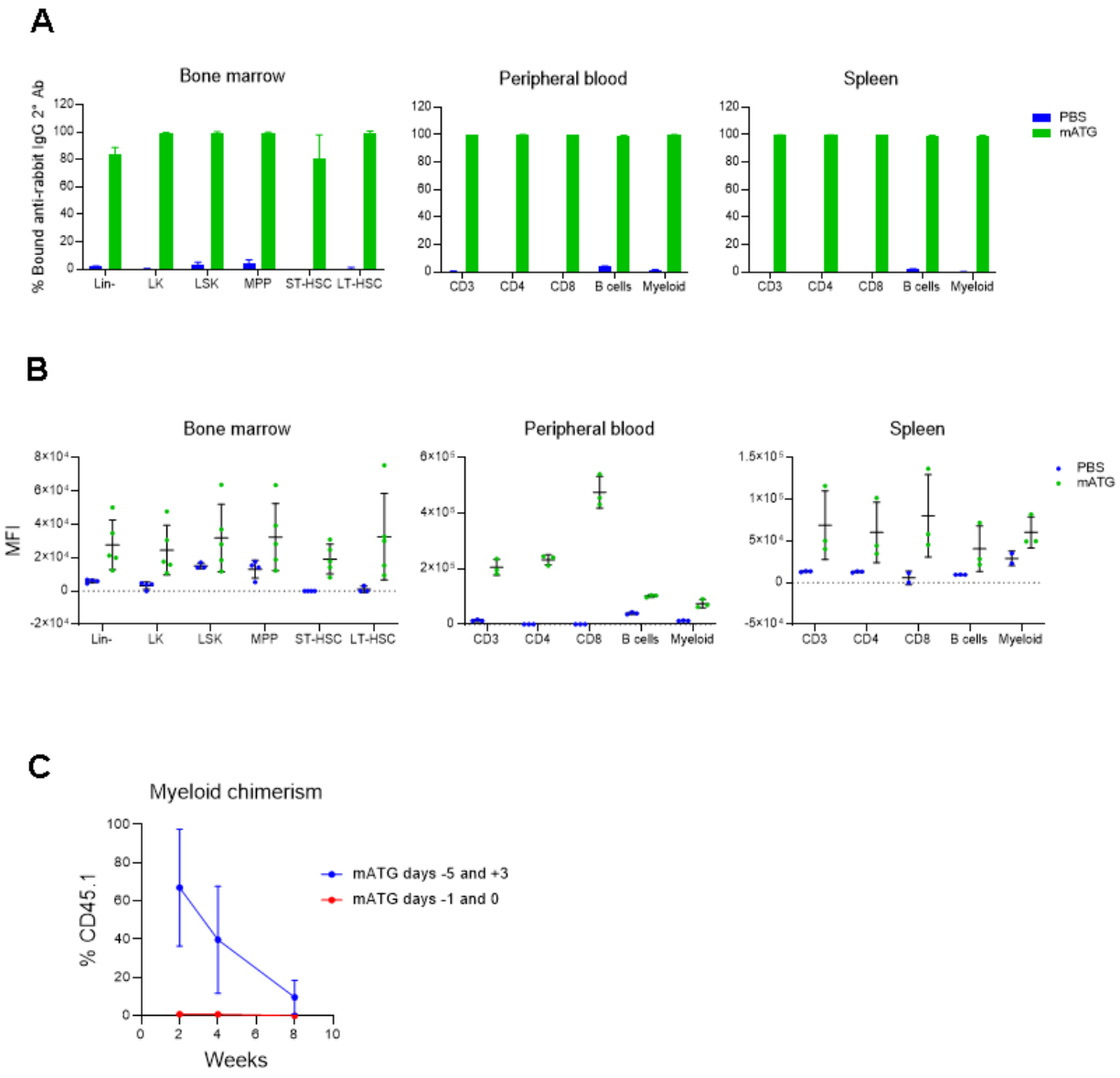


Figure S9. (**A and B**) In vitro binding studies using mATG and bone marrow, peripheral blood and spleen cells mirrored results observed in vivo (see Figure 16A and 16B). (**C**) HSCT-LV studies were performed to test alternative timing and dosing schedules of mATG. Two doses of mATG given at days -5 and +3 resulted in early chimerism that was eventually lost. Two doses of mATG given at days -1 and 0 resulted in complete abrogation of engraftment.

Figure S10

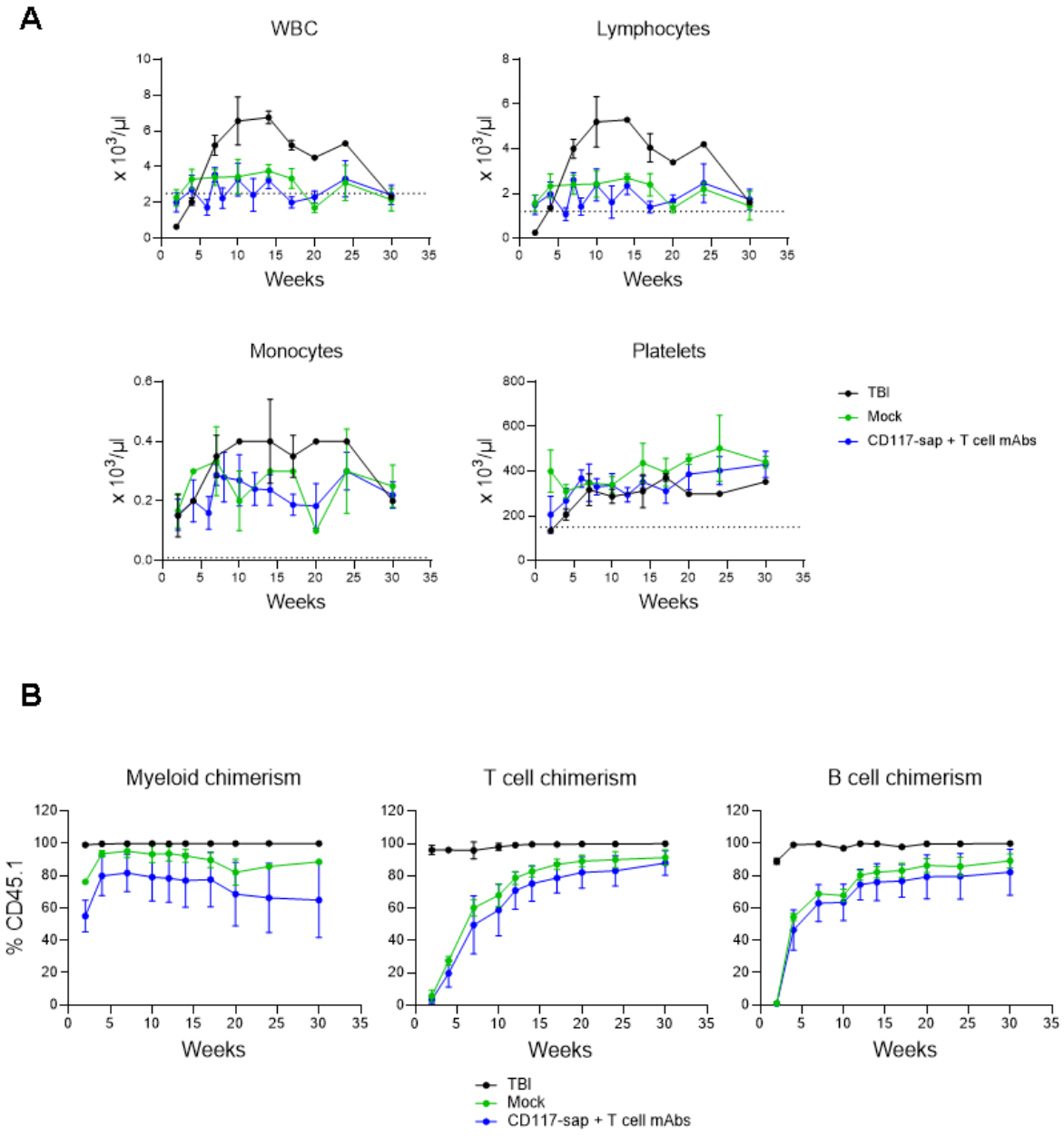


Figure S10. (**A**) Lymphocytes, monocytes and platelets measured in the normal range by 2 weeks post-transplantation in CD117-sap + T cell mAbs conditioned mice that received either ET3-modified cells or mock transduced HSPCs. Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. TBI mice had low lymphocytes and platelets until 4 weeks post-transplantation. See also Figure 17B. (**B**) High-level multilineage chimerism was achieved in primary recipients following TBI (n = 2), CD117-sap + T cell mAbs (n = 13), or CD117-sap + T cell mAbs and mock transduced HSCT (n = 3). Data represent mean ± sample SD. See also Figure 17C.

Figure S11

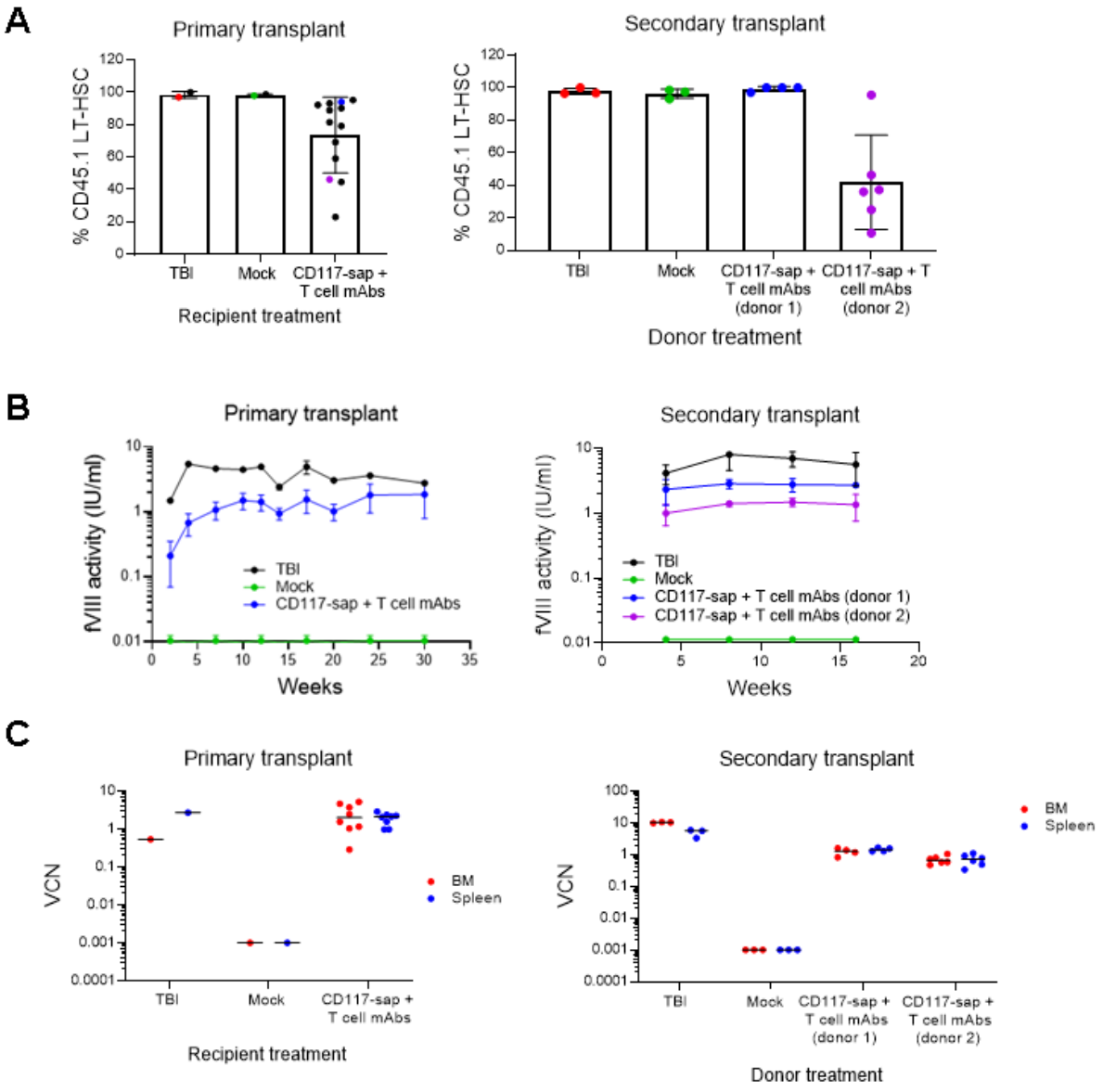


Figure S11. (A) Engraftment of donor-derived CD68-ECO-ET3-LV transduced LT-HSCs in chimeric HA mice conditioned with CD117-sap + T cell mAbs was 73.6 ± 23.5% (range: 23.0 – 95.2%). Bone marrow from two primary recipients that received CD117-sap + T cell mAbs conditioning and ET3-modified HSPCs (LT-HSC chimerism 94.1% and 46.2%; blue and purple dots, left panel) was harvested and transplanted into lethally irradiated secondary HA recipients (n = 6). CD45.1^{+} LT-HSC engraftment in secondary recipients measured 99.3 ± 1.5% in recipients of donor 1 (blue dots, right panel) and 41.8 ± 29.1% in recipients of donor 2 (purple dots, right panel). Mice treated with TBI (n = 2) were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction. LT-HSC engraftment in secondary recipients of TBI-treated mice (n = 3) and mice that received mock transduced cells (n = 3) was consistent with primary recipients (red and green dots, respectively). See also Figure 17D. **(B)** Curative ET3 levels of 0.49 ± 0.31 IU/ml were achieved in CD117-sap + T cell mAbs

conditioned mice, steadily increasing to 1.76 ± 1.0 IU/ml by 30 weeks. No ET3 activity was detected in animals receiving mock transduced cells. ET3 activity in secondary recipients of donor 1 bone marrow reached 2.70 ± 0.24 IU/ml and 1.35 ± 0.59 IU/ml in secondary recipients of donor 2 marrow. Mice treated with TBI for the primary transplant were included to ensure viability and engraftment potential of HSPCs following isolation, ex vivo culture and transduction (n = 2 primary recipients, n = 3 secondary recipients). See also Figures 18A and 18C. **(C)** Copies of CD68-ECO-ET3-LV proviral DNA were measured from bone marrow and spleen. In mice receiving the CD117-sap + T cell mAbs regimen, VCN measured 2.5 ± 1.8 copies per genome in bone marrow and 1.9 ± 0.68 in spleen. For secondary recipients of CD117-sap + T cell mAbs treated mice, copies of CD68-ECO-ET3-LV proviral DNA in bone marrow measured 1.3 ± 0.33 (donor 1) and 0.71 ± 0.21 (donor 2) and spleen measured 1.47 ± 0.20 (donor 1) and 0.72 ± 0.29 (donor 2). No proviral DNA was measured in animals transplanted with mock transduced cells. See also Figure 18B.

Figure S12

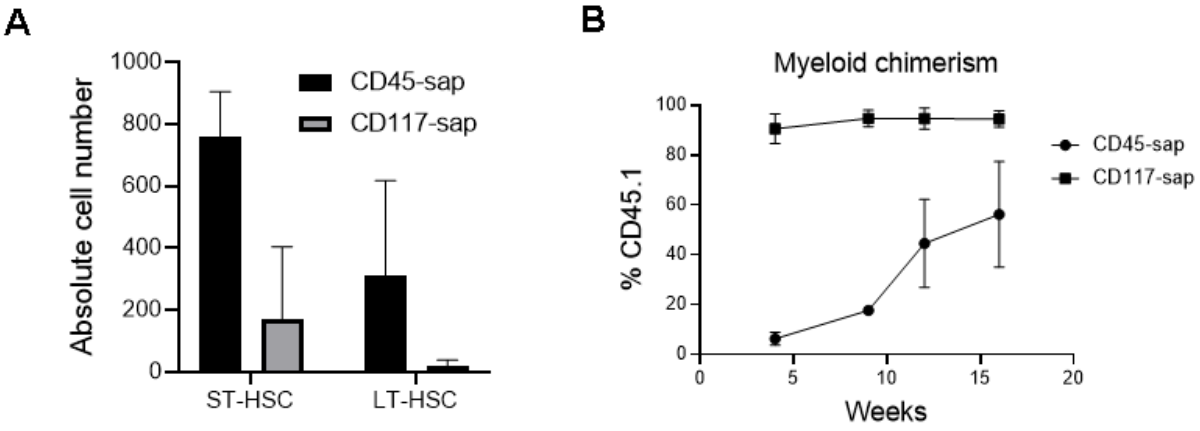


Figure S12. (A) In pilot studies, HA mice were conditioned with 0.5 mg/kg CD117-sap or 3.0 mg/kg CD45-sap and sacrificed after 7 days. Bone marrow was harvested, and depletion of ST-HSC and LT-HSC populations was analyzed by flow cytometry. **(B)** A pilot study was performed in which C57BL/6 mice were conditioned with either 0.5 mg/kg CD117-sap or 3.0 mg/kg CD45-sap, and then transplanted with 1 x 10^7 CD45.1$^+$ whole bone marrow cells after 5 days. Donor myeloid chimerism was consistently higher when CD117-sap was used.

Table S1

Conditioning reagents

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD117 (c-kit)	Biotin	2B8	Biolegend	105804
ZAP (saporin)	Streptavidin	NA	ATS	IT-27
CD4	NA	GK1.5	Bio X Cell	BE0003-1
CD8a	NA	YTS169.4	Bio X Cell	BE0117
CD40L	NA	MR-1	Bio X Cell	BE0017-1
Rabbit anti-mouse anti-thymocyte globulin	NA	NA	Cedarlane	CLAG3940T

Bone marrow flow cytometry antibodies

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
Lineage cocktail	FITC	NA	Biolegend	78022
CD117 (c-kit)	APC	ACK2	Biolegend	135108
Sca-1 (Ly-6A/E)	PE-Cy7	D7	Biolegend	108114
CD48	APC-Cy7	HM48-1	Biolegend	103432
CD150	BV421	Q38-480	BD	562811
CD45.1	PE	A20	BD	553776
CD45.2	Alexa Fluor 594	104	Biolegend	109850

Peripheral blood flow cytometry antibodies

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD45.1	PE	A20	BD	553776
CD45.2	APC	104	BD	558702
CD3	V450	500A2	BD	560801
CD45R/B220	PerCP	RA3-6B2	Biolegend	103234
Gr-1 (Ly-6G/Ly-6C)	BV605	RB6-8C5	Biolegend	108440

Spleen flow cytometry antibodies

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD3	V450	500A2	BD	560801
CD4	FITC	RM4-5	BD	553046
CD8a	PE	53-6.7	BD	553032
CD45R/B220	PE-Cy7	RA3-6B2	BD	552772
Gr-1 (Ly-6G/Ly-6C)	BV605	RB6-8C5	Biolegend	108440

ATG binding studies antibodies

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
Rabbit anti-mouse anti-thymocyte globulin	NA	NA	Cedarlane	CLAG3940T
Mouse anti-rabbit IgG	PE	NA	Santa Cruz Biotechnology	sc-3753

Chapter 3

Hematopoietic stem cell transplantation using non-genotoxic anti-CD117 immunotoxin conditioning corrects immune deficiencies and restores immune function in ataxia telangiectasia mice

This research was presented as an oral abstract at the
Clinical Immunology Society Annual Meeting 2021

Russell AL, Prince C, Knight KA, Lundgren TS, Rayaprolu S, Xiao H, Kumar D, Alexander JS, Rangajaru S, Spencer HT, Doering CB, Chandrakasan S

Athena Russell designed and performed experiments, wrote and edited the manuscript.

Chengyu Prince, Kristopher Knight, Taran Lundgren, Sruti Rayaprolu, Hailian Xiao, and Deepak Kumar performed experiments.

Jordan S. Alexander developed experimental techniques.

Shanmuganathan Chandrakasan, Christopher Doering, Srikant Rangajaru and Trent Spencer conceived experiments and reviewed the manuscript.

3.1 Abstract

Ataxia telangiectasia (AT) is a chromosomal instability syndrome caused by mutation in ATM kinase, a protein critical for DNA double strand break repair and cell cycle control. AT is characterized by cerebellar neurodegeneration, immune dysfunction and predisposition to malignancy. Like several other immune deficiency disorders with DNA repair defect, AT patients could benefit from allogeneic hematopoietic stem cell transplantation (HSCT) and immune reconstitution with ATM-competent cells. However, increased sensitivity to DNA damaging agents and progressive cerebellar degeneration in AT are major barriers precluding HSCT using traditional genotoxic conditioning agents. Conditioning with an antibody-based, non-genotoxic immunotoxin targeting CD117 on hematopoietic stem cells may offer a safer approach to allogeneic HSCT in this setting. *Atm*$^{-/-}$ mice were conditioned with CD117-saporin immunotoxin, then transplanted congenic GFP^{+} *Atm*$^{+/+}$ whole bone marrow. Multilineage donor chimerism, body weight, leukocyte counts, immunoglobulins, cytokine levels, and immunization responses in transplanted *Atm*$^{-/-}$ mice versus *Atm*$^{+/+}$ and non-transplanted *Atm*$^{-/-}$ controls were analyzed. CD117-saporin conditioning enabled high-level myeloid chimerism (97.5 ± 0.8%) within six weeks that was sustained for 26 weeks. Chimerism reached 80.1 ± 0.9% and 93.6 ± 1.0% in T and B cell compartments, respectively. *Atm*$^{-/-}$ mice have a growth defect that was not corrected by HSCT. However, CD4^{+} and CD8^{+} T cells were increased in chimeric *Atm*$^{-/-}$ mice compared with age-matched *Atm*$^{-/-}$ controls. Following immunization with a T cell-dependent immunogen, chimeric *Atm*$^{-/-}$ mice mounted measurable antigen-specific IgM and IgG immune responses after HSCT similar to *Atm*$^{+/+}$ mice, whereas *Atm*$^{-/-}$ controls did not. IgA levels were significantly increased in chimeric *Atm*$^{-/-}$ after transplant. One mouse that received HSCT after CD117-saporin conditioning alone developed host-derived T cell lymphoma at 29 weeks of age. After including antibodies to

deplete host lymphocytes to the conditioning regimen, we observed high levels of durable multilineage chimerism and prevention of malignancy in *Atm*$^{-/-}$ mice up to 45 weeks of age. Taken together, these findings are strong proof-of-concept to support the use of non-genotoxic antibody-based conditioning to facilitate allogeneic HSCT in AT patients.

3.2 Introduction

Ataxia telangiectasia (AT) is a rare, autosomal recessive, multisystem disorder classified as a chromosomal instability syndrome or DNA damage response syndrome. In its classical form, the disease is characterized by progressive neurodegeneration and cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, recurrent sinopulmonary infection, predisposition to malignancy, and sensitivity to radiation and radiomimetic chemicals (e.g., alkylating chemotherapeutics). Lymphoid cancer and respiratory failure are the main causes of morbidity and mortality in patients with AT. Disease management is centered on providing supportive care, as there is no cure and no current therapies significantly impact the course of the disease.[125, 226]

AT is caused by mutation in the ataxia telangiectasia mutated (*ATM*) gene. ATM is a serine/threonine protein best known for its central role in DNA double strand break (DSB) repair. Immunodeficiency in AT is caused by impaired DSB repair, which is required to resolve programmed genomic rearrangements that occur during T and B cell maturation (i.e., V(D)J recombination) leading to reductions in number of circulating T and B cells.[126] Impaired DSB repair causes defective B cell class-switch recombination, often leading to hyper IgM phenotype and decreased or absent serum IgA, IgE, and IgG2.[109, 126, 147] Elevated risk of malignancy may be attributed to DSB repair defects, decreased immune surveillance or both.[227] In addition to its apical

role in the DSB repair pathway, proteomic screens have identified over 1000 other putative substrates, involving ATM in a large variety of diverse cellular pathways, including cell cycle control, stress responses, metabolism and apoptosis.[114] This substantial diversity of ATM function may therefore explain the largely pleiotropic and multisystemic manifestations of AT.

Neurodegeneration of the cerebellar cortex, specifically loss of Purkinje neurons and granule cells,[125] is a principal hallmark of AT, although the exact mechanism or molecular pathogenesis underlying this process in AT is still largely unknown.[228] Impairment of DSB repair is likely a major contributor, since other related DNA repair deficiency syndromes such as Nijmegen breakage syndrome, AT-like disorder and Fanconi anemia exhibit broad neurological involvement, suggesting proper responses to DNA damage are critical for nervous system homeostasis.[107, 110, 140] However there has been considerable debate about whether defective DNA repair completely accounts for the neurodegenerative aspects of AT and whether these features can be attributed to solely one factor.[228] Many other mechanisms have been proposed, including defective responses to oxidative stress and elevation of reactive oxygen species, mitochondrial dysfunction, synaptic vesicle dysregulation, disruption of glucose homeostasis in the brain, and abnormalities in the interplay between neurons and glial cells.[107, 228]

There is a growing body of evidence that points to inflammation as a key contributor to AT pathogenesis and neurodegeneration. Many of the symptoms, systemic complications and/or secondary diseases associated with AT, such as arthritis, multiple sclerosis, premature aging, insulin resistance, cardiovascular disease, and elevated pro-inflammatory cytokines, are strongly suggestive of widespread pathological inflammatory processes.[229] Neuroinflammation is a

prominent pathological attribute in other neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, and alterations in neuronal/glial cell crosstalk and chronic activation of microglia, the innate immune cells of the central nervous system, play a major role in the progression of degenerative processes in the brain.[230-232] Previous studies have demonstrated the effect of ATM deficiency and unrepaired DNA damage in the priming of innate immune responses.[233] Accumulation of cytoplasmic DNA fragments triggers activation of the DNA sensor STING and the AIM2-containing inflammasome, as well as enhancement of Toll-like receptor signaling, leading to increased production of proinflammatory, neurotoxic cytokines by ATM-deficient microglia, likely contributing to the neuronal death and neuropathology observed in AT.[233-235] The notion that neuroinflammation plays a part in the onset and progression of neurological symptoms in AT is supported by reports that corticosteroid treatment can ameliorate these symptoms in AT patients.[130, 236-239]

Hematopoietic stem cell transplantation (HSCT) with ATM-competent bone marrow has been shown to restore T cell populations, immune function and prevent thymic lymphoma in ATM deficient mice.[148, 240, 241] Although there are fewer than ten published case reports of allogeneic HSCT performed in human patients with AT, in at least 3 surviving HSCT recipients, mixed donor chimerism was achieved, resulting in lymphocyte reconstitution, immunoglobulin production and lack of severe infection or lung disease post-transplantation.[151] Allogeneic HSCT has been used successfully to correct hematopoietic defects and immune deficiencies in other DSB repair disorders or chromosomal instability syndromes such as Fanconi anemia and Nijmegen breakage syndrome.[116, 242] Since these patients are particularly susceptible to DNA damaging agents conventionally used for HSCT conditioning (i.e., ionizing radiation and/or alkylating

chemotherapeutics), reduced intensity conditioning (RIC) regimens must be employed. Despite very limited clinical data reported in the literature, it is clear that AT patients are particularly sensitive to myeloablative conditioning, and overall survival in this population is improved with RIC.[149] Still, life-threatening toxicities in multiple organ systems have been reported in an AT patient even when a reduced intensity modified Fanconi protocol was used.[243] Due to uncertain outcomes associated in part with toxicity of the conditioning regimen, allogeneic HSCT is a controversial treatment option for AT.[149, 151] AT patients could therefore benefit from next generation, non-genotoxic antibody-based conditioning regimens to facilitate engraftment of ATM-competent donor cells in allogeneic HSCT.

3.3 Materials and Methods

Mice. CD45.2^{+} *Atm* knockout mice (B6.129S6-Atm^{tm1Awb}/J; JAX stock #008536) were used as transplantation recipients in this study. Donor mice were EGFP-transgenic mice[244] (C57BL/6-Tg(Act-EGFP)C14-Y01-FM131Osb; JAX stock #006567) or CD45.1 mice (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ; JAX stock #002014). Mouse strains were bred and maintained at an Emory University Division of Animal Resources facility. Transplant recipients were male and female mice 3 – 9 weeks of age. Animal studies were approved by Emory University Institutional Animal Care and Use Committee.

Genotyping. Mouse ear punch tissue was digested in 100 – 200 µl DirectPCR lysis reagent (Viagen Biotech, Los Angeles, CA) combined with 10 mg/ml proteinase K solution and incubated overnight in a 56 °C heat block. Samples were then incubated for 45 minutes in an 85 °C heat block, and then centrifuged for 15 minutes at 13000 rpm. Genomic DNA in the supernatant was

transferred to a clean microcentrifuge tube and stored at -80 °C until use. Genotyping was performed by PCR for wild-type or mutant *Atm* alleles according to protocols established by The Jackson Laboratory using the following primers:

Wild-type forward	5' – TGA TCT GTC AGG GGA ATG AAC – 3'
Mutant forward	5' – AGA CTG CCT TGG GAA AAG CG – 3'
Common reverse	5' – GCT GAG ATG CTA ACG GTA TAA ACA – 3'

Immunotoxin preparation and antibody conditioning. Biotinylated anti-CD117 (Clone 2B8, Biolegend, San Diego, CA) was combined at a 1:1 molar ratio with either streptavidin-ZAP (ATS, San Diego, CA) or an in-house developed streptavidin-saporin product.[179] CD117-saporin immunotoxin was delivered by retro-orbital injection at a dose of 0.5 mg/kg on day -5 before HSCT. Anti-CD4 (Clone GK1.5, Bio X Cell, Lebanon, NH) and anti-CD8 (Clone YTS 169.4, Bio X Cell, Lebanon, NH) were administered in 100 μg doses by intraperitoneal injection on day -2 and day -1. Anti-CD20 (Clone SA271G2, Biolegend, San Diego, CA) was administered as a 250 μg dose by intraperitoneal injection on day -2. Further reagent details are provided in Table S2.

Hematopoietic stem cell transplants. For bone marrow cell transplantation, 5 – 13-week-old donor EGFP mice were sacrificed, and femurs were dissected and crushed using a sterilized mortar and pestle. Bone marrow was strained through a 40 μm cell strainer and cells were counted using a Cellometer® Auto T4 automated cell counter (Nexcelom Bioscience, Lawrence, MA). Recipient mice were transplanted with 1 x 10^7 whole bone marrow cells by retro-orbital injection 5 days after immunotoxin conditioning.

Peripheral blood collection and analysis. Peripheral blood was periodically collected from the retro-orbital sinus in 3.8% w/v sodium citrate anticoagulant. CBC analysis was performed each sample using a HemaTrue Veterinary Hematology Analyzer (Heska, Loveland, CO). Remaining whole blood was centrifuged at 2000 x *g* for 15 minutes at 4 °C. Plasma was removed and frozen at -80 °C to be used for downstream analyses. Cell pellets were lysed using Hybri-Max RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) and peripheral blood leukocytes were stained for flow cytometric detection of donor- and host-derived cells using monoclonal antibodies against the following surface markers: CD45.2, CD3, CD4, CD8, B220, Gr-1 (Ly-6G/Ly-6C) and NK1.1 (CD161). Data were acquired using a Cytek® Aurora (Fremont, CA) flow cytometer or BD FACSymphony (Franklin Lakes, NJ). Flow cytometric analysis was performed using FlowJo v10.7.1 (BD, Ashland, OR). Further reagent details are provided in Table S2.

Immunoglobulin isotyping and quantification. Immunoglobulin analysis was performed by multiplex bead assay using the LEGENDplex™ Mouse Immunoglobulin Isotyping Panel kit (Biolegend, San Diego, CA). Mouse plasma samples were prepared according to manufacturer's instructions. Data were acquired using a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN). Data were analyzed using LEGENDplex™ Cloud-based Data Analysis Software (Qognit, San Carlos, CA).

Immunization response studies. Naïve *Atm*$^{-/-}$ mice, naïve *Atm*$^{+/+}$ littermates, and chimeric *Atm*$^{-/-}$ mice were immunized with recombinant factor VIII (fVIII) protein (Takeda, Lexington, MA) and plasma subsequently analyzed for the presence of anti-fVIII IgG and IgM antibodies to assess T

cell-dependent immune responses. Briefly, mice were immunized with 1 μg recombinant fVIII by retro-orbital injection weekly for 28 days. Plasma was collected at various timepoints, beginning 5 days after the immunization regimen and analyzed by ELISA. Microtiter plates were coated with 1.5 μg/ml recombinant fVIII antigen in coating buffer (20 mM bicine, 2 mM $CaCl_2$, pH 9.0) overnight at 4 °C. Plates were washed twice with wash buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, 0.05% sodium azide, pH 7.4) and then incubated in blocking buffer (wash buffer plus 2.0% bovine serum albumin) overnight at 4 °C. Plates were washed twice with wash buffer immediately before the assay. Mouse plasma samples were diluted 1:20 in blocking buffer and 25 μl plasma dilution was added to fVIII-coated wells. Plates were incubated at room temperature for 1 hour, and then washed twice with wash buffer. Goat anti-mouse IgG-alkaline phosphatase (AP) was diluted 1:1000 or IgM-AP was diluted 1:500 and 25 μl added to each well. Plates were incubated at room temperature for 1 hour, and then washed twice with wash buffer. AP substrate was reconstituted according to manufacturer's instructions (AP Substrate Kit, Cat #1721063, Bio-Rad, Hercules, CA) and 40 μl was added to each well. Plates were incubated room temperature for 20 minutes, and reaction was then quenched by adding 0.4 M NaOH. Absorbance at 405 nm was measured using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA).

Statistical analysis. Shapiro-Wilk test was used for normality testing of continuous variables. When comparing two groups, Student's t-test or Mann-Whitney U test was used depending on normality of data. When comparing more than two groups, one-way ANOVA with Tukey's multiple comparison tests was performed for normally distributed data, and Kruskal-Wallis test with Dunn's multiple comparison test was performed for nonparametric analyses. Data are

reported as mean ± sample standard deviation (SD). Statistical analysis was performed using GraphPad Prism v9.0.0 (San Diego, CA).

3.4 Results

HSCT using CD117-saporin conditioning enabled high-level multilineage engraftment in $Atm^{-/-}$ mice. We first evaluated whether CD117-saporin (sap) immunotoxin was effective at conditioning $Atm^{-/-}$ mice to allow engraftment of bone marrow from GFP^+ donor mice. Mutant $Atm^{-/-}$ mice, as well as heterozygous $Atm^{+/-}$ and wild-type $Atm^{+/+}$ littermates, were conditioned with 0.5 mg/kg CD117-sap, and then transplanted with 1 x 10^7 GFP^+ whole bone marrow cells. Donor chimerism was followed up to 26 weeks post-transplantation across four independent experimental cohorts ($Atm^{-/-}$: n = 10, $Atm^{+/-}$: n = 12, $Atm^{+/+}$: n = 10). Myeloid chimerism reached 97.5 ± 1.4% (mean ± sample SD) as early as 6 weeks post-transplantation in $Atm^{-/-}$ mice and was maintained at this level throughout the study. T cell and B cell chimerism in this group reached 58.9 ± 6.3% and 59.7 ± 8.9% at 6 weeks, continually increasing up to 80.1 ± 1.6% and 93.6 ± 1.8%, respectively, by 26 weeks post-transplantation (**Figure 20A**). High levels of multilineage chimerism were also achieved in the majority of $Atm^{+/-}$ (myeloid: 98.9 ± 0.4%, T cell: 59.5 ± 8.1%, B cell: 90.2 ± 2.4%) and $Atm^{+/+}$ (myeloid: 97.8 ± 2.0%, T cell: 50.4 ± 5.2%, B cell: 85.3 ± 2.0%) littermates by 26 weeks, although GFP^+ donor cells did not engraft in one $Atm^{+/-}$ mouse and three $Atm^{+/+}$ mice (**Figure S13**). This was not observed in any $Atm^{-/-}$ recipients. These data demonstrate that $Atm^{-/-}$ mice can successfully and durably engraft donor marrow under CD117-sap conditioning.

HSCT failed to correct growth deficiency in Atm$^{-/-}$ mice. Growth retardation and growth failure have long been recognized as common features in AT.[245] The reasons are multifactorial and include a variety of endocrinological disturbances such as growth hormone deficiency, low levels of insulin-like growth factor-1 (IGF-1), hypogonadism and infertility, insulin resistance and disrupted glucose metabolism.[246, 247] Direct roles of ATM kinase activity in growth factor signaling pathways have been demonstrated.[248] Consistent with these observation in humans, ATM-deficient mouse models are reported to exhibit decreased somatic growth.[249]

Body weight of naïve *Atm*$^{-/-}$ control mice and *Atm*$^{+/+}$ littermates were tracked from 3 weeks of age at the time of weaning for up to 42 weeks. Homozygous *Atm*$^{-/-}$ mutants consistently weighed less than their wild-type littermates, with the disparity being more pronounced in males (**Figure 20B, red vs. green lines**). Body weight of chimeric *Atm*$^{-/-}$ mice that had received HSCT following CD117-sap conditioning was also followed and compared with naïve age-matched controls. HSCT did not correct the somatic growth deficiency observed in untreated *Atm*$^{-/-}$ mice (**Figure 20B, blue line**). These findings suggest that hematopoietic reconstitution alone following HSCT using CD117-sap conditioning may not be sufficient to rectify the multifactorial growth impairment phenotype in AT.

Chimeric Atm$^{-/-}$ mice have increased CD4^{+} and CD8^{+} T cells after HSCT. ATM deficiency and associated impairment of DSB repair and V(D)J recombination leads to deficiencies in CD4^{+} and CD8^{+} T cell subsets in AT patients.[250] ATM deficient mice have likewise been shown to exhibit reductions in these T cell populations, with normal numbers of B cells and myeloid cells.[249] We performed complete blood count (CBC) and flow cytometric analysis on peripheral blood samples

of untreated *Atm*$^{-/-}$ and *Atm*$^{+/+}$ control mice, as well as chimeric *Atm*$^{-/-}$ mice after transplantation. *Atm*$^{-/-}$ untreated controls did not show deficiencies in overall white blood cells (WBC), lymphocytes, B cell, NK cell or granulocyte counts when compared with *Atm*$^{+/+}$ littermates (**Figure 20C, red vs. green lines**). However, CD3^{+} and CD3^{+} CD4^{+} T cells were markedly reduced, as well as CD3^{+} CD8^{+} to a lesser degree, in homozygous *Atm*$^{-/-}$ knockout mice (**Figure 20D, red vs. green lines**). *Atm*$^{-/-}$ mice that received HSCT had increased WBC, lymphocyte and B cell counts compared to controls (**Figure 20C, blue lines**). Moreover, CD3^{+}, CD3^{+} CD4^{+}, and CD3^{+} CD8^{+} T cells were increased similar to wild-type levels following HSCT (**Figure 20D, blue lines**). These data demonstrate that HSCT under CD117-sap conditioning is capable of correcting T cell deficiencies in *Atm*$^{-/-}$ mice.

Figure 23

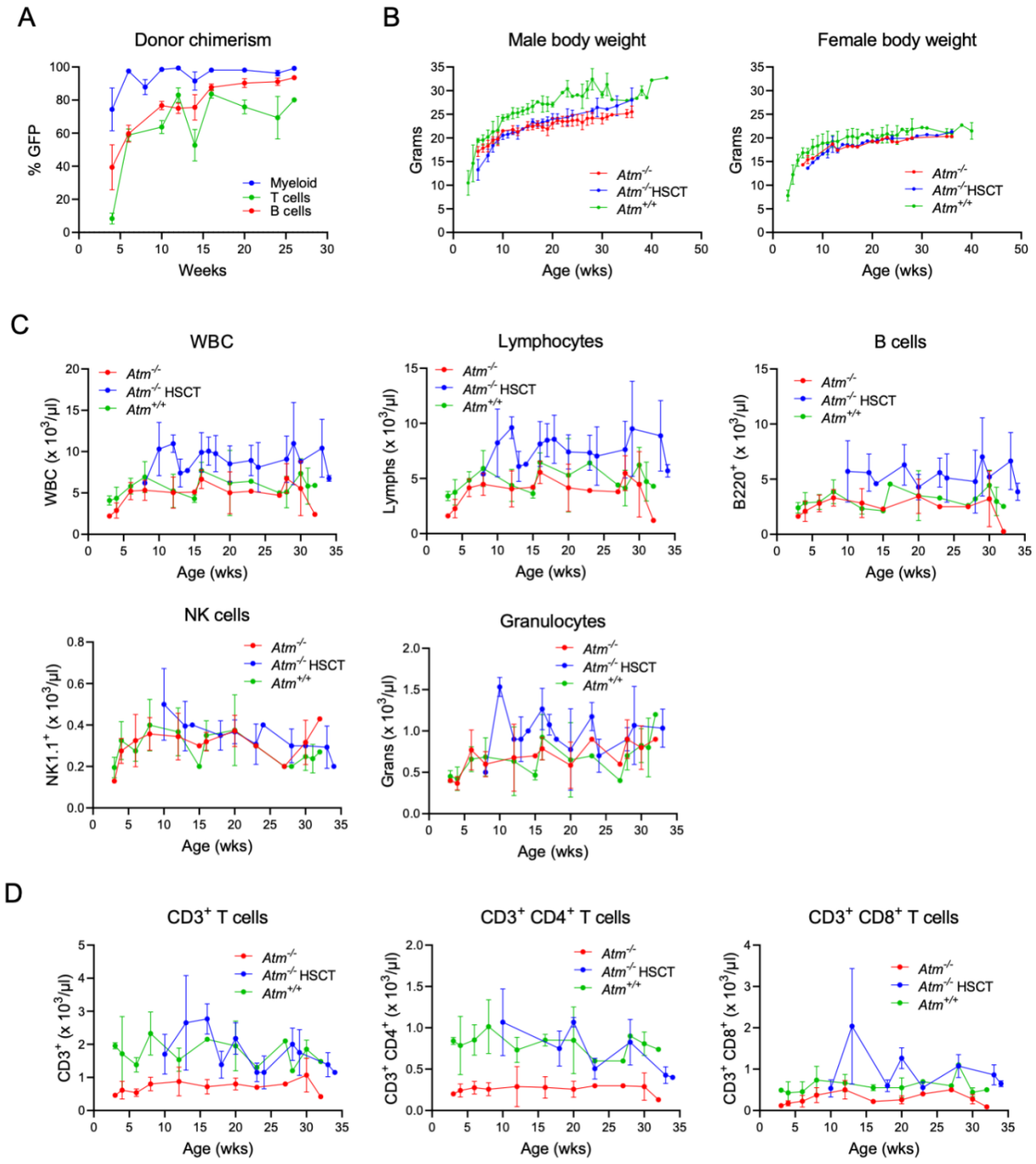


Figure 23. Multilineage chimerism and correction of T cell numbers, but not growth defect, is achieved after HSCT using CD117-sap

(**A**) Mutant *Atm*$^{-/-}$ mice were conditioned with 0.5 mg/kg CD117-sap and transplanted with 1 x 10^7 GFP$^+$ whole bone marrow cells. High multilineage donor chimerism was achieved in all *Atm*$^{-/-}$ transplant recipients. (**B**) Body weight of naïve *Atm*$^{-/-}$ control mice and *Atm*$^{+/+}$ littermates were tracked. Homozygous *Atm*$^{-/-}$ mutants consistently weighed less than their wild-type littermates, with the disparity being more pronounced in males (red vs. green line). Body weight of chimeric *Atm*$^{-/-}$ mice that had received HSCT

following CD117-sap conditioning was also followed and compared with naïve age-matched controls. HSCT did not correct the somatic growth defect observed in untreated *Atm*$^{-/-}$ mice (blue line). (**C**) CBC and flow cytometry on peripheral blood samples of untreated *Atm*$^{-/-}$, *Atm*$^{+/+}$ control mice, and chimeric *Atm*$^{-/-}$ mice after transplantation were performed. *Atm*$^{-/-}$ untreated controls did not show deficiencies in WBC, lymphocytes, B cell, NK cell or granulocyte counts compared with *Atm*$^{+/+}$ littermates (red vs. green lines). *Atm*$^{-/-}$ mice that received HSCT had increased WBC, lymphocyte and B cell counts compared to controls (blue lines). (**D**) $CD3^+$ and $CD3^+$ $CD4^+$ T cells were markedly reduced, as well as $CD3^+$ $CD8^+$ to a lesser degree, in *Atm*$^{-/-}$ knockout mice (red vs. green lines). $CD3^+$, $CD3^+$ $CD4^+$, and $CD3^+$ $CD8^+$ T cells were increased similar to wild-type levels following HSCT (blue lines).

HSCT improves IgA levels in Atm$^{-/-}$ mice. Lack of functional ATM kinase activity results in impaired B cell class-switch recombination. Dysgammaglobulinemia in AT patients is variable, but typically involves low or absent IgA, low or normal IgG, and elevated or normal IgM.[251] We analyzed plasma samples from 9-month-old untreated *Atm*$^{-/-}$ and *Atm*$^{+/+}$ control mice (n = 5 each group) and age-matched chimeric *Atm*$^{-/-}$ mice (n = 9) six months post-transplantation for IgA, IgG1 and IgM levels using a flow cytometry-based multiplex bead assay. Although statistically significant differences were not detected between untreated *Atm*$^{-/-}$ and *Atm*$^{+/+}$ control mice, IgA levels were significantly higher ($p = 0.029$) in *Atm*$^{-/-}$ mice receiving HSCT, suggesting that HSCT under CD117-sap conditioning has the potential to improve certain immunoglobulin deficiencies in *Atm*$^{-/-}$ mice (**Figure 24A**).

HSCT rescued defective T cell-dependent immune response in Atm$^{-/-}$ mice. *Atm* knockout mice recapitulate immune defects related to impaired T cell differentiation and T cell-dependent immune responses observed in AT patients.[252] In addition to hypogammaglobulinemia, impaired formation of T cell-dependent memory B cells is found in AT,[253] yet HSCT has been shown to increase production of pneumococcal vaccine antibodies.[152] We immunized untreated *Atm*$^{-/-}$, *Atm*$^{+/+}$, and transplanted chimeric *Atm*$^{-/-}$ mice (n = 3 each group) with the T cell-dependent

immunogen human factor VIII (fVIII) and assessed for the development of anti-fVIII specific antibodies by ELISA assay 70 days after the first immunization exposure. Our laboratory has extensive experience studying the immune response to fVIII as well as established immunization protocols, which influenced our selection of fVIII for these studies.

Anti-fVIII IgG or IgM responses were hampered in untreated *Atm*$^{-/-}$ knockout mice compared with *Atm*$^{+/+}$ littermates (**Figure 24B**). HSCT produced measurably increased anti-fVIII immune responses in *Atm*$^{-/-}$ mice, although statistical significance was not reached. One chimeric *Atm*$^{-/-}$ mouse generated a mild IgG response despite a robust IgM response at this time point. Nevertheless, HSCT and immune reconstitution with ATM competent cells positively impacted the overall T cell-dependent immune response in *Atm*$^{-/-}$ mice after transplantation.

Figure 24

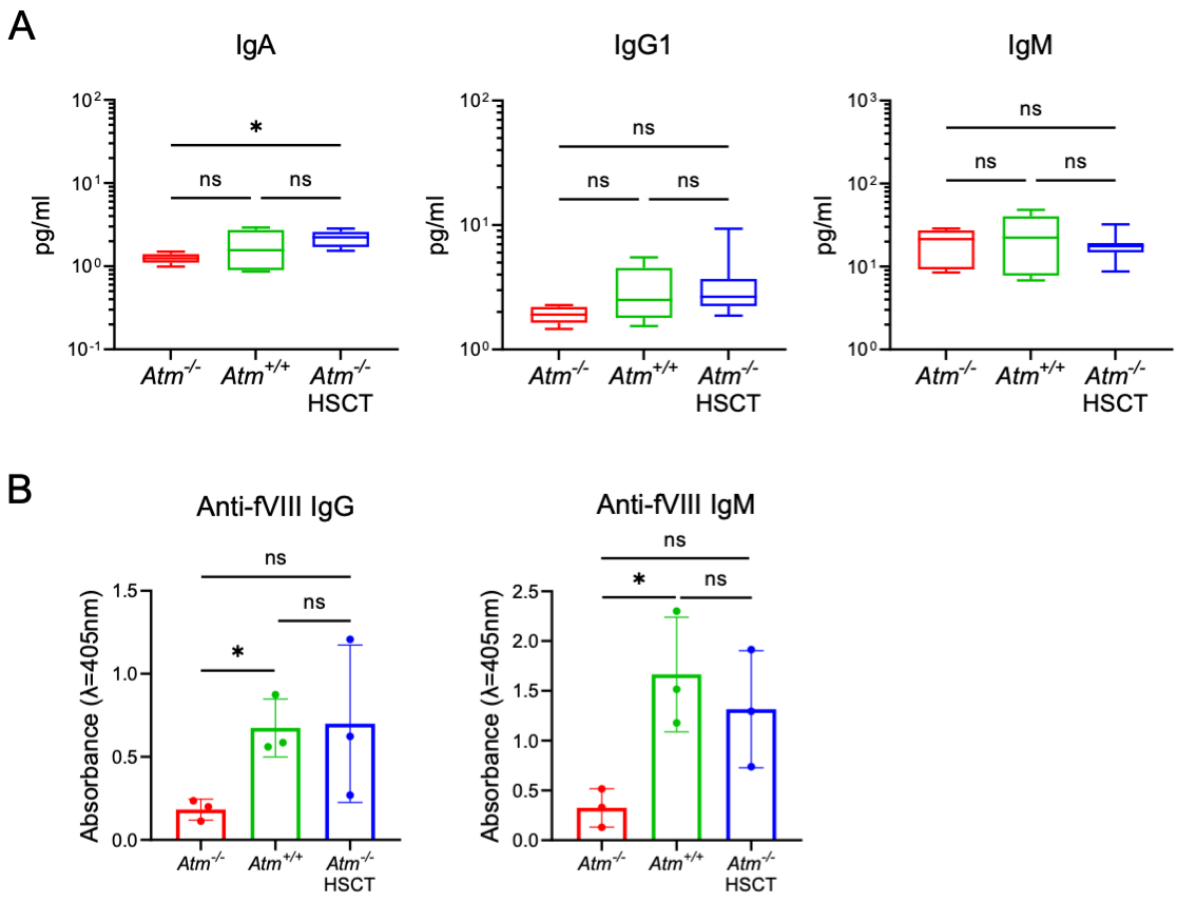


Figure 24. IgA levels and T cell-dependent immune responses corrected after HSCT using CD117-sap.
(**A**) IgA levels were significantly higher ($p = 0.029$) in *Atm*$^{-/-}$ mice receiving HSCT. (**B**) Anti-fVIII IgG or IgM responses were hampered in untreated *Atm*$^{-/-}$ knockout mice compared with *Atm*$^{+/+}$ littermates. HSCT produced measurably increased anti-fVIII immune responses in *Atm*$^{-/-}$ mice, although statistical significance was not reached.

Host-derived lymphoid malignancy despite HSCT. CD117-sap immunotoxin is a non-lymphoablative agent, specifically depleting ST-HSC and LT-HSC populations while sparing mature hematopoietic cells and other tissues.[189, 254] Since T lymphocytes are long-lived cells, endogenous ATM-deficient T cells may persist for up to one year in mice, even when full donor myeloid chimerism is achieved. These host-derived ATM-deficient cells retain their

predisposition for malignant transformation. In our initial studies using CD117-sap alone for conditioning, despite achieving 80.1% mean T cell chimerism six months after HSCT, one *Atm*$^{-/-}$ mouse (out of 10 *Atm*$^{-/-}$ mice that received HSCT under CD117-sap alone) became visibly ill and required euthanasia at 29 weeks of age. Donor T cell chimerism in this mouse measured 75.2% at 16 weeks post-transplantation but dropped to 10.3% at 24 weeks. WBC rose from 11.3 to 38.4 x 10^3/μl (ref. range: 2.6 – 10.1), lymphocytes from 9.2 to 29.9 x 10^3/μl (ref. range: 1.3 – 8.4), monocytes from 0.6 to 3.6 x 10^3/μl (ref. range: 0.0 – 0.3) and granulocytes from 1.5 to 4.9 x 10^3/μl (ref. range: 0.4 – 2.0) during this period (**Figure 25A**). Red blood cells (RBC) and hemoglobin fell from 9.4 to 5.2 x 10^6/μl (ref. range: 6.5 – 10.1) and 14.2 to 8.3 g/dl (ref. range: 10.0 – 16.1), and platelets from 446 to 84 x 10^3/μl (ref. range: 250 – 1540). Necropsy revealed marked splenomegaly (spleen weight: 0.51 g vs. normal spleen weight: ≈ 0.10 g) and thymic hyperplasia (thymus weight: 0.60 g vs. normal thymus weight: ≈ 0.04 g) (**Figure S14**). Flow cytometry was performed on peripheral blood (PB) leukocytes, splenocytes and thymocytes to determine which cell populations were increased and the cellular origin (**Figure 25B**). In all three tissues, the CD3$^+$ T cell population consisted predominantly of double-positive CD4$^+$ CD8$^+$ T cells (PB: 84.4%, spleen: 91.1%, thymus: 95.4%) and CD45.2$^+$ cells (PB: 89.4%, spleen: 94.3%, thymus: 96.9%), indicating the development of a host-derived T cell malignancy.

Figure 25

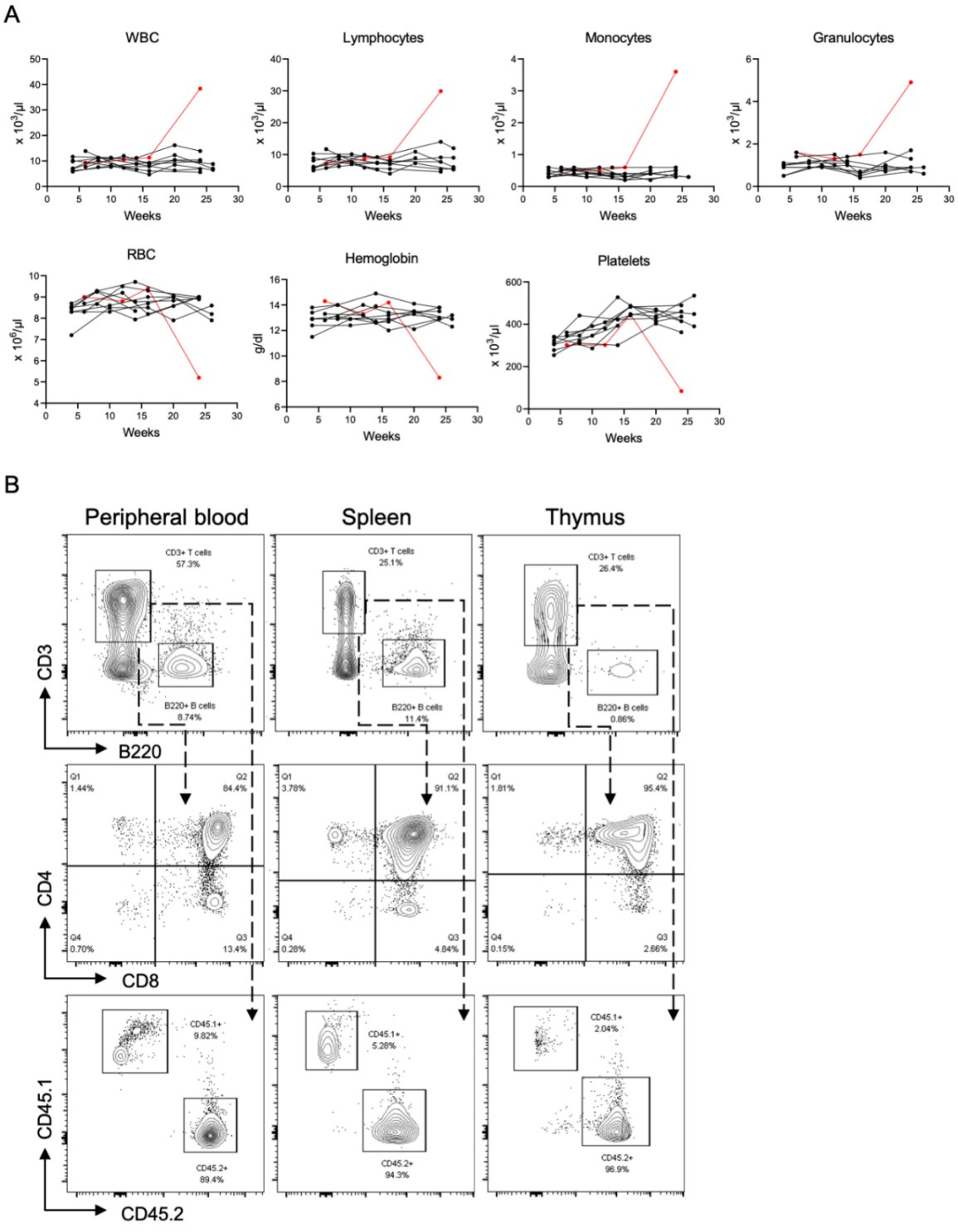


Figure 25. Host-derived T cell lymphoma detected in one *Atm*$^{-/-}$ recipient conditioned with CD117-sap alone

(**A**) One *Atm*$^{-/-}$ mouse (out of 10 *Atm*$^{-/-}$ mice that received HSCT under CD117-sap alone) required euthanasia at 29 weeks of age. Donor T cell chimerism in this mouse measured 75.2% at 16 weeks post-transplantation but dropped to 10.3% at 24 weeks. WBC rose from 11.3 to 38.4 x 10^3/μl, lymphocytes from 9.2 to 29.9 x 10^3/μl, monocytes from 0.6 to 3.6 x 10^3/μl and granulocytes from 1.5 to 4.9 x 10^3/μl. RBC and hemoglobin fell from 9.4 to 5.2 x 10^6/μl and 14.2 to 8.3 g/dl, and platelets from 446 to 84 x 10^3/μl. (**B**) In PB leukocytes, splenocytes and thymocytes, CD3$^+$ T cell population was predominantly double-positive CD4$^+$ CD8$^+$ T cells and CD45.2$^+$ cells, indicating host-derived T cell malignancy.

CD117-sap + lymphocyte depleting antibodies allowed multilineage engraftment in the absence of malignancy. Since host-derived ATM-deficient lymphocytes remain after HSCT and retain the propensity for malignant transformation, we next tested the hypothesis that addition of lymphocyte depleting antibodies to the conditioning regimen could permit high levels of multilineage donor chimerism while preventing the development of lymphoid malignancy. Three- to 6-week-old *Atm*$^{-/-}$ (n = 6) and *Atm*$^{+/+}$ (n = 4) mice in two independent experimental cohorts were conditioned with 0.5 mg/kg CD117-sap by retro-orbital injection five days before HSCT. On day -2 before HSCT, mice were administered an antibody cocktail of 100 μg anti-CD4, 100 μg anti-CD8 and 250 μg anti-CD20 intraperitoneally. On day -1, mice received a second dose of 100 μg each anti-CD4 and anti-CD8. Mice were transplanted with 1 x 10^7 GFP$^+$ whole bone marrow cells. Chimerism was followed for 32 weeks and high multilineage donor chimerism was achieved in *Atm*$^{-/-}$ mice (myeloid: 87.9 ± 6.4%, T cell: 90.4 ± 3.5%, B cell: 92.6 ± 3.2%) (**Figure 26A**). Donor chimerism in the T and B cell compartments was markedly higher in chimeric *Atm*$^{-/-}$ mice compared with chimeric *Atm*$^{+/+}$ littermates (myeloid: 88.4 ± 8.4%, T cell: 38.7 ± 9.8%, B cell: 71.5 ± 7.9%) (**Figure S15**). CBC analysis showed low WBC and lymphocyte counts in conditioned *Atm*$^{-/-}$ mice during the early post-transplantation period, consistent with lymphocyte depletion as part of the conditioning regimen (**Figure 26B**) but these counts returned to wild-type levels by 8 weeks post-transplant. The same trend was observed in CD4$^+$ and CD8$^+$ T cell subpopulations, which began

low due to lymphocyte depletion but gradually increased to wild-type levels throughout the study (**Figure 26C**). This was in contrast to T cell counts in naïve *Atm*$^{-/-}$ mice, which remained low. Moreover, no *Atm*$^{-/-}$ mice developed malignancy during the observation period up to 1 year of age and 11 months post-transplantation. These data illustrate that addition of lymphocyte depleting antibodies to CD117-sap conditioning enabled high levels of durable multilineage engraftment and restored T cell levels, while preventing neoplastic development in *Atm*$^{-/-}$ mice.

Figure 26

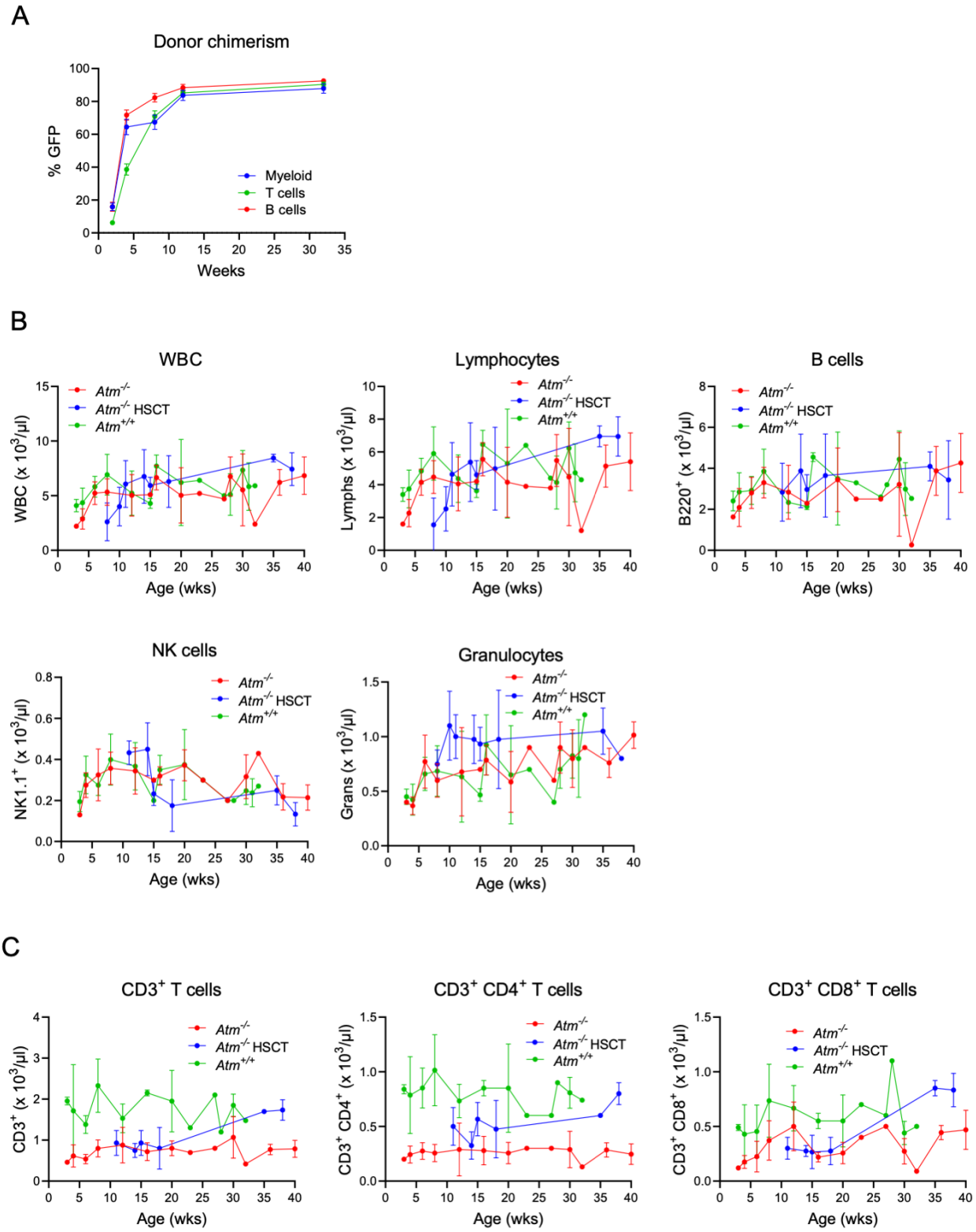

A
Donor chimerism
% GFP
Weeks
Myeloid
T cells
B cells
B
WBC
WBC (x 10³/µl)
Lymphocytes
Lymphs (x 10³/µl)
B cells
B220⁺ (x 10³/µl)
NK cells
NK1.1⁺ (x 10³/µl)
Granulocytes
Grans (x 10³/µl)
Age (wks)
Atm-/-
Atm-/- HSCT
Atm+/+
C
CD3⁺ T cells
CD3⁺ (x 10³/µl)
CD3⁺ CD4⁺ T cells
CD3⁺ CD4⁺ (x 10³/µl)
CD3⁺ CD8⁺ T cells
CD3⁺ CD8⁺ (x 10³/µl)

Figure 26. Multilineage chimerism and correction of T cell numbers is achieved after HSCT using CD117-sap plus lymphocyte depleting antibodies
(A) *Atm*$^{-/-}$ (n = 6) and *Atm*$^{+/+}$ (n = 4) mice from two independent experimental cohorts were conditioned with 0.5 mg/kg CD117-sap plus an antibody cocktail of CD4, CD8 and CD20 monoclonal antibodies and transplanted with 1 x 10^7 GFP$^+$ whole bone marrow cells. Chimerism was followed for 32 weeks and high multilineage donor chimerism was achieved in Atm$^{-/-}$ mice. (B) Low WBC and lymphocyte counts measured in conditioned Atm$^{-/-}$ mice during the early post-transplantation period due to lymphocyte depleting agents. Counts returned to wild-type levels by 8 weeks post-transplantation. (C) CD4$^+$ and CD8$^+$ T cell subpopulations were initially low and gradually increased to wild-type levels throughout the study.

HSCT under CD117-sap did not result in donor-derived microglia in brains of Atm$^{-/-}$ mice.

Although true brain-resident microglia are originally derived from a myelomonocytic lineage of the yolk sac, many studies have demonstrated the capacity of peripheral monocytes derived from adult hematopoietic stem cells to migrate to the brain and engraft as microglia or microglia-like cells with enhanced phagocytic functions in times of neurological injury or inflammation, and in the absence of myeloablative conditioning to disturb the blood-brain barrier.[255-259] We hypothesized that neuroinflammation and neurodegenerative processes resulting from ATM deficiency might promote recruitment and engraftment of bone marrow-derived cells to the brain after HSCT in our mouse model. We harvested brains from a naïve *Atm*$^{-/-}$ mouse, naïve *Atm*$^{+/+}$, and chimeric *Atm*$^{-/-}$ mouse 30 weeks post-transplantation under CD117-sap conditioning. The chimeric *Atm*$^{-/-}$ mouse was 39 weeks old and had 99.6% donor myeloid chimerism, 79.7% donor T cell chimerism, 94.6% B cell chimerism at the time of sacrifice. Brain tissue was dissociated, enriched for the microglial fraction, and analyzed by flow cytometry for markers of GFP$^+$ CD11b$^+$ donor-derived microglia, as well as GFP$^+$ CD45$^+$ peripherally derived, infiltrating immune cells. The chimeric mouse had higher percentages of CD45^{hi} CD11b$^+$ peripheral monocyte/macrophages as well as CD45^{hi} CD11b$^-$ peripheral lymphocytes than untransplanted *Atm*$^{-/-}$ and *Atm*$^{+/+}$ controls (**Figure 27A, first row**). Of the CD45^{hi} CD11b$^+$ peripheral monocyte/macrophage population

15.9% were Ly-6c^{hi} CD11c^{-}, which was a higher proportion of this subpopulation than observed in controls (*Atm*$^{-/-}$: 9.7% Ly-6c^{hi} CD11c^{-}; *Atm*$^{+/+}$: 6.1% Ly-6c^{hi} CD11c^{-}) (**Figure 27A, second row**). CD11b^{+} Ly-6c^{hi} expression in monocytes represents a subset of inflammatory monocytes that are recruited to sites of infection and inflammation.[260] These infiltrating inflammatory monocytes were derived from the GFP^{+} donor (**Figure 27A, third row**). Pattern of expression of Ly-6c and CD11c in CD45^{int} CD11b^{+} microglia populations and CD45^{hi} CD11b^{-} lymphocyte populations were similar across control and chimeric animals (**Figure S16**). No donor-derived GFP^{+} CD45^{int} CD11b^{+} microglia were detected in the chimeric animal by flow cytometry (**Figure 27A, fourth row**). Infiltrating GFP^{+} cells were clearly detected by immunofluorescence staining of cerebellum in the chimeric mouse. Consistent with observations by flow, GFP^{+} signal does not appear to colocalize with Iba1-stained microglia, yet GFP^{+} cells do appear to associate closely with Iba1^{+} microglia (**Figure 27B**). Cresyl violet staining did not reveal remarkable neuronal loss in cerebellum in naïve *Atm*$^{-/-}$ controls or chimeric mice. Taken together, these findings suggest that donor-derived ATM-competent, proinflammatory myeloid cells infiltrate the cerebellum of *Atm*$^{-/-}$ mice after HSCT, potentially in response to active neuroinflammatory processes occurring in the brain.

Figure 27

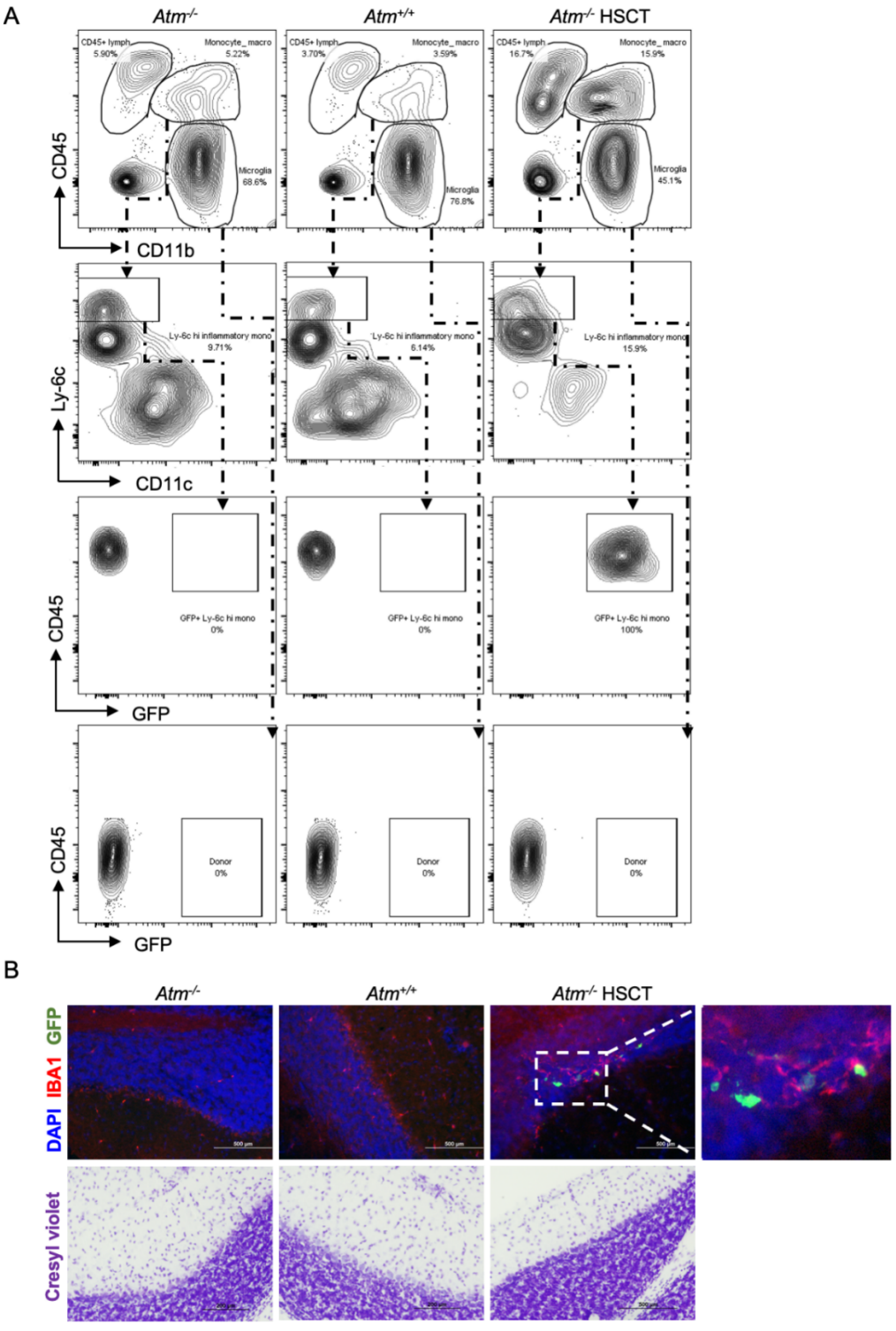

A
Atm-/-
Atm+/+
Atm-/- HSCT
CD45+ lymph
5.90%
Monocyte_ macro
5.22%
Microglia
68.6%
CD45+ lymph
3.70%
Monocyte_ macro
3.59%
Microglia
76.8%
CD45+ lymph
16.7%
Monocyte_ macro
15.9%
Microglia
45.1%
CD45
CD11b
Ly-6c hi inflammatory mono
9.71%
Ly-6c hi inflammatory mono
6.14%
Ly-6c hi inflammatory mono
15.9%
Ly-6c
CD11c
GFP+ Ly-6c hi mono
0%
GFP+ Ly-6c hi mono
0%
GFP+ Ly-6c hi mono
100%
CD45
GFP
Donor
0%
Donor
0%
Donor
0%
CD45
GFP
B
Atm-/-
Atm+/+
Atm-/- HSCT
GFP
IBA1
DAPI
Cresyl violet

Figure 27. Donor-derived GFP$^+$ microglia not detected in chimeric *Atm*$^{-/-}$ brain, however GFP$^+$ cells are present
Brain tissue was dissociated, enriched for the microglial fraction, and analyzed by flow cytometry for markers of GFP$^+$ CD11b$^+$ donor-derived microglia, as well as GFP$^+$ CD45$^+$ peripherally derived, infiltrating immune cells. (**A**) The chimeric mouse had higher percentages of CD45^{hi} CD11b$^+$ peripheral monocyte/macrophages as well as CD45^{hi} CD11b$^-$ peripheral lymphocytes than untransplanted *Atm*$^{-/-}$ and *Atm*$^{+/+}$ controls (first row). Of the CD45^{hi} CD11b$^+$ peripheral monocyte/macrophage population 15.9% were Ly-6c^{hi} CD11c$^-$, which was a higher proportion of this subpopulation than observed in controls (second row). CD11b$^+$ Ly-6c^{hi} expressing monocytes are inflammatory and are recruited to sites of infection and inflammation. These infiltrating inflammatory monocytes were derived from the GFP$^+$ donor (third row). No donor derived GFP$^+$ CD45^{int} CD11b$^+$ microglia were detected in the chimeric animal (fourth row). (**B**) Infiltrating GFP$^+$ cells were detected by immunofluorescence staining of cerebellum in the chimeric mouse. GFP$^+$ signal does not appear to colocalize with Iba1-stained microglia, yet GFP$^+$ cells do appear to associate closely with Iba1$^+$ microglia. Cresyl violet staining did not reveal remarkable neuronal loss in cerebellum in naïve *Atm*$^{-/-}$ controls or chimeric mice.

3.5 Discussion

Allogeneic HSCT has been used successfully in chromosomal instability disorders such as Nijmegen breakage syndrome and Fanconi anemia, however its use in AT is controversial and not routinely performed. Patients with AT are particularly susceptible to toxicities associated with genotoxic conditioning agents and, although published reports of allogeneic HSCT in AT are extremely limited, outcomes in this patient population are generally worse than those experienced in other DNA double strand break disorders.[149, 242] Myeloablative regimens are poorly tolerated in AT; HSCT survivors were transplanted under modified Fanconi anemia conditioning protocols with mixed results regarding regimen-related toxicities. Modified GEFA02 (IV busulfan, fludarabine, and ATG) or GEFA03 (IV busulfan, fludarabine, cyclophosphamide, and alemtuzumab or ATG) German Fanconi anemia protocols were well tolerated in three patients transplanted at age 3 or younger without significant adverse effects,[151] while a 13-year-old boy experienced life-threatening toxicity in multiple organ systems under a modified GEFA02 protocol (IV busulfan, fludarabine, cyclophosphamide, and rituximab).[153] Factors contributing to such variable outcomes are poorly understood, and lack of published reports preclude the ability to

establish clear guidelines. However, reconstitution of the hematopoietic system with HSCT has the potential to restore the hematopoietic system and immune function, prevent infections and lymphoid malignancy, ameliorating several of the primary disease manifestations of AT. Achieving a critical balance between minimizing regimen-related toxicity while maintaining ablative properties required to achieve donor HSC engraftment is key to successful HSCT for AT.

HSCT studies performed in *Atm*$^{-/-}$ mice using non-myeloablative conditioning regimens have demonstrated encouraging results. Bagley et al. pioneered a reduced-intensity conditioning approach in mice using a combination of depleting doses of anti-CD4 and anti-CD8 monoclonal antibodies with cyclophosphamide before HSCT.[240] Using this regimen, full donor chimerism in 7 of 9 recipients was reported, as well as restored frequency of CD4 and CD8 T cells in the peripheral blood and restored immune function in skin allograft rejection assays. Additionally, only 1 of 21 *Atm*$^{-/-}$ mice receiving HSCT under this non-myeloablative regimen developed thymic lymphoma. Schubert and colleagues have since used this conditioning regimen in multiple studies to demonstrate the ability of HSCT to normalize T cell populations, and improve motor function, growth and survival, although only mixed donor chimerism could be acheived.[148, 241, 261] Addition of low doses of irradiation significantly improved engraftment into multiple tissues, however even at 0.5 Gy, activation of dysfunctional DNA damage response pathways led to increased inflammation, oxidative stress and lethality,[261] highlighting the critical need for non-genotoxic strategies in this disease setting.

In the present study, conditioning was accomplished using a non-genotoxic immunotoxin targeting the CD117 receptor on HSCs. In contrast to the studies described above, CD117-sap conditioning

permitted universally high levels of multilineage donor chimerism in all *Atm*$^{-/-}$ recipients. We observed corrections in T cell counts and immune responses against alloantigen following HSCT, consistent with previous reports, as well as improved IgA levels. Using immunotoxin conditioning alone however, 1 of 10 *Atm*$^{-/-}$ mice developed T cell malignancy that was host-derived. Since CD117-sap specifically targets HSCs for killing, host lymphocytes persist after HSCT and the recipient remains vulnerable to development of lymphoid malignancy from ATM-deficient cells. To address this issue, we added a cocktail of lymphocyte depleting antibodies targeting CD4, CD8 and CD20 to the conditioning regimen. This approach led to even higher levels of donor T and B cell chimerism (90.4% and 92.6%, respectively) and prevented the development of lymphoma up to 1 year of age. Despite these high levels of chimerism of ATM-competent cells achieved under the combined CD117-sap + CD4/CD8/CD20 antibody regimen, it is possible that the ≈10% host-derived T cells that remain could still give rise to malignancy in a small fraction of recipients in larger cohorts. The non-myeloablative protocol established by Bagley et al. used significantly higher doses of depleting antibodies (total doses: 1.0 mg anti-CD4 and 2.0 mg anti-CD8) in 4–6-week-old mice; our regimen used total doses of only 200 μg anti-CD4 and anti-CD8 and 250 μg anti-CD20 in 3–6-week-old recipients. Therefore, antibody doses in our non-genotoxic regimen could likely be increased to achieve greater depletion of host lymphocytes and full donor chimerism with ATM-competent cells, effectively eliminating the risk of *Atm*$^{-/-}$-driven lymphoid malignancy.

Finally, in contrast to Pietzner et al.[241] who found no evidence of GFP^{+} and/or *Atm*$^{+/+}$ donor cells in brain by immunofluorescence or PCR, we detected the presence of GFP^{+} donor cells in brain by immunofluorescence and flow cytometry, indicating that bone marrow derived donor cells can

penetrate the blood-brain barrier and infiltrate the cerebellum in our model. While we found no evidence of donor-derived microglia engraftment in the brains of *Atm*$^{-/-}$ mice transplanted under CD117-sap conditioning, we observed increased levels of donor-derived, infiltrating peripheral leukocytes, of which a high proportion of myeloid cells represented a subset of activated, inflammatory monocytes known to be rapidly recruited to sites of inflammation.[260] Although the significance of these infiltrating cells in our model is yet to be determined, further studies will focus on the neuroinflammatory status of the brain in *Atm*$^{-/-}$ mice and whether HSCT under this non-genotoxic conditioning protocol has the potential to alleviate states of inflammation and oxidative stress that likely contribute to the neuropathology of AT.

In summary, we have developed a completely non-genotoxic, antibody-based conditioning regimen for HSCT with a significantly improved safety profile over traditional genotoxic conditioning agents that can be used to correct immune deficiencies, restore immune function, and prevent malignancy in a murine model of AT. These findings are proof-of-concept for the use of non-genotoxic conditioning to attenuate risks of toxicity and facilitate successful immune reconstitution in allogeneic HSCT for AT.

3.6 Acknowledgements

This work was supported by NHLBI 1K08HL141635-01A1, Atlanta Pediatric Scholars Program K12 Scholar supported by grant K12HD072245 and U54AI082973 (to S.C.) and Henagan Foundation (to S.C.). S.C serves on the advisory committee on emapalumab for Sobi. Research reported in this publication was supported in part by the Pediatrics/Winship Flow Cytometry Core of Winship Cancer Institute of Emory University, Children's Healthcare of Atlanta and NIH/NCI

under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

3.7 Supplemental Information

Figure S13

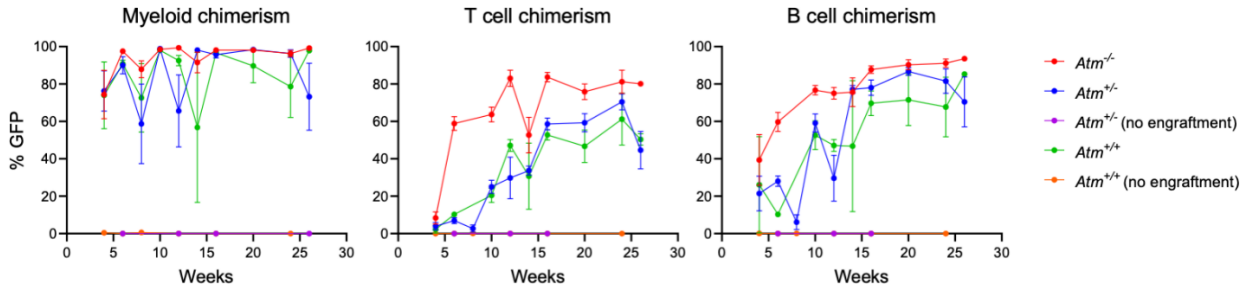


Figure S13. High levels of multilineage chimerism were achieved in the majority of $Atm^{+/-}$ (myeloid: 98.9 ± 0.4%, T cell: 59.5 ± 8.1%, B cell: 90.2 ± 2.4%) and $Atm^{+/+}$ (myeloid: 97.8 ± 2.0%, T cell: 50.4 ± 5.2%, B cell: 85.3 ± 2.0%) littermates by 26 weeks, although GFP^{+} donor cells did not engraft in one $Atm^{+/-}$ mouse and three $Atm^{+/+}$ mice. This was not observed in any $Atm^{-/-}$ recipients.

Figure S14

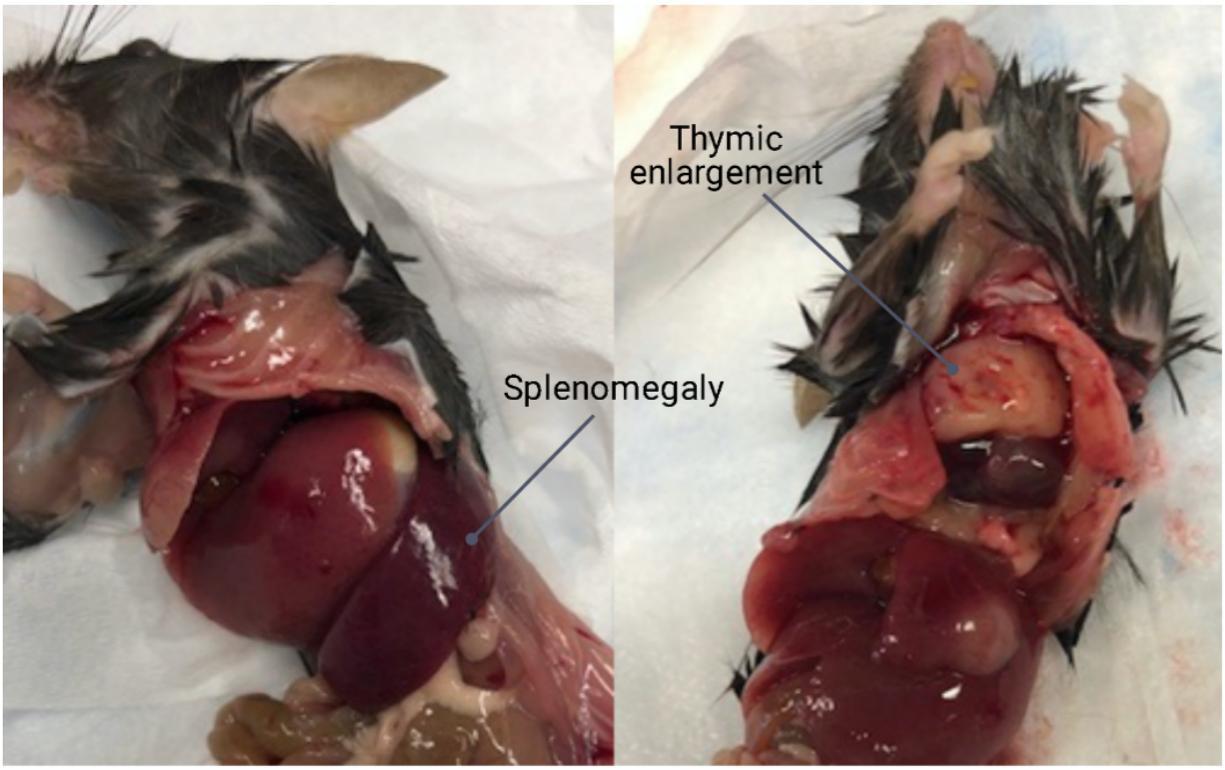


Figure S14. Necropsy revealed marked splenomegaly (spleen weight: 0.51 g vs. normal spleen weight: ≈0.10 g) and thymic hyperplasia (thymus weight: 0.60 g vs. normal thymus weight: ≈0.04 g).

Figure S15

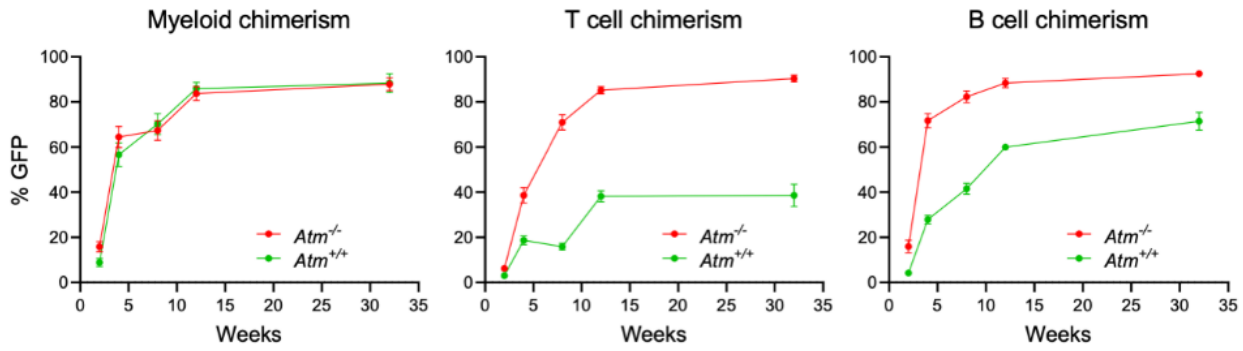


Figure S15. Donor chimerism in the T and B cell compartments was markedly higher in chimeric *Atm*$^{-/-}$ mice compared with chimeric *Atm*$^{+/+}$ littermates following HSCT.

Figure S16

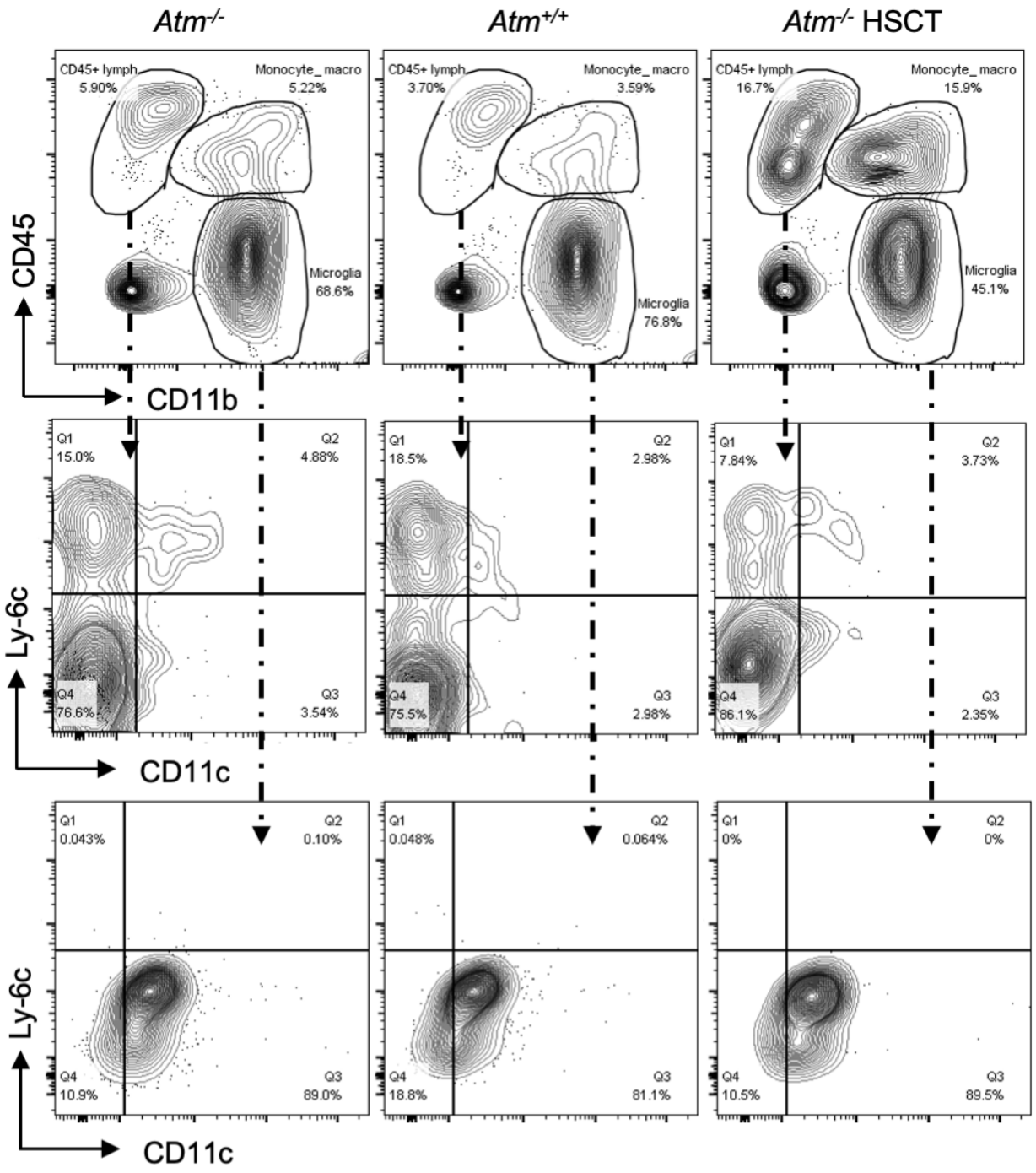


Figure S16. Pattern of expression of Ly-6c and CD11c in $CD45^{int}$ $CD11b^{+}$ microglia populations and $CD45^{hi}$ $CD11b^{-}$ lymphocyte populations were similar across control and chimeric animals.

Table S2

Conditioning reagents

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD117 (c-kit)	Biotin	2B8	Biolegend	105804
Saporin	Streptavidin	NA	In house	NA
CD4	NA	GK1.5	Bio X Cell	BE0003-1
CD8a	NA	YTS169.4	Bio X Cell	BE0117
CD20	NA	SA271G2	Biolegend	152104

Flow cytometry antibodies (blood, spleen or thymus)

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD45.2	APC-Cy7	104	BD	558702
CD45.1	PE	A20	BD	553776
CD3	V450	500A2	BD	560801
CD45R/B220	PE-Cy7	RA3-6B2	BD	552772
Gr-1 (Ly-6G/Ly-6C)	BV605	RB6-8C5	Biolegend	108440
CD4	Alexa Fluor 647	RM4-5	Biolegend	100530
CD8a	PE	53-6.7	BD	553032
NK1.1 (CD161)	PE-Cy5	PK136	Biolegend	108716

Flow cytometry antibodies (brain)

Antigen/reagent	Conjugate	Clone	Manufacturer	Catalog number
CD45	PE-Cy7	HI30	BD	560915
CD11b	APC-Cy7	M1/70	BD	557657
CD11c	BV421	N418	BD	565452
Ly-6C	PE	AL-21	BD	560592

Chapter 4

General Discussion

4.1 Summary of Results

Significant advances have been made in hematopoietic stem cell transplantation research and clinical practice in the nearly 65 years since it was first performed. To date, over a million HSCT procedures have been performed for indications ranging from hematologic malignancy and solid tumors to hemoglobinopathies and immune deficiency to inborn errors of metabolism and genomic instability syndromes. Despite its powerful therapeutic and curative potential, profound toxicities associated with standard conditioning regimens necessary for successful HSCT remain a significant hurdle to its more widespread application in non-malignant disease and autologous gene therapy. Progress has been made over the years in the development of regimens of reduced intensity and/or reduced toxicity. This has enabled the expansion of the patient population eligible to receive HSCT to include more vulnerable groups such as the elderly, who are more likely to be diagnosed with a hematological malignancy treatable with HSCT, and patients with DNA repair defects, e.g., Fanconi anemia, who are particularly susceptible to adverse effects attributable to the use of DNA damaging condition agents. However, these regimens still require the use of agents that are genotoxic, and despite their reduced intensity or non-myeloablative natures, the fact remains that short- and long-term risks associated with these regimens have been far from eliminated, which will have a substantial impact on the uptake of novel autologous LV-HSC gene therapies, as well as allogeneic HSCT for non-malignant diseases. Therefore, significant unmet need remains in the fields of both clinical HSCT and translational gene therapy. These hurdles must be surmounted so that these powerful therapies can become safe and realistic therapeutic options for many more patients who desperately need them.

Chapter two describes studies aimed at demonstrating the feasibility and efficacy of using a completely non-genotoxic, antibody-based conditioning regimen to accomplish successful LV-HSC gene therapy in a murine model of HA using a high-expression bioengineered fVIII transgene (ET3). In this model, a saporin-based immunotoxin targeting the CD117 (c-Kit) receptor on HSPCs was used to condition the bone marrow to engraft ET3 gene-modified congenic HSPCs, followed by establishment of donor-derived hematopoiesis and stable expression and secretion of ET3 from monocyte-lineage progeny. It is well-known that fVIII is a highly immunogenic protein. One of the key findings of this work was a demonstrated absolute requirement for immune suppression in this setting to enable engraftment of the gene-modified cells. Initial studies employed the use of mATG for immune suppression with mixed results. Although the majority of mice established and maintained high-level and sustained donor engraftment and circulating ET3 activity, a subset of mice failed to durably engraft gene-modified cells, with an even smaller subset developing fVIII inhibitors. Further investigation into the potential causes of this phenomenon when mATG was used revealed substantial binding of mATG antibodies to T cell populations, as expected, but also extensive binding to other mature leukocyte subsets, and perhaps more disconcertingly, to primitive HPC and HSC populations. The primary concern was whether binding of mATG antibodies to HSCs in the graft during transplantation could interfere with appropriate homing and engraftment, and indeed, additional studies showed when mATG was administered at the time of HSCT, engraftment was abrogated. Consistent with these observations, it has been demonstrated by another group that human-specific ATG can broadly bind leukocyte populations, as well as $CD34^+$ human HSPCs.[195] Similar to findings reported in chapter two, this group demonstrated impaired engraftment of human HSPCs in an NSG mouse model when human ATG was given at the time of HSCT.

As a result of these observations, a targeted non-genotoxic immune suppressive regimen was developed using a cocktail of T cell targeting monoclonal antibodies. This new regimen rectified the issues encountered using mATG, allowing for high-level donor engraftment of ET3 gene-modified cells and sustained ET3 hemostatic activity in 100% of gene therapy recipients in the absence of immunological rejection of the cells. Mice with stably engrafted ET3-modified cells did not develop anti-ET3 humoral responses and maintained durable, high-level ET3 expression. It is therefore posited that transient immune suppression during the early post-transplantation period allowed for the development of immunological tolerance to the ET3 neoantigen, although further studies will be necessary to elucidate the exact mechanism by which tolerance is established. Nevertheless, these preclinical proof-of-concept studies support the continued development of non-genotoxic antibody-based conditioning agents and regimens for use in autologous LV-HSC gene therapy applications for human translation.

Chapter three describes studies aimed at demonstrating the efficacy of using a similar non-genotoxic conditioning regimen in the setting of allogeneic HSCT for the chromosomal instability disorder ataxia telangiectasia. In these studies, the same CD117-saporin immunotoxin was used to condition AT mice and facilitate engraftment of donor cells, reconstituting a healthy ATM-competent immune system and restoring immune function. These observations represent the potential for significant improvements in the clinical management and quality of life of these patients. During these studies, however, it was determined that CD117-sap conditioning alone was not sufficient to prevent malignant transformation of long-lived endogenous ATM-deficient lymphocytes, which are not eliminated in the absence of genotoxic conditioning. Therefore, an

additional monoclonal antibody regimen was incorporated in order to deplete endogenous T and B lymphocytes, allowing complete immune reconstitution with ATM-competent cells following HSCT. Although high-level donor chimerism could be achieved with or without the inclusion of lymphocyte depleting antibodies, the inclusion of these antibodies allowed for the prevention of lymphoid malignancy in all recipients up to 1 year of age, whereas one mouse developed host-derived T cell lymphoma in the group conditioned with CD117-sap alone. In addition to high donor chimerism of ATM-competent cells, it was demonstrated that normal T cell numbers in the periphery were restored, as well as T cell function in immunization challenge assays, and IgA immunoglobulin levels. Additionally, although donor-derived microglia or microglia-like cells were not detected in the brain, donor-derived cells were found to migrate to the cerebellum and associate with endogenous microglia. The nature of this interaction will be the subject of future studies aimed at investigating whether HSCT under non-genotoxic conditioning has the potential to impart neuroprotection and ameliorate or slow neurodegenerative processes.

4.2 Implications of Findings

The observations reported herein support the hypothesis that non-genotoxic antibody-based conditioning agents and regimens can be used to safely and successfully implement autologous LV-HSC gene therapy or allogeneic HSCT. In addition to bone marrow conditioning using a robust and specific CD117-targeting immunotoxin, non-genotoxic regimens using naked monoclonal antibody cocktails can also be used to facilitate transient immune suppression where necessary in the setting of gene therapy, as well as other instances in which depletion of endogenous immune cells is advantageous for achieving optimal outcomes. These findings have the potential to propel the fields of HSCT and HSC-directed gene therapy forward dramatically. Non-genotoxic options

for pre-transplant conditioning would significantly expand accessibility of HSCT as a realistic therapeutic option in disease settings where HSCT is currently not routinely recommended (e.g., ataxia telangiectasia), would make novel LV-HSC gene therapies for non-malignant diseases significantly more attractive, and could even decrease the intensity and toxic burden of chemotherapy regimens in malignant disease settings, as the ability to deplete endogenous HSCs could be accomplished using immunotoxin, and therefore full chemotherapy-induced myeloablation may not be necessary in certain settings, such as multiple myeloma.

As discussed in chapter two, this work builds on previous work reported by other groups who have tested various CD117 and/or CD45 targeting antibodies and/or immunotoxins, all which have demonstrated promising results in both immune compromised and immune competent disease settings, as well as LV-HSC gene therapy. Collectively, these studies continue to lay a solid groundwork for catapulting these new non-genotoxic methodologies into clinical trials and towards commercialization. An anti-CD117 antibody, JSP191 developed by Jasper Therapeutics, Inc., has already entered human clinical trials for myelodysplastic syndrome/acute myeloid leukemia, severe combined immune deficiency, and most recently, Fanconi anemia. Additionally, a key component of the product portfolio of Magenta Therapeutics is MGTA-117, an anti-CD117 amanitin-based immunotoxin and the company's most advanced conditioning product candidate. Magenta is currently engaged in investigational new drug (IND)-enabling studies using MGTA-117 conditioning for multiple indications, including leukemia, lysosomal storage disorders, and hemoglobinopathies. With more ex vivo modified HSC gene therapies advancing into clinical testing, these non-genotoxic agents will prove critical to patient uptake and long-term market viability of LV-HSC gene therapy products. After nearly 65 years of clinical practice, these

biotechnological and pharmacological advances in next-generation HSCT conditioning agents represent the most dramatic and impactful leap forward for HSCT, offering the potential to create remarkable improvements in the safety profile of the procedure in both autologous and allogeneic settings.

4.3 Limitations and Future Directions

The findings reported herein provide proof-of-concept data for the use of antibody-based regimens to support successful autologous LV-HSC gene therapy and traditional allogeneic HSCT without the use of genotoxic agents, essentially eliminating the attendant toxicities and adverse effects associated with these drugs. However, these experiments were performed exclusively in mice, using mouse-specific reagents, and translation of this approach to humans will require extensive studies to identify the most efficacious human-specific reagents that will produce similar results in the human HSCT setting. As discussed in depth in chapter two, there are many options that can be explored for both the immunotoxin and the immune suppressive/immune cell depleting components of these protocols. Some of these candidate reagents are currently only available for research use and would require the development of clinical grade versions produced under current good manufacturing practices (cGMP) to be used in human studies. On the other hand, certain drugs that could be exploited for immune suppression/immune cell depletion are FDA-approved biologics that are currently used in clinical practice (e.g., ATG, alemtuzumab, abatacept). The availability of these options could simplify and accelerate clinical testing and translation. Nevertheless, comprehensive studies will need to be performed to determine potency of candidate reagents against human cells for HSC and immune cell depletion, as well as optimization of dosage and timing of drug delivery.

Subsequent preclinical or IND-enabling studies will need to be carried out in larger mammals, such as non-human primates (NHP) using reagents that are cross-reactive between NHPs and humans. Robust results have been reported using various antibody-based conditioning regimens in mice by several groups, however limited data are available on whether these observations in mice will translate to successful outcomes in NHPs and humans. In the context of LV-HSC gene therapy, available data using antibody-based conditioning is particularly scarce. When MGTA-117 was applied as single agent conditioning to facilitate engraftment of autologous β-globin gene-modified cells in NHPs, Magenta reported maximum vector copy number of only 0.05 in peripheral blood. Whether this level of engraftment of gene-modified cells is sufficient to elicit phenotypic correction in the setting of hemoglobinopathy is yet to be shown. It is well-established that mouse studies can be poorly predictive of outcomes in humans, and even large animal studies may not provide an accurate picture of what will be observed in human trials. However, these studies remain essential to the clinical translational process and must be meticulously performed to demonstrate safety, feasibility and efficacy of these novel regimens before advancing into human testing.

The scope of the LV-HSC gene therapy experiments described in chapter two was limited to demonstrating proof-of-concept that a non-genotoxic antibody-based conditioning approach could be employed for HA gene therapy. Mice that maintained stable engraftment of gene-modified cells and durable fVIII activity after HSCT using antibody-based conditioning did not develop humoral anti-fVIII responses, suggesting immune tolerance was induced in these mice by gene therapy. These studies were not designed to investigate the mechanisms by which immune tolerance were achieved in our system, although potential mechanisms include central tolerance involving

elimination of fVIII-reactive lymphocytes or peripheral induction of fVIII-specific T regulatory cells mediated by donor-derived fVIII-expressing antigen presenting cells. Future studies will investigate the mechanistic underpinnings of tolerance induction following ex vivo LV-HSC gene therapy for HA.

The studies described in chapter three were limited to demonstrating feasibility and efficacy of HSCT under antibody-based conditioning in AT, and whether this approach could restore immune functionality and prevent development of leukemia/lymphoma due to immune reconstitution with ATM-competent donor cells. Of considerable interest is whether bone marrow derived cells that traffic to the brain can be neuroprotective, either by reducing neuroinflammation or replacing damaged glial cells, as has been demonstrated in other models of neurodegeneration or neuropathology.[262-266] The studies described in chapter three confirmed that donor-derived bone marrow cells can home to the brain in AT mice, however the implications of these observations remain unclear. These initial studies provide the basis for further in-depth studies to examine what, if any, effects these migrating cells have on brain pathology. Additionally, follow-up studies are currently underway in which fetal liver-derived cells were used as source material for HSCT in AT mice. The intent of these studies is to determine if these more primitive HSC populations have increased potential for brain homing and engraftment, as well as protective and/or regenerative functions in the AT brain. Although available AT mouse models closely recapitulate the immune dysfunction phenotype of AT disease, the majority of these models do not well model the neurodegenerative manifestations observed in human AT patients, which is a notable limitation of these studies.[228] At least one study has reported detectable perturbation in Purkinje cell/glial cell architecture in AT mice upon close microscopic evaluation,[241] however differences observed in

behavioral studies or in cerebellar atrophy by magnetic resonance imaging, if detected, are often subtle, and there is a high degree of variability in findings reported in the current literature. A newer rat model of AT was described in 2017 that displays some neurological impairments, namely spinal cord atrophy and paralysis, which are reminiscent of outcomes seen in mild AT or older AT patients.[267] This new model may be more suitable for the study of AT neurodegeneration and could be explored for future projects.

Though other studies employing the same mouse model used in the studies described in chapter three have reported near universal development of malignant thymic lymphoma and death before 30 weeks of age in AT mice,[240, 241] in our hands there was large variability in the incidence of lymphoma development as well as age of death in *Atm* knockout mice. Naïve homozygous mutant mice in our colony died anywhere between 19 weeks and 66 weeks of age. Cause of death was not determined in every case, but necropsy in some mice revealed absence of advanced thymic malignancy. In our studies using CD117-sap conditioning paired with T and B lymphocyte depleting antibodies, we observed lack of development of lymphoid malignancy in all mice receiving HSCT up to 1 year of age in a relatively small cohort (n = 11). Additional long-term follow up on these mice and survival tracking compared with untreated controls, and repeating these studies on larger cohorts, will be needed to confirm the effect of HSCT under antibody-based conditioning on malignancy prevention and overall survival in this model.

An important future direction for this work on non-genotoxic antibody-based conditioning for HSCT will be to identify novel surface proteins on HSCs that can be explored for antibody targeting. CD117 expression is predominantly found on HSPCs, and although CD117 can be found

on cells and tissues outside the hematopoietic compartment, our findings demonstrated that these tissues were not negatively impacted by CD117-targeted immunotoxin. Nonetheless, our findings suggest that successful HSCT can be accomplished when only ST- and LT-HSC populations are depleted in bone marrow during conditioning, and therefore identifying highly specific targets that could limit cytotoxicity to the cell types of interest and reduce the possibility of off-target effects would be an ideal approach. Our group identified and began preliminary testing on an HSC target, the thrombopoietin receptor CD110 (MPL). CD110 is an important signal for HSC maintenance, proliferation and megakaryocyte differentiation, and is enriched on a subset of HSCs capable of long-term multilineage repopulation.[268] In addition to CD110, many other potential HSC targets, such as CD34, CD90 (Thy-1), or CD133 (PROM1) could be explored for immunotoxin development.

Finally, several characteristics of the immunotoxin can be modified for optimal efficacy. Different payloads can be tested to ensure the safest and most effective drugs or toxins are used. The antibody-payload linker chemistry can be optimized to ensure the most efficient intracellular payload release. Strategies to improve endosomal escape can be examined to maximize efficiency of cytoplasmic entry of the payload. The antibody can be modified to fine tune half-life and time in circulation as needed. Alternatively, novel components, such as cell-internalizing aptamers, could be considered in the search for targeting moieties with increased specificity and optimal drug delivery to HSCs.[269-272]

4.4 Conclusions

In summary, a body of work has been presented herein in support of the further exploration of next generation, non-genotoxic antibody-based conditioning regimens for use in HSCT applications. We described the successful implementation of a CD117-saporin immunotoxin along with transient antibody-based immune suppression to accomplish HSCT conditioning prior to ex vivo lentivirus HSC-directed gene therapy for hemophilia A. This regimen, or a customized iteration of it, could likely be applied to many other LV-HSC gene therapies, involving both secreted and non-secreted transgenic proteins, depending on the immunogenicity of the neoantigen that is expressed. We also described the successful use of this CD117-saporin immunotoxin along with antibody-based immune depletion of ATM-deficient lymphocytes to accomplish allogeneic HSCT, after which immune deficiencies were corrected and immune function restored in ataxia telangiectasia. The observations reported here provide a strong case that a similar regimen could also be applied for allogeneic HSCT in the setting of other chromosomal instability disorders such as Fanconi anemia, AT-like syndrome, or Nijmegen breakage syndrome to ameliorate immune manifestations. This regimen would also likely be useful to facilitate allogeneic HSCT for other diseases characterized by or associated with immune deficiency, as well as other non-malignant genetic diseases. The work presented in this dissertation, along with complementary work being performed by other groups across the globe (e.g., Stanford University, Harvard University, Washington University, Jikei University and others) and private industry (i.e., Magenta Therapeutics, Jasper Therapeutics), represent the dawn of a new era of possibilities for the fields of hematopoietic stem cell transplantation and ex vivo lentivirus gene therapy.

References

1. Jacobson, LO, Marks, EK, Gaston, EO, Robson, M, and Zirkle, RE (1949). The Role of the Spleen in Radiation Injury. *Proceedings of the Society for Experimental Biology and Medicine* **70**: 740-742.
2. Lorenz, E, Uphoff, D, Reid, TR, and Shelton, E (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* **12**: 197-201.
3. Loutit, JF (1954). Protection against ionizing radiation: The "recovery factor" in spleen and bone-marrow. *Journal of Nuclear Energy (1954)* **1**: 87-91.
4. Main, JM, and Prehn, RT (1955). Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J Natl Cancer Inst* **15**: 1023-1029.
5. Epstein, RB, Storb, R, Ragde, H, and Thomas, ED (1968). Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation* **6**: 45-58.
6. Gatti, RA, Meuwissen, HJ, Allen, HD, Hong, R, and Good, RA (1968). Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* **2**: 1366-1369.
7. Bach, FH, Albertini, RJ, Joo, P, Anderson, JL, and Bortin, MM (1968). Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet* **2**: 1364-1366.
8. De Koning, J, Van Bekkum, DW, Dicke, KA, Dooren, LJ, Rádl, J, and Van Rood, JJ (1969). Transplantation of bone-marrow cells and fetal thymus in an infant with lymphopenic immunological deficiency. *Lancet* **1**: 1223-1227.
9. Granot, N, and Storb, R (2020). History of hematopoietic cell transplantation: challenges and progress. *Haematologica* **105**: 2716-2729.
10. Balassa, K, Danby, R, and Rocha, V (2019). Haematopoietic stem cell transplants: principles and indications. *Br J Hosp Med (Lond)* **80**: 33-39.
11. Bazinet, A, and Popradi, G (2019). A general practitioner's guide to hematopoietic stem-cell transplantation. *Current oncology (Toronto, Ont)* **26**: 187-191.
12. Morrison, SJ, and Scadden, DT (2014). The bone marrow niche for haematopoietic stem cells. *Nature* **505**: 327-334.
13. Suárez-Álvarez, B, López-Vázquez, A, and López-Larrea, C (2012). Mobilization and homing of hematopoietic stem cells. *Adv Exp Med Biol* **741**: 152-170.
14. Chabannon, C, Kuball, J, Bondanza, A, Dazzi, F, Pedrazzoli, P, Toubert, A, Ruggeri, A, Fleischhauer, K, and Bonini, C (2018). Hematopoietic stem cell transplantation in its 60s: A platform for cellular therapies. *Sci Transl Med* **10**.
15. Gyurkocza, B, and Sandmaier, BM (2014). Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood* **124**: 344-353.
16. Blaise, D, and Castagna, L (2012). Do different conditioning regimens really make a difference? *Hematology* **2012**: 237-245.
17. Bacigalupo, A, Ballen, K, Rizzo, D, Giralt, S, Lazarus, H, Ho, V, Apperley, J, Slavin, S, Pasquini, M, Sandmaier, BM*, et al.* (2009). Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant* **15**: 1628-1633.
18. Champlin, R (2003). Selection of Autologous or Allogeneic Transplantation. In: Kufe DW, PR, Weichselbaum RR, et al. (ed). *Holland-Frei Cancer Medicine*, 6 edn. BC Decker: Hamilton, ON.
19. Copelan, EA (2006). Hematopoietic stem-cell transplantation. *N Engl J Med* **354**: 1813-1826.

20. Yeşilipek, MA (2014). Hematopoetic stem cell transplantation in children. *Turk Pediatri Ars* **49**: 91-98.
21. Giralt, S, and Bishop, MR (2009). Principles and overview of allogeneic hematopoietic stem cell transplantation. *Cancer Treat Res* **144**: 1-21.
22. Gluckman, E, Broxmeyer, HA, Auerbach, AD, Friedman, HS, Douglas, GW, Devergie, A, Esperou, H, Thierry, D, Socie, G, Lehn, P*, et al.* (1989). Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* **321**: 1174-1178.
23. Mehta, RS, Rezvani, K, and Shpall, EJ (2019). Cord Blood Expansion: A Clinical Advance. *Journal of Clinical Oncology* **37**: 363-366.
24. Pineault, N, and Abu-Khader, A (2015). Advances in umbilical cord blood stem cell expansion and clinical translation. *Exp Hematol* **43**: 498-513.
25. Cohen, S, Roy, J, Lachance, S, Delisle, JS, Marinier, A, Busque, L, Roy, DC, Barabé, F, Ahmad, I, Bambace, N*, et al.* (2020). Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1-2 safety and feasibility study. *Lancet Haematol* **7**: e134-e145.
26. Stefanski, H, Brunstein, CG, McKenna, DH, Sumstad, D, Miller, JS, Blazar, BR, DeFor, TE, Boitano, AE, Cooke, MP, Raffel, GD*, et al.* (2019). Mgta-456, an Aryl Hydrocarbon Receptor (AHR) Antagonist Based Expansion of CD34+ Hematopoietic Stem Cells (HSC), Permits Selection of Better HLA Matched Cord Blood Units (CBUs) and Promotes Faster Neutrophil Recovery and Uniform Engraftment with Potentially Less Acute Graft-Vs-Host Disease (GVHD). *Blood* **134**: 804-804.
27. Sabloff, M, Chandy, M, Wang, Z, Logan, BR, Ghavamzadeh, A, Li, CK, Irfan, SM, Bredeson, CN, Cowan, MJ, Gale, RP*, et al.* (2011). HLA-matched sibling bone marrow transplantation for β-thalassemia major. *Blood* **117**: 1745-1750.
28. Bernaudin, F, Socie, G, Kuentz, M, Chevret, S, Duval, M, Bertrand, Y, Vannier, J-P, Yakouben, K, Thuret, I, Bordigoni, P*, et al.* (2007). Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. *Blood* **110**: 2749-2756.
29. Locatelli, F, Kabbara, N, Ruggeri, A, Ghavamzadeh, A, Roberts, I, Li, CK, Bernaudin, F, Vermylen, C, Dalle, JH, Stein, J*, et al.* (2013). Outcome of patients with hemoglobinopathies given either cord blood or bone marrow transplantation from an HLA-identical sibling. *Blood* **122**: 1072-1078.
30. Soncini, E, Slatter, MA, Jones, LBKR, Hughes, S, Hodges, S, Flood, TJ, Barge, D, Spickett, GP, Jackson, GH, Collin, MP*, et al.* (2009). Unrelated donor and HLA-identical sibling haematopoietic stem cell transplantation cure chronic granulomatous disease with good long-term outcome and growth. *British Journal of Haematology* **145**: 73-83.
31. Czechowicz, A, Kraft, D, Weissman, IL, and Bhattacharya, D (2007). Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science* **318**: 1296-1299.
32. Derderian, SC, Togarrati, PP, King, C, Moradi, PW, Reynaud, D, Czechowicz, A, Weissman, IL, and MacKenzie, TC (2014). In utero depletion of fetal hematopoietic stem cells improves engraftment after neonatal transplantation in mice. *Blood* **124**: 973-980.
33. Xue, X, Pech, NK, Shelley, WC, Srour, EF, Yoder, MC, and Dinauer, MC (2010). Antibody targeting KIT as pretransplantation conditioning in immunocompetent mice. *Blood* **116**: 5419-5422.

34. Chhabra, A, Ring, AM, Weiskopf, K, Schnorr, PJ, Gordon, S, Le, AC, Kwon, HS, Ring, NG, Volkmer, J, Ho, PY*, et al.* (2016). Hematopoietic stem cell transplantation in immunocompetent hosts without radiation or chemotherapy. *Sci Transl Med* **8**: 351ra105.
35. George, BM, Kao, KS, Kwon, HS, Velasco, BJ, Poyser, J, Chen, A, Le, AC, Chhabra, A, Burnett, CE, Cajuste, D*, et al.* (2019). Antibody Conditioning Enables MHC-Mismatched Hematopoietic Stem Cell Transplants and Organ Graft Tolerance. *Cell Stem Cell* **25**: 185-192 e183.
36. Beck, A, Goetsch, L, Dumontet, C, and Corvaïa, N (2017). Strategies and challenges for the next generation of antibody–drug conjugates. *Nature Reviews Drug Discovery* **16**: 315-337.
37. Pasqualucci, L, Flenghi, L, Terenzi, A, Bolognesi, A, Stirpe, F, Bigerna, B, and Falini, B (1995). Immunotoxin therapy of hematological malignancies. *Haematologica* **80**: 546-556.
38. Lanieri, L, Lamothe, TL, Miske, O, McDonough, SM, Sarma, GN, Bhattarai, P, Latimer, K, Dushime, J, Jain, N, Palchaudhuri, R*, et al.* (2020). A Single Dose of a Novel Anti-Human CD117-Amanitin Antibody Drug Conjugate (ADC) Engineered for a Short Half-Life Provides Dual Conditioning and Anti-Leukemic Activity and Extends Survival Compared to Standard of Care in Multiple Preclinical Models of Acute Myeloid Leukemia (AML). *Blood* **136**: 47-48.
39. Khongorzul, P, Ling, CJ, Khan, FU, Ihsan, AU, and Zhang, J (2020). Antibody–Drug Conjugates: A Comprehensive Review. *Molecular Cancer Research* **18**: 3.
40. De Cecco, M, Galbraith, DN, and McDermott, LL (2021). What makes a good antibody-drug conjugate? *Expert Opin Biol Ther*: 1-7.
41. Russkamp, NF, Myburgh, R, Kiefer, JD, Neri, D, and Manz, MG (2021). Anti-CD117 immunotherapy to eliminate hematopoietic and leukemia stem cells. *Experimental Hematology* **95**: 31-45.
42. Sahoo, RK, and Kumar, L (2016). Chemo-radiotherapy free conditioning regimen: immunotoxin at its magical best. *Translational Cancer Research*: S438-S443.
43. Abadir, E, Bryant, C, Larsen, S, and Clark, GJ (2019). Targeting the niche: depleting haemopoietic stem cells with targeted therapy. *Bone Marrow Transplantation* **54**: 961-968.
44. Ali, AM, Dehdashti, F, DiPersio, JF, and Cashen, AF (2016). Radioimmunotherapy-based conditioning for hematopoietic stem cell transplantation: Another step forward. *Blood Rev* **30**: 389-399.
45. Lum, SH, Hoenig, M, Gennery, AR, and Slatter, MA (2019). Conditioning Regimens for Hematopoietic Cell Transplantation in Primary Immunodeficiency. *Curr Allergy Asthma Rep* **19**: 52-52.
46. Palchaudhuri, R, Saez, B, Hoggatt, J, Schajnovitz, A, Sykes, DB, Tate, TA, Czechowicz, A, Kfoury, Y, Ruchika, F, Rossi, DJ*, et al.* (2016). Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. *Nat Biotechnol* **34**: 738-745.
47. Li, Z, Czechowicz, A, Scheck, A, Rossi, DJ, and Murphy, PM (2019). Hematopoietic chimerism and donor-specific skin allograft tolerance after non-genotoxic CD117 antibody-drug-conjugate conditioning in MHC-mismatched allotransplantation. *Nat Commun* **10**: 616.

48. Gao, C, Schroeder, JA, Xue, F, Jing, W, Cai, Y, Scheck, A, Subramaniam, S, Rao, S, Weiler, H, Czechowicz, A*, et al.* (2019). Nongenotoxic antibody-drug conjugate conditioning enables safe and effective platelet gene therapy of hemophilia A mice. *Blood Adv* **3**: 2700-2711.
49. Castiello, MC, Bosticardo, M, Sacchetti, N, Calzoni, E, Fontana, E, Yamazaki, Y, Draghici, E, Corsino, C, Bortolomai, I, Sereni, L*, et al.* (2020). Efficacy and safety of anti-CD45-saporin as conditioning agent for RAG deficiency. *J Allergy Clin Immunol*.
50. Srikanthan, MA, Humbert, O, Haworth, KG, Ironside, C, Rajawat, YS, Blazar, BR, Palchaudhuri, R, Boitano, AE, Cooke, MP, Scadden, DT*, et al.* (2020). Effective Multi-lineage Engraftment in a Mouse Model of Fanconi Anemia Using Non-genotoxic Antibody-Based Conditioning. *Mol Ther Methods Clin Dev* **17**: 455-464.
51. Chandrakasan, S, Jayavaradhan, R, Ernst, J, Shrestha, A, Loberg, A, Dexheimer, P, Jordan, M, Pang, Q, Aronow, B, and Malik, P (2017). KIT blockade is sufficient for donor hematopoietic stem cell engraftment in Fanconi anemia mice. *Blood* **129**: 1048-1052.
52. Palchaudhuri, R, Hyzy, SL, Proctor, JL, Adams, HL, Pearse, BR, Sarma, G, Aslanian, S, Gillard, G, Lamothe, TL, Burenkova, O*, et al.* (2018). Antibody Drug Conjugates Targeted to CD45 or CD117 Enable Allogeneic Hematopoietic Stem Cell Transplantation in Animal Models. *Blood* **132**: 3324-3324.
53. Proctor, JL, Hyzy, SL, Adams, HL, Brooks, ML, Gabros, AD, McDonough, SM, Kien, L, Aslanian, S, Pearse, BR, Palchaudhuri, R*, et al.* (2018). Single Doses of Antibody Drug Conjugates (ADCs) Targeted to CD117 or CD45 Have Potent In Vivo Anti-Leukemia Activity and Survival Benefit in Patient Derived AML Models. *Blood* **132**: 3316-3316.
54. Persaud, SP, Cooper, ML, Rettig, MP, and DiPersio, JF (2018). Conditioning for Hematopoietic Stem Cell Transplantation Using Antibody-Drug Conjugate Targeting CD45 Permits Engraftment across Immunologic Barriers. *Blood* **132**: 2035-2035.
55. Palchaudhuri, R, Pearse, BR, Proctor, JL, Hyzy, SL, Aslanian, S, McDonough, SM, Sarma, G, Brooks, ML, Bhat, A, Ladwig, D*, et al.* (2018). Targeting CD45 with an Amanitin Antibody-Drug Conjugate Effectively Depletes Human HSCs and Immune Cells for Transplant Conditioning. *Blood* **132**: 4526-4526.
56. Palchaudhuri, R, Pearse, BR, Proctor, JL, Hyzy, SL, Aslanian, S, Bhat, A, Gillard, GO, McDonough, SM, Sarma, GN, Ladwig, D*, et al.* (2019). Non-Genotoxic Conditioning Using Amanitin Antibody-Drug Conjugates Targeting CD45 Effectively Deplete Human and Non-Human Primate Hematopoietic Stem Cells and Immune Cells. *Biology of Blood and Marrow Transplantation* **25**: S32.
57. Pearse, BR, McDonough, SM, Proctor, JL, Panwar, R, Sarma, G, McShea, MA, Kien, L, Dushime, J, Adams, HL, Hyzy, SL*, et al.* (2018). CD117-Amanitin Antibody Drug Conjugates Effectively Deplete Human and Non-Human Primate HSCs: Proof of Concept As a Targeted Strategy for Conditioning Patients for Bone Marrow Transplant. *Blood* **132**: 3314-3314.
58. Tisdale, JF, Donahue, RE, Uchida, N, Pearse, BR, McDonough, SM, Proctor, JL, Krouse, A, Linde, NS, Bonifacino, AC, Panwar, R*, et al.* (2019). A Single Dose of CD117 Antibody Drug Conjugate Enables Autologous Gene-Modified Hematopoietic Stem Cell Transplant (Gene Therapy) in Nonhuman Primates. *Blood* **134**: 610-610.
59. Persaud, SP, Cooper, ML, Ritchey, JK, Rettig, MP, and DiPersio, JF (2019). CD45-ADC Plus Janus Kinase (JAK) Inhibitors As Conditioning for MHC-Mismatched Murine

Hematopoietic Stem Cell Transplantation Is Associated with Minimal Toxicity and Graft Versus Host Disease. *Blood* **134**: 3200-3200.

60. Persaud, SP, Ritchey, JK, Choi, J, Ruminski, PG, Cooper, ML, Rettig, MP, and DiPersio, JF (2020). Antibody-drug conjugates targeting CD45 plus Janus kinase inhibitors effectively condition for allogeneic hematopoietic stem cell transplantation. *bioRxiv*: 2020.2010.2002.324475.
61. Persaud, SP, Cooper, ML, Ritchey, JK, Rettig, MP, and DiPersio, JF (2020). Antibody-Drug Conjugates Targeting CD45 Plus Janus Kinase (JAK) Inhibitors As Conditioning for Allogeneic Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation* **26**: S150-S151.
62. Tiercy, J-M (2016). How to select the best available related or unrelated donor of hematopoietic stem cells? *Haematologica* **101**: 680-687.
63. Morgan, RA, Gray, D, Lomova, A, and Kohn, DB (2017). Hematopoietic Stem Cell Gene Therapy: Progress and Lessons Learned. *Cell Stem Cell* **21**: 574-590.
64. Aiuti, A, Roncarolo, MG, and Naldini, L (2017). Gene therapy for ADA-SCID, the first marketing approval of an ex vivo gene therapy in Europe: paving the road for the next generation of advanced therapy medicinal products. *EMBO Mol Med* **9**: 737-740.
65. Cavazzana, M, Six, E, Lagresle-Peyrou, C, André-Schmutz, I, and Hacein-Bey-Abina, S (2016). Gene Therapy for X-Linked Severe Combined Immunodeficiency: Where Do We Stand? *Hum Gene Ther* **27**: 108-116.
66. Naldini, L, Blömer, U, Gallay, P, Ory, D, Mulligan, R, Gage, FH, Verma, IM, and Trono, D (1996). In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. *Science* **272**: 263.
67. Naldini, L, Trono, D, and Verma, IM (2016). Lentiviral vectors, two decades later. *Science* **353**: 1101.
68. Dull, T, Zufferey, R, Kelly, M, Mandel, RJ, Nguyen, M, Trono, D, and Naldini, L (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**: 8463-8471.
69. Tucci, F, Scaramuzza, S, Aiuti, A, and Mortellaro, A (2021). Update on Clinical Ex Vivo Hematopoietic Stem Cell Gene Therapy for Inherited Monogenic Diseases. *Molecular Therapy* **29**: 489-504.
70. Staal, FJT, Aiuti, A, and Cavazzana, M (2019). Autologous Stem-Cell-Based Gene Therapy for Inherited Disorders: State of the Art and Perspectives. *Front Pediatr* **7**.
71. Biffi, A, Bartolomae, CC, Cesana, D, Cartier, N, Aubourg, P, Ranzani, M, Cesani, M, Benedicenti, F, Plati, T, Rubagotti, E*, et al.* (2011). Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood* **117**: 5332-5339.
72. (2021). bluebird bio Announces Temporary Suspension on Phase 1/2 and Phase 3 Studies of LentiGlobin Gene Therapy for Sickle Cell Disease (bb1111) https://investor.bluebirdbio.com/news-releases/news-release-details/bluebird-bio-announces-temporary-suspension-phase-12-and-phase-3.
73. (2021). bluebird bio Provides Updated Findings from Reported Case of Acute Myeloid Leukemia (AML) in LentiGlobin for Sickle Cell Disease (SCD) Gene Therapy Program.
74. Naldini, L (2019). Genetic engineering of hematopoiesis: current stage of clinical translation and future perspectives. *EMBO Molecular Medicine* **11**: e9958.
75. Salen, P, and Babiker, HM (2021). Hemophilia A. *StatPearls*: Treasure Island (FL).

76. Zanon, E, and Pasca, S (2019). Intracranial haemorrhage in children and adults with haemophilia A and B: a literature review of the last 20 years. *Blood Transfus* **17**: 378-384.
77. Mehta, P, and Reddivari, AKR (2021). Hemophilia. *StatPearls*: Treasure Island (FL).
78. Mazurkiewicz-Pisarek, A, Plucienniczak, G, Ciach, T, and Plucienniczak, A (2016). The factor VIII protein and its function. *Acta Biochim Pol* **63**: 11-16.
79. Tantawy, AAG (2010). Molecular genetics of hemophilia A: Clinical perspectives. *Egyptian Journal of Medical Human Genetics* **11**: 105-114.
80. Gale, AJ (2011). Continuing education course #2: current understanding of hemostasis. *Toxicol Pathol* **39**: 273-280.
81. Fay, PJ (2006). Factor VIII structure and function. *Int J Hematol* **83**: 103-108.
82. Saenko, EL, Shima, M, and Sarafanov, AG (1999). Role of activation of the coagulation factor VIII in interaction with vWf, phospholipid, and functioning within the factor Xase complex. *Trends Cardiovasc Med* **9**: 185-192.
83. Moreira, A, and Das, H (2018). Acute Life-Threatening Hemorrhage in Neonates With Severe Hemophilia A: A Report of 3 Cases. *J Investig Med High Impact Case Rep* **6**: 2324709618800349.
84. Mannucci, PM (2020). Hemophilia therapy: the future has begun. *Haematologica* **105**: 545-553.
85. Thorat, T, Neumann, PJ, and Chambers, JD (2018). Hemophilia Burden of Disease: A Systematic Review of the Cost-Utility Literature for Hemophilia. *Journal of Managed Care & Specialty Pharmacy* **24**: 632-642.
86. Iorio, A, Stonebraker, JS, Brooker, M, and Soucie, JM (2017). Measuring the quality of haemophilia care across different settings: a set of performance indicators derived from demographics data. *Haemophilia* **23**: e1-e7.
87. Seth Chhabra, E, Liu, T, Kulman, J, Patarroyo-White, S, Yang, B, Lu, Q, Drager, D, Moore, N, Liu, J, Holthaus, AM*, et al.* (2020). BIVV001, a new class of factor VIII replacement for hemophilia A that is independent of von Willebrand factor in primates and mice. *Blood* **135**: 1484-1496.
88. Rayes, J, and Lacroix-Desmazes, S (2020). A molecular jewel for hemophilia A treatment. *Blood* **135**: 1417-1419.
89. Weyand, AC, and Pipe, SW (2019). New therapies for hemophilia. *Blood* **133**: 389-398.
90. Meeks, SL, and Batsuli, G (2016). Hemophilia and inhibitors: current treatment options and potential new therapeutic approaches. *Hematology Am Soc Hematol Educ Program* **2016**: 657-662.
91. Schep, SJ, Schutgens, REG, Fischer, K, and Boes, ML (2018). Review of immune tolerance induction in hemophilia A. *Blood Rev* **32**: 326-338.
92. Merlin, S, and Follenzi, A (2020). Escape or Fight: Inhibitors in Hemophilia A. *Frontiers in Immunology* **11**.
93. Shapiro, AD, Mitchell, IS, and Nasr, S (2018). The future of bypassing agents for hemophilia with inhibitors in the era of novel agents. *J Thromb Haemost* **16**: 2362-2374.
94. Gringeri, A, Mantovani, LG, Scalone, L, and Mannucci, PM (2003). Cost of care and quality of life for patients with hemophilia complicated by inhibitors: the COCIS Study Group. *Blood* **102**: 2358-2363.

95. Kizilocak, H, Marquez-Casas, E, Phei Wee, C, Malvar, J, Carmona, R, and Young, G (2021). Comparison of bypassing agents in patients on emicizumab using global hemostasis assays. *Haemophilia* **27**: 164-172.
96. Shapiro, AD, Mitchell, IS, and Nasr, S (2018). The future of bypassing agents for hemophilia with inhibitors in the era of novel agents. *Journal of Thrombosis and Haemostasis* **16**: 2362-2374.
97. Samelson-Jones, BJ, and Arruda, VR (2019). Protein-Engineered Coagulation Factors for Hemophilia Gene Therapy. *Molecular Therapy - Methods & Clinical Development* **12**: 184-201.
98. Nathwani, AC, Tuddenham, EGD, Rangarajan, S, Rosales, C, McIntosh, J, Linch, DC, Chowdary, P, Riddell, A, Pie, AJ, Harrington, C*, et al.* (2011). Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B. *New England Journal of Medicine* **365**: 2357-2365.
99. Batty, P, and Lillicrap, D (2021). Hemophilia Gene Therapy: Approaching the First Licensed Product. *Hemasphere* **5**: e540.
100. Sheridan, C (2020). A reprieve from hemophilia A, but for how long? *Nat Biotechnol* **38**: 1107-1109.
101. Pierce, GF (2020). Gene Therapy for Hemophilia: Are Expectations Matching Reality? *Mol Ther* **28**: 2097-2098.
102. Nguyen, GN, Everett, JK, Kafle, S, Roche, AM, Raymond, HE, Leiby, J, Wood, C, Assenmacher, CA, Merricks, EP, Long, CT*, et al.* (2021). A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat Biotechnol* **39**: 47-55.
103. Wilson, JM, and Flotte, TR (2020). Moving Forward After Two Deaths in a Gene Therapy Trial of Myotubular Myopathy. *Hum Gene Ther* **31**: 695-696.
104. Shieh, PB, Bönnemann, CG, Müller-Felber, W, Blaschek, A, Dowling, JJ, Kuntz, NL, and Seferian, AM (2020). Re: "Moving Forward After Two Deaths in a Gene Therapy Trial of Myotubular Myopathy" by Wilson and Flotte. *Hum Gene Ther* **31**: 787.
105. Zakas, PM, Spencer, HT, and Doering, CB (2011). Engineered Hematopoietic Stem Cells as Therapeutics for Hemophilia A. *J Genet Syndr Gene Ther* **1**.
106. Chang, LJ (2010). Lentiviral vector transduction of dendritic cells for novel vaccine strategies. *Methods Mol Biol* **614**: 161-171.
107. Rothblum-Oviatt, C, Wright, J, Lefton-Greif, MA, McGrath-Morrow, SA, Crawford, TO, and Lederman, HM (2016). Ataxia telangiectasia: a review. *Orphanet J Rare Dis* **11**: 159.
108. Alyasin, S, Esmaeilzadeh, H, Ebrahimi, N, Nabavizadeh, SH, and Nemati, H (2019). Clinical Presentation of Ataxia-Telangiectasia. *Arch Iran Med* **22**: 682-686.
109. Frappart, PO, and McKinnon, PJ (2006). Ataxia-telangiectasia and related diseases. *Neuromolecular Med* **8**: 495-511.
110. McKinnon, PJ (2012). ATM and the molecular pathogenesis of ataxia telangiectasia. *Annu Rev Pathol* **7**: 303-321.
111. Teive, HA, Moro, A, Moscovich, M, Arruda, WO, Munhoz, RP, Raskin, S, and Ashizawa, T (2015). Ataxia-telangiectasia - A historical review and a proposal for a new designation: ATM syndrome. *J Neurol Sci* **355**: 3-6.
112. Hoche, F, Seidel, K, Theis, M, Vlaho, S, Schubert, R, Zielen, S, and Kieslich, M (2012). Neurodegeneration in ataxia telangiectasia: what is new? What is evident? *Neuropediatrics* **43**: 119-129.

113. Kastan, MB, and Lim, D-s (2000). The many substrates and functions of ATM. *Nature Reviews Molecular Cell Biology* **1**: 179-186.
114. Shiloh, Y, and Ziv, Y (2013). The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* **14**: 197-210.
115. Matsuoka, S, Ballif, BA, Smogorzewska, A, McDonald, ER, Hurov, KE, Luo, J, Bakalarski, CE, Zhao, Z, Solimini, N, Lerenthal, Y*, et al.* (2007). ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. *Science* **316**: 1160-1166.
116. Taylor, AMR, Rothblum-Oviatt, C, Ellis, NA, Hickson, ID, Meyer, S, Crawford, TO, Smogorzewska, A, Pietrucha, B, Weemaes, C, and Stewart, GS (2019). Chromosome instability syndromes. *Nature Reviews Disease Primers* **5**: 64.
117. Yang, C, Tang, X, Guo, X, Niikura, Y, Kitagawa, K, Cui, K, Wong, ST, Fu, L, and Xu, B (2011). Aurora-B mediated ATM serine 1403 phosphorylation is required for mitotic ATM activation and the spindle checkpoint. *Mol Cell* **44**: 597-608.
118. Rhodes, N, D'Souza, T, Foster, CD, Ziv, Y, Kirsch, DG, Shiloh, Y, Kastan, MB, Reinhart, PH, and Gilmer, TM (1998). Defective potassium currents in ataxia telangiectasia fibroblasts. *Genes & Development* **12**: 3686-3692.
119. Famulski, KS, Al-Hijailan, RS, Dobler, K, Pienkowska, M, Al-Mohanna, F, and Paterson, MC (2003). Aberrant sensing of extracellular Ca2+ by cultured ataxia telangiectasia fibroblasts. *Oncogene* **22**: 471-475.
120. Liang, N, He, Q, Liu, X, and Sun, H (2019). Multifaceted roles of ATM in autophagy: From nonselective autophagy to selective autophagy. *Cell Biochem Funct* **37**: 177-184.
121. Lee, JH, and Paull, TT (2020). Mitochondria at the crossroads of ATM-mediated stress signaling and regulation of reactive oxygen species. *Redox Biol* **32**: 101511.
122. Cosentino, C, Grieco, D, and Costanzo, V (2011). ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *Embo j* **30**: 546-555.
123. Qin, X, Jiang, B, and Zhang, Y (2016). 4E-BP1, a multifactor regulated multifunctional protein. *Cell Cycle* **15**: 781-786.
124. Halaby, MJ, Hibma, JC, He, J, and Yang, DQ (2008). ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. *Cell Signal* **20**: 1555-1563.
125. Nissenkorn, A, and Ben-Zeev, B (2015). Ataxia telangiectasia. *Handb Clin Neurol* **132**: 199-214.
126. van Os, NJH, Jansen, AFM, van Deuren, M, Haraldsson, A, van Driel, NTM, Etzioni, A, van der Flier, M, Haaxma, CA, Morio, T, Rawat, A*, et al.* (2017). Ataxia-telangiectasia: Immunodeficiency and survival. *Clin Immunol* **178**: 45-55.
127. Lavin, MF, Gueven, N, Bottle, S, and Gatti, RA (2007). Current and potential therapeutic strategies for the treatment of ataxia-telangiectasia. *Br Med Bull* **81-82**: 129-147.
128. Perlman, S, Becker-Catania, S, and Gatti, RA (2003). Ataxia-telangiectasia: diagnosis and treatment. *Semin Pediatr Neurol* **10**: 173-182.
129. Zannolli, R, Buoni, S, Betti, G, Salvucci, S, Plebani, A, Soresina, A, Pietrogrande, MC, Martino, S, Leuzzi, V, Finocchi, A*, et al.* (2012). A randomized trial of oral betamethasone to reduce ataxia symptoms in ataxia telangiectasia. *Mov Disord* **27**: 1312-1316.
130. Chessa, L, Leuzzi, V, Plebani, A, Soresina, A, Micheli, R, D'Agnano, D, Venturi, T, Molinaro, A, Fazzi, E, Marini, M*, et al.* (2014). Intra-erythrocyte infusion of

dexamethasone reduces neurological symptoms in ataxia teleangiectasia patients: results of a phase 2 trial. *Orphanet J Rare Dis* **9**: 5.

131. Menotta, M, Biagiotti, S, Bianchi, M, Chessa, L, and Magnani, M (2012). Dexamethasone partially rescues ataxia telangiectasia-mutated (ATM) deficiency in ataxia telangiectasia by promoting a shortened protein variant retaining kinase activity. *J Biol Chem* **287**: 41352-41363.
132. Pozzi, E, Giorgio, E, Mancini, C, Lo Buono, N, Augeri, S, Ferrero, M, Di Gregorio, E, Riberi, E, Vinciguerra, M, Nanetti, L*, et al.* (2020). In vitro dexamethasone treatment does not induce alternative ATM transcripts in cells from Ataxia–Telangiectasia patients. *Scientific Reports* **10**: 20182.
133. van Os, NJH, Haaxma, CA, van der Flier, M, Merkus, P, van Deuren, M, de Groot, IJM, Loeffen, J, van de Warrenburg, BPC, and Willemsen, M (2017). Ataxia-telangiectasia: recommendations for multidisciplinary treatment. *Dev Med Child Neurol* **59**: 680-689.
134. Riboldi, GM, Samanta, D, and Frucht, S (2021). Ataxia Telangiectasia. *StatPearls*: Treasure Island (FL).
135. Jerzak, KJ, Mancuso, T, and Eisen, A (2018). Ataxia-telangiectasia gene (ATM) mutation heterozygosity in breast cancer: a narrative review. *Curr Oncol* **25**: e176-e180.
136. van Os, NJH, Haaxma, CA, van der Flier, M, Merkus, P, van Deuren, M, de Groot, IJM, Loeffen, J, van de Warrenburg, BPC, Willemsen, M, and Group, ATS (2017). Ataxia-telangiectasia: recommendations for multidisciplinary treatment. *Dev Med Child Neurol* **59**: 680-689.
137. Fiore, K (2019). Desperate Families Pursue 'N-of-1' Trials for Ultra-Rare Diseases https://www.medpagetoday.com/special-reports/exclusives/81725.
138. Du, L, Pollard, JM, and Gatti, RA (2007). Correction of prototypic ATM splicing mutations and aberrant ATM function with antisense morpholino oligonucleotides. *Proc Natl Acad Sci U S A* **104**: 6007-6012.
139. Margus, B (2020). A-T ASO Gene Therapy Trial Continues! https://www.atcp.org/a-t-aso-gene-therapy-trial-continues/.
140. McKinnon, PJ (2004). ATM and ataxia telangiectasia. *EMBO Rep* **5**: 772-776.
141. Carranza, D, Torres-Rusillo, S, Ceballos-Pérez, G, Blanco-Jimenez, E, Muñoz-López, M, García-Pérez, JL, and Molina, IJ (2018). Reconstitution of the Ataxia-Telangiectasia Cellular Phenotype With Lentiviral Vectors. *Frontiers in Immunology* **9**.
142. Qi, J, Shackelford, R, Manuszak, R, Cheng, D, Smith, M, Link, CJ, and Wang, S (2004). Functional expression of ATM gene carried by HSV amplicon vector in vitro and in vivo. *Gene Therapy* **11**: 25-33.
143. Cortés, ML, Oehmig, A, Perry, KF, Sanford, JD, and Breakefield, XO (2006). Expression of human ATM cDNA in Atm-deficient mouse brain mediated by HSV-1 amplicon vector. *Neuroscience* **141**: 1247-1256.
144. Cortés, ML, Oehmig, A, Saydam, O, Sanford, JD, Perry, KF, Fraefel, C, and Breakefield, XO (2008). Targeted integration of functional human ATM cDNA into genome mediated by HSV/AAV hybrid amplicon vector. *Mol Ther* **16**: 81-88.
145. Lim, F, Khalique, H, Ventosa, M, and Baldo, A (2013). Biosafety of gene therapy vectors derived from herpes simplex virus type 1. *Curr Gene Ther* **13**: 478-491.
146. Abu-Awwad, HAM, Thiagarajan, L, and Dixon, JE (2017). Controlled release of GAG-binding enhanced transduction (GET) peptides for sustained and highly efficient intracellular delivery. *Acta Biomater* **57**: 225-237.

147. Nowak-Wegrzyn, A, Crawford, TO, Winkelstein, JA, Carson, KA, and Lederman, HM (2004). Immunodeficiency and infections in ataxia-telangiectasia. *J Pediatr* **144**: 505-511.
148. Duecker, R, Baer, PC, Buecker, A, Huenecke, S, Pfeffermann, LM, Modlich, U, Bakhtiar, S, Bader, P, Zielen, S, and Schubert, R (2019). Hematopoietic Stem Cell Transplantation Restores Naive T-Cell Populations in Atm-Deficient Mice and in Preemptively Treated Patients With Ataxia-Telangiectasia. *Front Immunol* **10**: 2785.
149. Slack, J, Albert, MH, Balashov, D, Belohradsky, BH, Bertaina, A, Bleesing, J, Booth, C, Buechner, J, Buckley, RH, Ouachée-Chardin, M*, et al.* (2018). Outcome of hematopoietic cell transplantation for DNA double-strand break repair disorders. *J Allergy Clin Immunol* **141**: 322-328.e310.
150. Ussowicz, M, Musiał, J, Duszeńko, E, Haus, O, and Kałwak, K (2013). Long-term survival after allogeneic-matched sibling PBSC transplantation with conditioning consisting of low-dose busilvex and fludarabine in a 3-year-old boy with ataxia-telangiectasia syndrome and ALL. *Bone Marrow Transplant* **48**: 740-741.
151. Ussowicz, M, Wawrzyniak-Dzierzek, E, Mielcarek-Siedziuk, M, Salamonowicz, M, Fraczkiewicz, J, Rybka, B, Ryczan-Krawczyk, R, and Kalwak, K (2018). Allogeneic Stem Cell Transplantation after Fanconi Anemia Conditioning in Children with Ataxia-Telangiectasia Results in Stable T Cell Engraftment and Lack of Infections despite Mixed Chimerism. *Biol Blood Marrow Transplant* **24**: 2245-2249.
152. Bakhtiar, S, Woelke, S, Huenecke, S, Kieslich, M, Taylor, AM, Schubert, R, Zielen, S, and Bader, P (2018). Pre-emptive Allogeneic Hematopoietic Stem Cell Transplantation in Ataxia Telangiectasia. *Front Immunol* **9**: 2495.
153. Beier, R, Sykora, KW, Woessmann, W, Maecker-Kolhoff, B, Sauer, M, Kreipe, HH, Dork-Bousset, T, Kratz, C, and Lauten, M (2016). Allogeneic-matched sibling stem cell transplantation in a 13-year-old boy with ataxia telangiectasia and EBV-positive non-Hodgkin lymphoma. *Bone Marrow Transplant* **51**: 1271-1274.
154. Pelletier, W, Hinds, PS, Alderfer, MA, Fairclough, DL, Stegenga, K, and Pentz, RD (2014). Themes reported by families as important when proceeding with pediatric hematopoietic stem cell transplantation. *Pediatr Blood Cancer* **61**: 1625-1631.
155. Ide, LM, Gangadharan, B, Chiang, KY, Doering, CB, and Spencer, HT (2007). Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. *Blood* **110**: 2855-2863.
156. Auerswald, G, Dolan, G, Duffy, A, Hermans, C, Jimenez-Yuste, V, Ljung, R, Morfini, M, Lambert, T, and Salek, SZ (2016). Pain and pain management in haemophilia. *Blood Coagul Fibrinolysis* **27**: 845-854.
157. Doering, CB, and Spencer, HT (2014). Replacing bad (F)actors: hemophilia. *Hematology Am Soc Hematol Educ Program* **2014**: 461-467.
158. Doering, CB, and Spencer, HT (2009). Advancements in gene transfer-based therapy for hemophilia A. *Expert Rev Hematol* **2**: 673-683.
159. Cantore, A, and Naldini, L (2021). WFH State-of-the-art paper 2020: In vivo lentiviral vector gene therapy for haemophilia. *Haemophilia* **27 Suppl 3**: 122-125.
160. Joglekar, AV, and Sandoval, S (2017). Pseudotyped Lentiviral Vectors: One Vector, Many Guises. *Hum Gene Ther Methods* **28**: 291-301.

161. Lytle, AM, Brown, HC, Paik, NY, Knight, KA, Wright, JF, Spencer, HT, and Doering, CB (2016). Effects of FVIII immunity on hepatocyte and hematopoietic stem cell-directed gene therapy of murine hemophilia A. *Mol Ther Methods Clin Dev* **3**: 15056.
162. Patel, SR, Lundgren, TS, Spencer, HT, and Doering, CB (2020). The Immune Response to the fVIII Gene Therapy in Preclinical Models. *Front Immunol* **11**: 494.
163. Hahn, K, Pollmann, L, Nowak, J, Nguyen, AHH, Haake, K, Neehus, AL, Waqas, SFH, Pessler, F, Baumann, U, Hetzel, M*, et al.* (2020). Human Lentiviral Gene Therapy Restores the Cellular Phenotype of Autosomal Recessive Complete IFN-gammaR1 Deficiency. *Mol Ther Methods Clin Dev* **17**: 785-795.
164. Ferrua, F, Cicalese, MP, Galimberti, S, Giannelli, S, Dionisio, F, Barzaghi, F, Migliavacca, M, Bernardo, ME, Calbi, V, Assanelli, AA*, et al.* (2019). Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol* **6**: e239-e253.
165. Brown, HC, Gangadharan, B, and Doering, CB (2011). Enhanced biosynthesis of coagulation factor VIII through diminished engagement of the unfolded protein response. *J Biol Chem* **286**: 24451-24457.
166. Doering, CB, Denning, G, Dooriss, K, Gangadharan, B, Johnston, JM, Kerstann, KW, McCarty, DA, and Spencer, HT (2009). Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Mol Ther* **17**: 1145-1154.
167. Doering, CB, Healey, JF, Parker, ET, Barrow, RT, and Lollar, P (2004). Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J Biol Chem* **279**: 6546-6552.
168. Johnston, JM, Denning, G, Doering, CB, and Spencer, HT (2013). Generation of an optimized lentiviral vector encoding a high-expression factor VIII transgene for gene therapy of hemophilia A. *Gene Ther* **20**: 607-615.
169. Spencer, HT, Denning, G, Gautney, RE, Dropulic, B, Roy, AJ, Baranyi, L, Gangadharan, B, Parker, ET, Lollar, P, and Doering, CB (2011). Lentiviral vector platform for production of bioengineered recombinant coagulation factor VIII. *Mol Ther* **19**: 302-309.
170. Zakas, PM, Brown, HC, Knight, K, Meeks, SL, Spencer, HT, Gaucher, EA, and Doering, CB (2017). Enhancing the pharmaceutical properties of protein drugs by ancestral sequence reconstruction. *Nat Biotechnol* **35**: 35-37.
171. Doering, CB, Denning, G, Shields, JE, Fine, EJ, Parker, ET, Srivastava, A, Lollar, P, and Spencer, HT (2018). Preclinical Development of a Hematopoietic Stem and Progenitor Cell Bioengineered Factor VIII Lentiviral Vector Gene Therapy for Hemophilia A. *Hum Gene Ther* **29**: 1183-1201.
172. Bazinet, A, and Popradi, G (2019). A general practitioner's guide to hematopoietic stem-cell transplantation. *Curr Oncol* **26**: 187-191.
173. Diaconescu, R, Flowers, CR, Storer, B, Sorror, ML, Maris, MB, Maloney, DG, Sandmaier, BM, and Storb, R (2004). Morbidity and mortality with nonmyeloablative compared with myeloablative conditioning before hematopoietic cell transplantation from HLA-matched related donors. *Blood* **104**: 1550-1558.
174. Omori, M, Yamashita, H, Shinohara, A, Kurokawa, M, Takita, J, Hiwatari, M, and Nakagawa, K (2013). Eleven secondary cancers after hematopoietic stem cell

transplantation using a total body irradiation-based regimen in 370 consecutive pediatric and adult patients. *Springerplus* **2**: 424.

175. Shimoni, A, Shem-Tov, N, Chetrit, A, Volchek, Y, Tallis, E, Avigdor, A, Sadetzki, S, Yerushalmi, R, and Nagler, A (2013). Secondary malignancies after allogeneic stem-cell transplantation in the era of reduced-intensity conditioning; the incidence is not reduced. *Leukemia* **27**: 829-835.
176. Tabbara, IA, Zimmerman, K, Morgan, C, and Nahleh, Z (2002). Allogeneic Hematopoietic Stem Cell Transplantation: Complications and Results. *Archives of Internal Medicine* **162**: 1558-1566.
177. Akahoshi, Y, Kimura, SI, Gomyo, A, Hayakawa, J, Tamaki, M, Harada, N, Kusuda, M, Kameda, K, Ugai, T, Wada, H*, et al.* (2018). Delayed platelet recovery after allogeneic hematopoietic stem cell transplantation: Association with chronic graft-versus-host disease and survival outcome. *Hematol Oncol* **36**: 276-284.
178. Bi, L, Lawler, AM, Antonarakis, SE, High, KA, Gearhart, JD, and Kazazian, HH, Jr. (1995). Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* **10**: 119-121.
179. Gunhan, E, Swe, M, Palazoglu, M, Voss, JC, and Chalupa, LM (2008). Expression and purification of cysteine introduced recombinant saporin. *Protein Expr Purif* **58**: 203-209.
180. Gangadharan, B, Parker, ET, Ide, LM, Spencer, HT, and Doering, CB (2006). High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood* **107**: 3859-3864.
181. Kasper, CK, Aledort, L, Aronson, D, Counts, R, Edson, JR, van Eys, J, Fratantoni, J, Green, D, Hampton, J, Hilgartner, M*, et al.* (1975). Proceedings: A more uniform measurement of factor VIII inhibitors. *ThrombDiathHaemorrh* **34**: 612.
182. Parker, ET, and Lollar, P (2003). A quantitative measure of the efficacy of factor VIII in hemophilia A mice. *Thromb Haemost* **89**: 480-485.
183. Miettinen, M, and Lasota, J (2005). KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. *Appl Immunohistochem Mol Morphol* **13**: 205-220.
184. Mohty, M (2007). Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* **21**: 1387-1394.
185. Mohty, M, and Gaugler, B (2008). Mechanisms of action of antithymocyte globulin: old dogs with new tricks! *Leuk Lymphoma* **49**: 1664-1667.
186. Jacquemin, M, and Saint-Remy, JM (2016). T cell response to FVIII. *Cell Immunol* **301**: 8-11.
187. Lacroix-Desmazes, S, Voorberg, J, Lillicrap, D, Scott, DW, and Pratt, KP (2019). Tolerating Factor VIII: Recent Progress. *Front Immunol* **10**: 2991.
188. Elgueta, R, Benson, MJ, de Vries, VC, Wasiuk, A, Guo, Y, and Noelle, RJ (2009). Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* **229**: 152-172.
189. Czechowicz, A, Palchaudhuri, R, Scheck, A, Hu, Y, Hoggatt, J, Saez, B, Pang, WW, Mansour, MK, Tate, TA, Chan, YY*, et al.* (2019). Selective hematopoietic stem cell ablation using CD117-antibody-drug-conjugates enables safe and effective transplantation with immunity preservation. *Nat Commun* **10**: 617.
190. Polito, L, Bortolotti, M, Mercatelli, D, Battelli, MG, and Bolognesi, A (2013). Saporin-S6: a useful tool in cancer therapy. *Toxins (Basel)* **5**: 1698-1722.

191. Siegall, CB, Liggitt, D, Chace, D, Tepper, MA, and Fell, HP (1994). Prevention of immunotoxin-mediated vascular leak syndrome in rats with retention of antitumor activity. *Proc Natl Acad Sci U S A* **91**: 9514-9518.
192. Moicean, AD, Popp, AM, and Sinescu, I (2009). Thymoglobulin--new approaches to optimal outcomes. *J Med Life* **2**: 319-324.
193. Clemmons, AB, Hartranft, ME, Kota, V, Pantin, J, Awan, FT, Salman, H, and Jillella, AP (2014). Administration of anti-thymocyte globulin: a comparison of two protocols. *Bone Marrow Transplant* **49**: 1535-1537.
194. Mueller, TF (2007). Mechanisms of Action of Thymoglobulin. *Transplantation* **84**: S5-S10.
195. Jin, F, He, J, Jin, C, Fan, W, Shan, Y, Zhang, Z, Sun, L, Hu, Z, and Yang, Y-G (2017). Antithymocyte globulin treatment at the time of transplantation impairs donor hematopoietic stem cell engraftment. *Cellular & Molecular Immunology* **14**: 443-450.
196. Samelson-Jones, BJ, Finn, JD, Favaro, P, Wright, JF, and Arruda, VR (2020). Timing of Intensive Immunosuppression Impacts Risk of Transgene Antibodies after AAV Gene Therapy in Nonhuman Primates. *Mol Ther Methods Clin Dev* **17**: 1129-1138.
197. L'Italien, L, Orozco, O, Abrams, T, Cantagallo, L, Connor, A, Desai, J, Ebersbach, H, Gelderblom, H, Hoffmaster, K, Lees, E*, et al.* (2018). Mechanistic Insights of an Immunological Adverse Event Induced by an Anti-KIT Antibody Drug Conjugate and Mitigation Strategies. *Clinical Cancer Research* **24**: 3465-3474.
198. Pang, WW, Czechowicz, A, Logan, AC, Bhardwaj, R, Poyser, J, Park, CY, Weissman, IL, and Shizuru, JA (2019). Anti-CD117 antibody depletes normal and myelodysplastic syndrome human hematopoietic stem cells in xenografted mice. *Blood* **133**: 2069-2078.
199. Kwon, HS, Logan, AC, Chhabra, A, Pang, WW, Czechowicz, A, Tate, K, Le, A, Poyser, J, Hollis, R, Kelly, BV*, et al.* (2019). Anti-human CD117 antibody-mediated bone marrow niche clearance in nonhuman primates and humanized NSG mice. *Blood* **133**: 2104-2108.
200. Agarwal, R, Dvorak, CC, Prohaska, S, Long-Boyle, J, Kwon, H-S, Brown, JM, Weinberg, KI, Le, A, Guttman-Klein, A, Logan, AC*, et al.* (2019). Toxicity-Free Hematopoietic Stem Cell Engraftment Achieved with Anti-CD117 Monoclonal Antibody Conditioning. *Biology of Blood and Marrow Transplantation* **25**: S92.
201. Ashman, LK, Buhring, HJ, Aylett, GW, Broudy, VC, and Muller, C (1994). Epitope mapping and functional studies with three monoclonal antibodies to the c-kit receptor tyrosine kinase, YB5.B8, 17F11, and SR-1. *J Cell Physiol* **158**: 545-554.
202. Gandhi, S, Kulasekararaj, AG, Mufti, GJ, and Marsh, JC (2013). Allogeneic stem cell transplantation using alemtuzumab-containing regimens in severe aplastic anemia. *Int J Hematol* **97**: 573-580.
203. Finazzi, MC, Boschini, C, Craddock, C, Rambaldi, A, Ward, J, and Malladi, RK (2020). Characteristics of graft-versus-host disease occurring after alemtuzumab-containing allogeneic stem cell transplants: incidence, organ involvement, risk factors and survival. *Br J Haematol* **188**: 550-559.
204. Marsh, JC, Pearce, RM, Koh, MB, Lim, Z, Pagliuca, A, Mufti, GJ, Perry, J, Snowden, JA, Vora, AJ, Wynn, RT*, et al.* (2014). Retrospective study of alemtuzumab vs ATG-based conditioning without irradiation for unrelated and matched sibling donor transplants in acquired severe aplastic anemia: a study from the British Society for Blood and Marrow Transplantation. *Bone Marrow Transplant* **49**: 42-48.

205. Poire, X, and van Besien, K (2011). Alemtuzumab in allogeneic hematopoetic stem cell transplantation. *Expert Opin Biol Ther* **11**: 1099-1111.
206. Benekli, M, Hahn, T, Williams, BT, Cooper, M, Roy, HN, Wallace, P, Stewart, C, Bambach, B, and McCarthy, PL, Jr. (2006). Muromonab-CD3 (Orthoclone OKT3), methylprednisolone and cyclosporine for acute graft-versus-host disease prophylaxis in allogeneic bone marrow transplantation. *Bone Marrow Transplant* **38**: 365-370.
207. Miller, ML, and Smith, GH (1988). Muromonab-CD3 in Acute Graft-Versus-Host Disease. *Drug Intelligence & Clinical Pharmacy* **22**: 236-237.
208. Slack, J, Albert, MH, Balashov, D, Belohradsky, BH, Bertaina, A, Bleesing, J, Booth, C, Buechner, J, Buckley, RH, Ouachee-Chardin, M*, et al.* (2018). Outcome of hematopoietic cell transplantation for DNA double-strand break repair disorders. *J Allergy Clin Immunol* **141**: 322-328 e310.
209. Stahl, L, Duenkel, A, Hilger, N, Tretbar, US, and Fricke, S (2019). The Epitope-Specific Anti-human CD4 Antibody MAX.16H5 and Its Role in Immune Tolerance. *Front Immunol* **10**: 1035.
210. Horneff, G, Guse, AH, Schulze-Koops, H, Kalden, JR, Burmester, GR, and Emmrich, F (1993). Human CD4 modulation in vivo induced by antibody treatment. *Clin Immunol Immunopathol* **66**: 80-90.
211. Shitara, K, Ueha, S, Shichino, S, Aoki, H, Ogiwara, H, Nakatsura, T, Suzuki, T, Shimomura, M, Yoshikawa, T, Shoda, K*, et al.* (2019). First-in-human phase 1 study of IT1208, a defucosylated humanized anti-CD4 depleting antibody, in patients with advanced solid tumors. *J Immunother Cancer* **7**: 195.
212. Kuhn, C, and Weiner, HL (2016). Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. *Immunotherapy* **8**: 889-906.
213. Ferrua, F, Cicalese, MP, Galimberti, S, Giannelli, S, Dionisio, F, Barzaghi, F, Migliavacca, M, Bernardo, ME, Calbi, V, Assanelli, AA*, et al.* (2019). Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *The Lancet Haematology* **6**: e239-e253.
214. Aiuti, A, Biasco, L, Scaramuzza, S, Ferrua, F, Cicalese, MP, Baricordi, C, Dionisio, F, Calabria, A, Giannelli, S, Castiello, MC*, et al.* (2013). Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* **341**: 1233151.
215. Langford-Smith, KJ, Sandiford, Z, Langford-Smith, A, Wilkinson, FL, Jones, SA, Wraith, JE, Wynn, RF, and Bigger, BW (2013). Signal One and Two Blockade Are Both Critical for Non-Myeloablative Murine HSCT across a Major Histocompatibility Complex Barrier. *PLoS ONE* **8**: e77632.
216. Oh, AL, Mahmud, D, Nicolini, B, Mahmud, N, Senyuk, V, Patel, PR, Bonetti, E, Arpinati, M, Ferrara, JLM, and Rondelli, D (2017). T Cell–Mediated Rejection of Human CD34+ Cells Is Prevented by Costimulatory Blockade in a Xenograft Model. *Biology of Blood and Marrow Transplantation* **23**: 2048-2056.
217. Patel, A, and Yong, J (2019). Elimination of Acute GvHD with Three Doses of Abatacept Prophylaxis Combined with Post-Transplant Cyclophosphamide after Myeloablative Treosulfan-Based Conditioning in the HLA Identical Sibling and Unrelated (10-12/12) Peripheral Blood Stem Cell Donor Setting: Improved Safety and Efficacy Compared to Alemtuzumab Prophylaxis. *Blood* **134**: 4540-4540.

218. Khandelwal, P, Yeh, RF, Yu, L, Lane, A, Dandoy, CE, El-Bietar, J, Davies, SM, and Grimley, MS (2020). Graft Versus Host Disease Prophylaxis With Abatacept Reduces Severe Acute Graft Versus Host Disease in Allogeneic Hematopoietic Stem Cell Transplant for Beta Thalassemia Major with Busulfan, Fludarabine, and Thiotepa. *Transplantation*.
219. Khoury, R, Grimley, MS, Davies, SM, and Khandelwal, P (2020). Abatacept Decreases the Rate of Acute and Chronic Gvhd in Children with Sickle Cell Disease Undergoing Matched Sibling Transplants. *Biology of Blood and Marrow Transplantation* **26**: S218.
220. Frentsch, M, Japp, AS, Dingeldey, M, Matzmohr, N, Thiel, A, Scheiflinger, F, Reipert, BM, and de la Rosa, M (2020). Blockade of the costimulatory CD28-B7 family signal axis enables repeated application of AAV8 gene vectors. *Journal of Thrombosis and Haemostasis* **18**: 1075-1080.
221. Pilat, N, Sayegh, MH, and Wekerle, T (2011). Costimulatory pathways in transplantation. *Semin Immunol* **23**: 293-303.
222. Vincenti, F, Klintmalm, G, Yang, H, Ram Peddi, V, Blahunka, P, Conkle, A, Santos, V, and Holman, J (2020). A randomized, phase 1b study of the pharmacokinetics, pharmacodynamics, safety, and tolerability of bleselumab, a fully human, anti-CD40 monoclonal antibody, in kidney transplantation. *Am J Transplant* **20**: 172-180.
223. Okimura, K, Maeta, K, Kobayashi, N, Goto, M, Kano, N, Ishihara, T, Ishikawa, T, Tsumura, H, Ueno, A, Miyao, Y*, et al.* (2014). Characterization of ASKP1240, a fully human antibody targeting human CD40 with potent immunosuppressive effects. *Am J Transplant* **14**: 1290-1299.
224. Anil Kumar, MS, Papp, K, Tainaka, R, Valluri, U, Wang, X, Zhu, T, and Schwabe, C (2018). Randomized, controlled study of bleselumab (ASKP1240) pharmacokinetics and safety in patients with moderate-to-severe plaque psoriasis. *Biopharm Drug Dispos* **39**: 245-255.
225. Zhang, R, Shi, W, Wang, HF, You, Y, Zhong, ZD, Li, WM, Zhang, C, Lu, X, Wang, YD, Zheng, P*, et al.* (2017). Idarubicin-intensified haploidentical HSCT with GvHD prophylaxis of ATG and basiliximab provides comparable results to sibling donors in high-risk acute leukemia. *Bone Marrow Transplantation* **52**: 1253-1260.
226. Chaudhary, MW, and Al-Baradie, RS (2014). Ataxia-telangiectasia: future prospects. *Appl Clin Genet* **7**: 159-167.
227. Bakhtiar, S, Woelke, S, Huenecke, S, Kieslich, M, Taylor, AM, Schubert, R, Zielen, S, and Bader, P (2018). Pre-emptive Allogeneic Hematopoietic Stem Cell Transplantation in Ataxia Telangiectasia. *Frontiers in immunology* **9**: 2495-2495.
228. Choy, KR, and Watters, DJ (2018). Neurodegeneration in ataxia-telangiectasia: Multiple roles of ATM kinase in cellular homeostasis. *Dev Dyn* **247**: 33-46.
229. Zaki-Dizaji, M, Akrami, SM, Azizi, G, Abolhassani, H, and Aghamohammadi, A (2018). Inflammation, a significant player of Ataxia-Telangiectasia pathogenesis? *Inflamm Res* **67**: 559-570.
230. Amor, S, Peferoen, LA, Vogel, DY, Breur, M, van der Valk, P, Baker, D, and van Noort, JM (2014). Inflammation in neurodegenerative diseases--an update. *Immunology* **142**: 151-166.
231. Chitnis, T, and Weiner, HL (2017). CNS inflammation and neurodegeneration. *The Journal of Clinical Investigation* **127**: 3577-3587.

232. Guzman-Martinez, L, Maccioni, RB, Andrade, V, Navarrete, LP, Pastor, MG, and Ramos-Escobar, N (2019). Neuroinflammation as a Common Feature of Neurodegenerative Disorders. *Frontiers in Pharmacology* **10**.
233. Härtlova, A, Erttmann, SF, Raffi, FAM, Schmalz, AM, Resch, U, Anugula, S, Lienenklaus, S, Nilsson, LM, Kröger, A, Nilsson, JA*, et al.* (2015). DNA Damage Primes the Type I Interferon System via the Cytosolic DNA Sensor STING to Promote Anti-Microbial Innate Immunity. *Immunity* **42**: 332-343.
234. Song, X, Ma, F, and Herrup, K (2019). Accumulation of Cytoplasmic DNA Due to ATM Deficiency Activates the Microglial Viral Response System with Neurotoxic Consequences. *J Neurosci* **39**: 6378-6394.
235. Hui, CW, and Herrup, K (2015). Individual Cytokines Modulate the Neurological Symptoms of ATM Deficiency in a Region Specific Manner. *eNeuro* **2**.
236. Broccoletti, T, Del Giudice, E, Cirillo, E, Vigliano, I, Giardino, G, Ginocchio, VM, Bruscoli, S, Riccardi, C, and Pignata, C (2011). Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia. *Eur J Neurol* **18**: 564-570.
237. Broccoletti, T, Del Giudice, E, Amorosi, S, Russo, I, Di Bonito, M, Imperati, F, Romano, A, and Pignata, C (2008). Steroid-induced improvement of neurological signs in ataxia-telangiectasia patients. *Eur J Neurol* **15**: 223-228.
238. Buoni, S, Zannolli, R, Sorrentino, L, and Fois, A (2006). Betamethasone and Improvement of Neurological Symptoms in Ataxia-Telangiectasia. *Archives of Neurology* **63**: 1479-1482.
239. Leuzzi, V, Micheli, R, D'Agnano, D, Molinaro, A, Venturi, T, Plebani, A, Soresina, A, Marini, M, Ferremi Leali, P, Quinti, I*, et al.* (2015). Positive effect of erythrocyte-delivered dexamethasone in ataxia-telangiectasia. *Neurol Neuroimmunol Neuroinflamm* **2**: e98.
240. Bagley, J, Cortes, ML, Breakefield, XO, and Iacomini, J (2004). Bone marrow transplantation restores immune system function and prevents lymphoma in Atm-deficient mice. *Blood* **104**: 572-578.
241. Pietzner, J, Baer, PC, Duecker, RP, Merscher, MB, Satzger-Prodinger, C, Bechmann, I, Wietelmann, A, Del Turco, D, Doering, C, Kuci, S*, et al.* (2013). Bone marrow transplantation improves the outcome of Atm-deficient mice through the migration of ATM-competent cells. *Hum Mol Genet* **22**: 493-507.
242. Wolska-Kuśnierz, B, and Gennery, AR (2020). Hematopoietic Stem Cell Transplantation for DNA Double Strand Breakage Repair Disorders. *Front Pediatr* **7**: 557-557.
243. Beier, R, Sykora, KW, Woessmann, W, Maecker-Kolhoff, B, Sauer, M, Kreipe, HH, Dörk-Bousset, T, Kratz, C, and Lauten, M (2016). Allogeneic-matched sibling stem cell transplantation in a 13-year-old boy with ataxia telangiectasia and EBV-positive non-Hodgkin lymphoma. *Bone Marrow Transplantation* **51**: 1271-1274.
244. Okabe, M, Ikawa, M, Kominami, K, Nakanishi, T, and Nishimune, Y (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* **407**: 313-319.
245. Gershanik, JJ, and James, V (1971). Ataxia Telangiectasia and Growth Failure. *American Journal of Diseases of Children* **122**: 538-540.
246. Stewart, E, Prayle, AP, Tooke, A, Pasalodos, S, Suri, M, Bush, A, and Bhatt, JM (2016). Growth and nutrition in children with ataxia telangiectasia. *Archives of Disease in Childhood* **101**: 1137.

247. Woelke, S, Pommerening, H, Kieslich, M, Schubert, R, and Zielen, S (2017). Growth hormone treatment in patients with ataxia telangiectasia. *Growth Factors* **35**: 125-130.
248. Voss, S, Pietzner, J, Hoche, F, Taylor, AM, Last, JI, Schubert, R, and Zielen, S (2014). Growth retardation and growth hormone deficiency in patients with Ataxia telangiectasia. *Growth Factors* **32**: 123-129.
249. Barlow, C, Hirotsune, S, Paylor, R, Liyanage, M, Eckhaus, M, Collins, F, Shiloh, Y, Crawley, JN, Ried, T, Tagle, D*, et al.* (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* **86**: 159-171.
250. Schubert, R, Reichenbach, J, and Zielen, S (2002). Deficiencies in CD4+ and CD8+ T cell subsets in ataxia telangiectasia. *Clin Exp Immunol* **129**: 125-132.
251. Azarsiz, E, Karaca, NE, Gunaydin, NC, Gulez, N, Ozturk, C, Aksu, G, Genel, F, and Kutukculer, N (2014). Do elevated serum IgM levels have to be included in probable diagnosis criteria of patients with ataxia-telangiectasia? *Int J Immunopathol Pharmacol* **27**: 421-427.
252. Chao, C, Yang, EM, and Xu, Y (2000). Rescue of defective T cell development and function in Atm-/- mice by a functional TCR alpha beta transgene. *J Immunol* **164**: 345-349.
253. Driessen, GJ, Ijspeert, H, Weemaes, CM, Haraldsson, Á, Trip, M, Warris, A, van der Flier, M, Wulffraat, N, Verhagen, MM, Taylor, MA*, et al.* (2013). Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. *J Allergy Clin Immunol* **131**: 1367-1375.e1369.
254. Czechowicz, A, Palchaudhuri, R, Scheck, A, Hoggatt, J, Saez, B, Scadden, DT, and Rossi, DJ (2016). Completely Non-Myeloablative/Non-Lymphoablative Conditioning for BMT/HSCT Using Anti-Ckit Immunotoxins. *Blood* **128**: 493-493.
255. Leone, C, Le Pavec, G, Même, W, Porcheray, F, Samah, B, Dormont, D, and Gras, G (2006). Characterization of human monocyte-derived microglia-like cells. *Glia* **54**: 183-192.
256. Lund, H, Pieber, M, Parsa, R, Han, J, Grommisch, D, Ewing, E, Kular, L, Needhamsen, M, Espinosa, A, Nilsson, E*, et al.* (2018). Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells. *Nature Communications* **9**: 4845.
257. Cronk, JC, Filiano, AJ, Louveau, A, Marin, I, Marsh, R, Ji, E, Goldman, DH, Smirnov, I, Geraci, N, Acton, S*, et al.* (2018). Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia. *J Exp Med* **215**: 1627-1647.
258. Larochelle, A, Bellavance, M-A, Michaud, J-P, and Rivest, S (2016). Bone marrow-derived macrophages and the CNS: An update on the use of experimental chimeric mouse models and bone marrow transplantation in neurological disorders. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1862**: 310-322.
259. Li, C, Chen, Y-H, and Zhang, K (2020). Neuroprotective Properties and Therapeutic Potential of Bone Marrow–Derived Microglia in Alzheimer's Disease. *American Journal of Alzheimer's Disease & Other Dementiasr* **35**: 1533317520927169.
260. Shi, C, and Pamer, EG (2011). Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology* **11**: 762-774.
261. Pietzner, J, Merscher, BM, Baer, PC, Duecker, RP, Eickmeier, O, Fussbroich, D, Bader, P, Del Turco, D, Henschler, R, Zielen, S*, et al.* (2016). Low-dose irradiation prior to bone

marrow transplantation results in ATM activation and increased lethality in Atm-deficient mice. *Bone Marrow Transplant* **51**: 560-567.

262. Costa-Marques, L, Arnold, K, Pardon, M-C, Leovsky, C, Swarbrick, S, Fabian, C, and Stolzing, A (2019). Transplantation of bone marrow derived macrophages reduces markers of neuropathology in an APP/PS1 mouse model. *Translational Neurodegeneration* **8**: 33.
263. Biju, KC, Santacruz, RA, Chen, C, Zhou, Q, Yao, J, Rohrabaugh, SL, Clark, RA, Roberts, JL, Phillips, KA, Imam, SZ*, et al.* (2013). Bone marrow-derived microglia-based neurturin delivery protects against dopaminergic neurodegeneration in a mouse model of Parkinson's disease. *Neurosci Lett* **535**: 24-29.
264. Sun, JM, and Kurtzberg, J (2018). Cell therapy for diverse central nervous system disorders: inherited metabolic diseases and autism. *Pediatric Research* **83**: 364-371.
265. Burman, J, Tolf, A, Hägglund, H, and Askmark, H (2018). Autologous haematopoietic stem cell transplantation for neurological diseases. *Journal of Neurology, Neurosurgery & Psychiatry* **89**: 147.
266. Wang, Y, Ji, X, Leak, RK, Chen, F, and Cao, G (2017). Stem cell therapies in age-related neurodegenerative diseases and stroke. *Ageing Res Rev* **34**: 39-50.
267. Quek, H, Luff, J, Cheung, K, Kozlov, S, Gatei, M, Lee, CS, Bellingham, MC, Noakes, PG, Lim, YC, Barnett, NL*, et al.* (2017). A rat model of ataxia-telangiectasia: evidence for a neurodegenerative phenotype. *Hum Mol Genet* **26**: 109-123.
268. Ninos, JM, Jefferies, LC, Cogle, CR, and Kerr, WG (2006). The thrombopoietin receptor, c-Mpl, is a selective surface marker for human hematopoietic stem cells. *Journal of Translational Medicine* **4**: 9.
269. Uemachi, H, Kasahara, Y, Tanaka, K, Okuda, T, Yoneda, Y, and Obika, S (2020). Discovery of cell-internalizing artificial nucleic acid aptamers for lung fibroblasts and targeted drug delivery. *Bioorganic Chemistry* **105**: 104321.
270. Ray, P, and White, RR (2010). Aptamers for Targeted Drug Delivery. *Pharmaceuticals (Basel)* **3**: 1761-1778.
271. He, F, Wen, N, Xiao, D, Yan, J, Xiong, H, Cai, S, Liu, Z, and Liu, Y (2020). Aptamer-Based Targeted Drug Delivery Systems: Current Potential and Challenges. *Curr Med Chem* **27**: 2189-2219.
272. Goyal, M, Citu, Singh, N, Singla, V, and Singla, A (2019). Aptamer: Apt System for Target-Specific Drug Delivery. In: Yadav, GS, Kumar, V and Aggarwal, NK (eds). *Aptamers: Biotechnological Applications of a Next Generation Tool*. Springer Singapore: Singapore. pp 59-71.