INVESTIGATION OF HIV CURE STRATEGIES IN VIVO IN BLT HUMANIZED MICE

Perry Tsai

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology in the School of Medicine.

Chapel Hill
2016

Approved by:
J. Victor Garcia-Martinez
Kristina De Paris
Joseph Eron
Nilu Goonetilleke
Edward Miao
Jonathan Serody
ABSTRACT

Perry Tsai: Investigation of HIV cure strategies in vivo in BLT humanized mice
(Under the direction of J. Victor Garcia-Martinez)

There is no cure yet for HIV. The development of HIV cure strategies will be accelerated by the use of animal models for HIV infection and treatment. Here, we investigated several HIV cure strategies in BLT humanized mice. First, we sought to explore the use of CCR5delta32 transplant to cure HIV-infected BLT mice. We found that CCR5delta32 stem cells engrafted in NSG mice and rendered them resistant to HIV infection. However, we were not able to successfully engraft CCR5delta32 cells in infected, suppressed mice.

Alternatively, a cure might be achieved by reversing HIV latency, so we tested a latency-reversing agent: histone deacetylase inhibitor panobinostat. Panobinostat treatment resulted in increased histone acetylation in BLT mice, but did not significantly change levels of HIV cell-associated RNA, DNA, or latently infected cells in infected, suppressed BLT mice. Such an approach may require combination with other latency-reversing or cell-killing strategies.

Next, we investigated the ability of CD8+ T cells to control HIV infection in BLT mice generated from donors with protective HLA alleles. While these mice did not control wildtype infection, several mice were able to control infection with a nef-deleted virus; and peak viral loads and average viral loads were significantly lower in two cohorts infected with nef-deleted virus. We also detected functional, HLA-
restricted, HIV-specific CD8+ T cells in the mice; and viremia increased rapidly in several mice after CD8-depletion. These studies suggest that CD8+ T cells are able to control infection in BLT mice only with nef-deficient virus.

A targeted immunological agent may be necessary for CD8+ T cells to kill infected cells. We tested a novel CD19xCD3 dual affinity retargeting (DART) molecule for redirected lysis in BLT mice. DART molecule administration resulted in profound depletion of CD19+ cells in both peripheral blood and tissues, and this depletion was dependent on the presence of CD8+ T cells.

Overall, I present herein results from four approaches in HIV cure — allogeneic transplant, latency reversal, CD8+ T cell control, redirected lysis — in BLT humanized mice. This work represents significant progress in the development of BLT humanized mice for use in research toward an HIV cure.
To my coworkers, family, friends, and everyone at UNC who has guided me, and to all the mice and humans that have contributed to the search for a cure
ACKNOWLEDGEMENTS

I must express profound gratitude to my research mentor, Dr. J. Victor Garcia-Martinez. He has been my teacher, my role model, my coach, and my cheerleader over the past five years. I have had unprecedented opportunities and experiences learning and working in his laboratory; and his patience, his insight, and his enthusiasm have kept me going on my path toward becoming a physician-scientist.

I would like to thank the other members of my thesis committee, Dr. Kristina De Paris, Dr. Joseph Eron, Dr. Edward Miao, and Dr. Jonathan Serody, for providing valuable feedback and always believing in me. I would also like to thank Dr. Nancie Archin, Dr. David Margolis, and Dr. Nilu Goonetilleke for numerous scientific discussions and experimental input throughout my graduate career.

I would like to thank the members of the Garcia lab, past and present. We worked together, we laughed together, and we had cake together.

Finally, thank you to all of my family and friends for the advice, the encouragement, the food, and the stories. And thank you to Shayla Birath, Michael Haas, Jenna Honeycutt, Lee Hong, Sian Lewis-Bevan, Matthew Moy, Erica Pettigrew, Monica Rizk, and Orrin Thayer for the final read-throughs.
# TABLE OF CONTENTS

**LIST OF TABLES** ................................................................................. xiii

**LIST OF FIGURES** .............................................................................. xiv

**LIST OF ABBREVIATIONS** ................................................................. xvi

**CHAPTER 1: INTRODUCTION** ............................................................... 1

- HIV EPIDEMIOLOGY ........................................................................... 1
- HIV ORIGIN ......................................................................................... 1
- HIV ENTRY AND REPLICATION ......................................................... 1
- HIV PATHOGENESIS .......................................................................... 3
- ANTIRETROVIRAL THERAPY ............................................................... 4
- HIV PERSISTENCE .............................................................................. 6
- ACTIVE HIV RESERVOIRS ................................................................. 7
- LATENT HIV RESERVOIRS ................................................................. 8
- A NEED FOR AN HIV CURE ................................................................. 10
- THE BERLIN PATIENT ........................................................................ 11
- THE BOSTON PATIENTS ..................................................................... 12
- THE MISSISSIPPI BABY .................................................................... 13
- THE VISCONTI COHORT .................................................................... 13
- ELITE HIV CONTROLLERS ................................................................. 14
- APPROACHES TO HIV CURE OR CONTROL ...................................... 14
- MECHANISMS OF HIV LATENCY ....................................................... 17
- LATENCY-REVERSING AGENTS ......................................................... 19
CHAPTER 3: IN VIVO ANALYSIS OF THE EFFECT OF PANOBINOSTAT ON CELL-ASSOCIATED HIV RNA AND DNA LEVELS AND LATENT HIV INFECTION

SUMMARY

INTRODUCTION

METHODS

ETHICS STATEMENT

ISOLATION OF RESTING HUMAN CD4+ T CELLS FOR RNA INDUCTION AND QUANTITATIVE VIRAL OUTGROWTH ASSAY

MEASUREMENT OF RNA INDUCTION AND QUANTITATIVE VIRAL OUTGROWTH FROM RESTING CELLS

GENERATION OF BLT HUMANIZED MICE

ANALYSIS OF HISTONE ACETYLATION

HIV INFECTION AND TREATMENT OF BLT MICE

STATISTICAL TESTS

RESULTS

INDUCTION OF HIV EXPRESSION WITH PANOBINOSTAT FROM RESTING CD4+ T CELLS ISOLATED FROM HIV-INFECTED PATIENTS ON SUPPRESSIVE ANTIRETROVIRAL THERAPY

IN VIVO HISTONE ACETYLATION IN TISSUES AFTER TREATMENT WITH PANOBINOSTAT

ANALYSIS OF THE EFFECT OF PANOBINOSTAT TREATMENT IN HIV-INFECTED, ART-SUPPRESSED BLT MICE

ANALYSIS OF THE EFFECT OF PANOBINOSTAT ON THE LEVELS OF LATENTLY INFECTED RESTING HUMAN CD4+ T CELLS
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>106</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>107</td>
</tr>
<tr>
<td>METHODS</td>
<td>110</td>
</tr>
<tr>
<td>ETHICS STATEMENT</td>
<td>110</td>
</tr>
<tr>
<td>GENERATION OF BLT HUMANIZED MICE</td>
<td>110</td>
</tr>
<tr>
<td>DART MOLECULES</td>
<td>110</td>
</tr>
<tr>
<td>TREATMENT OF BLT MICE</td>
<td>111</td>
</tr>
<tr>
<td>ANALYSIS OF BLT MICE</td>
<td>111</td>
</tr>
<tr>
<td>STATISTICAL ANALYSIS</td>
<td>112</td>
</tr>
<tr>
<td>RESULTS</td>
<td>112</td>
</tr>
<tr>
<td>DEPLETION OF HUMAN CD19⁺ B CELLS IN PERIPHERAL BLOOD AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN</td>
<td>112</td>
</tr>
<tr>
<td>DEPLETION OF HUMAN CD19⁺ B CELLS IN TISSUES AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN</td>
<td>115</td>
</tr>
<tr>
<td>REGENERATION OF HUMAN CD19⁺ B CELLS AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN</td>
<td>116</td>
</tr>
<tr>
<td>EFFECT OF CD19xCD3 DART PROTEIN ADMINISTRATION ON THE LEVELS OF HUMAN T CELLS IN VIVO</td>
<td>117</td>
</tr>
<tr>
<td>DEPENDENCE OF CD19xCD3 DART PROTEIN-MEDIATED DEPLETION ON THE PRESENCE OF HUMAN CD8⁺ T CELLS</td>
<td>118</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>120</td>
</tr>
<tr>
<td>CONTRIBUTIONS</td>
<td>123</td>
</tr>
<tr>
<td>FIGURES</td>
<td>124</td>
</tr>
<tr>
<td>CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS</td>
<td>135</td>
</tr>
<tr>
<td>STUDY SUMMARY</td>
<td>135</td>
</tr>
<tr>
<td>SUMMARY OF STUDIES ON CCR5DELTA32 TRANSPLANTATION</td>
<td>136</td>
</tr>
<tr>
<td>SUMMARY OF STUDIES ON PANOBINOSTAT</td>
<td>137</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 3.1. Patient characteristics ................................................................. 81
Table 4.1. Cohort characteristics ................................................................. 105
LIST OF FIGURES

Figure 2.1: Reconstitution in CCR5WT and CCR5delta32 NSG-Hu mice ................................................................. 49

Figure 2.2. Susceptibility of CCR5WT and CCR5delta32 mice to HIV infection .......................................................... 50

Figure 2.3. CCR5 genotyping by PCR and restriction digest .......................................................... 51

Figure 2.4. Reconstitution of irradiated and non-irradiated BLT mice ................................................................. 52

Figure 2.5. Conditioning of BLT mice by busulfan and antithymocyte globulin ......................................................... 53

Figure 2.6. CCR5WT or CCR5delta32 allogeneic transplant in infected, ART-treated BLT mice ........................................ 54

Figure 2.7. CCR5 genotyping of tissues from infected, ART-treated BLT mouse transplanted with CCR5delta32 stem cells .......................................................... 55

Figure 3.1. Effect of panobinostat on histone acetylation, HIV RNA, and viral outgrowth from patient cells .................................................. 75

Figure 3.2. Panobinostat administration induces systemic histone acetylation .......................................................... 76

Figure 3.3. Outline of panobinostat treatment of HIV-infected, ART-suppressed BLT mice .................................................. 77

Figure 3.4. Analysis of cell-associated HIV RNA levels in the tissues of infected, suppressed, panobinostat-treated BLT mice .................................................. 78

Figure 3.5. Analysis of HIV DNA levels in the tissues of infected, suppressed, panobinostat-treated BLT mice .................................................. 79

Figure 3.6. Analysis of panobinostat treatment on HIV latency in infected, suppressed BLT mice .................................................. 80

Figure 4.1. Flow cytometric analysis of HLA alleles ........................................................................ 99

Figure 4.2. Viral loads of JRCSF and JRCSF*Nef*dd in BLT mice with protective HLA alleles .................................................. 100

Figure 4.3. Analysis of peak viral loads and average viral loads in protective-HLA BLT mice infected with JRCSF or JRCSF*Nef*dd .................................................. 101
Figure 4.4. Pentamer staining of HIV-specific CD8$^+$ T cells from tissues of infected HLAB*2705 BLT mice .................................................. 102

Figure 4.5. Functional assessment of HIV-specific CD8$^+$ T cells from tissues of infected HLAB*2705 BLT mice .................................................. 103

Figure 4.6. Effect of CD8$^+$ T cell depletion in infected BLT mice .................................................. 104

Figure 5.1. CD19xCD3 DART protein administration depletes human CD19$^+$ B cells from the peripheral blood .................................................. 124

Figure 5.2. Gating scheme for flow cytometry analysis .................................................. 125

Figure 5.3. The percent human CD19$^+$ cells out of human CD45$^+$ cells decreases as percent human CD3$^+$ cells increases over time in the peripheral blood of NSG/BLT humanized mice .................................................. 126

Figure 5.4. 4420xCD3 DART protein administration does not deplete human CD19$^+$ B cells from the peripheral blood .................................................. 127

Figure 5.5. CD19xCD3 DART protein administration depletes human CD19$^+$ B cells from the tissues .................................................. 128

Figure 5.6. Immature human CD19$^+$ B cells regenerate in NSG/BLT mice after CD19xCD3 DART protein administration .................................................. 129

Figure 5.7. CD19xCD3 DART protein administration results in transient differences in the levels of human T cells in the peripheral blood of NSG/BLT mice, and over time there are no significant differences in absolute numbers of human CD8$^+$ T cells in the peripheral blood or tissues as compared to vehicle-treated mice .......... 131

Figure 5.8. Human CD8$^+$ T cells are depleted after administration of CD8-depleting antibody .................................................. 133

Figure 5.9. Human CD19$^+$ B cell depletion by CD19xCD3 DART protein is dependent on the presence of human CD8$^+$ T cells ............... 134
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APOBEC3G</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>ATG</td>
<td>Antithymocyte globulin</td>
</tr>
<tr>
<td>BCL</td>
<td>B cell line</td>
</tr>
<tr>
<td>BiTE</td>
<td>Bispecific T cell engager</td>
</tr>
<tr>
<td>BLT</td>
<td>Bone marrow / liver / thymus</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Brd4</td>
<td>Bromodomain 4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CCR5delta32</td>
<td>CCR5 delta32 mutant</td>
</tr>
<tr>
<td>CCR5WT</td>
<td>CCR5 wildtype</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD19</td>
<td>Cluster of differentiation 19</td>
</tr>
<tr>
<td>Cdk9</td>
<td>Cyclin-dependent kinase 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CycT1</td>
<td>Cyclin T1</td>
</tr>
<tr>
<td>DART</td>
<td>Dual affinity retargeting</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSIF</td>
<td>DRB sensitivity-inducing factor</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EC</td>
<td>Elite controller</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunospot</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>FTC</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific antigen</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HEXIM</td>
<td>Hexamethylene bisacetamide-inducible protein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMBA</td>
<td>Hexamethylene bisacetamide</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IUPB</td>
<td>Infectious units per billion</td>
</tr>
<tr>
<td>IUPM</td>
<td>Infectious units per million</td>
</tr>
<tr>
<td>Liv</td>
<td>Liver</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>LRA</td>
<td>Latency-reversing agent</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>Nef&lt;sup&gt;dd&lt;/sup&gt;</td>
<td>Nef-deleted</td>
</tr>
<tr>
<td>NELF</td>
<td>Negative elongation factor</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primates</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRG</td>
<td>NOD-Rag2&lt;sup&gt;−/−&lt;/sup&gt;-gammachain&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside/nucleotide reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD/SCID-gammachain&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>QVOA</td>
<td>Quantitative viral outgrowth assay</td>
</tr>
<tr>
<td>R5-tropic</td>
<td>CCR5-tropic</td>
</tr>
<tr>
<td>Rad</td>
<td>Radiation absorbed dose</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of the virion</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>Sterile alpha motif-domain and histidine aspartic-domain containing protein 1</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFU</td>
<td>Spot-forming units</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SPT5</td>
<td>Suppressor of Ty homolog-5</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans-activation response</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDF</td>
<td>Tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>Thy</td>
<td>Thymus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UNC</td>
<td>University of North Carolina</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VISCONTI</td>
<td>Virological and Immunological Studies in CONtrollers after Treatment Interruption</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Vpx</td>
<td>Viral protein X</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>X4-tropic</td>
<td>CXCR4-tropic</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

HIV EPIDEMIOLOGY

The global HIV/AIDS pandemic began over thirty years ago. The first reported cases of HIV/AIDS appeared in the Centers for Disease Control’s *Morbidity and Mortality Weekly Report* on June 5, 1981 [1]. Five men in Los Angeles had been treated for *Pneumocystis carinii* pneumonia, an infection typically found only in patients that were severely immunocompromised. These unusual cases pointed to the possibility of a “cellular-immune dysfunction related to a common exposure that predisposes individuals to opportunistic infections” like *Pneumocystis*. Over the following year, the number of cases of this immune deficiency rose, spanning the country – 158 in New York City, 10 elsewhere in New York State, 14 in New Jersey, and 71 in California [2] – and including occurrences of rare Kaposi’s sarcoma as well [3]. The syndrome, initially referred to as gay-related immune deficiency because most of those affected were men who have sex with men, came to be known as acquired immune deficiency syndrome, or AIDS [4]. Soon after, two groups led by Robert Gallo [5] and by Luc Montagnier [6] discovered that the causative agent was a virus, first called human T-lymphotropic virus-III or lymphadenopathy-associated virus. Later, this pathogen would be renamed the human immunodeficiency virus, or HIV [7].

Since the beginning of the epidemic, almost 71 million people have been infected with the virus, and about 34 million people have died from the disease [8]. In
2014, the number of individuals infected with HIV was 36.9 million people, with 2 million people newly infected and 1.2 million having died from AIDS-related illnesses [9]. Sub-Saharan Africa continues to be the most severely affected area, where roughly 1 in 20 adults are infected, representing two thirds of all cases worldwide. Specific to the United States, about 1.2 million people are living with HIV, with approximately 50,000 new infections each year [10].

**HIV ORIGIN**

HIV originated from the simian immunodeficiency virus (SIV) found in non-human primates (NHP) [11]. The two types of HIV, HIV-1 and HIV-2, seem to have derived from species-crossover events, HIV-1 from SIV in chimpanzees and gorillas and HIV-2 from SIV in sooty mangabeys [11, 12]. HIV-1 can be further categorized into four groups, M, N, O, and P; and 95% of the viruses found worldwide fall within Group M and its nine subtypes (A-D, F-H, J, and K). Subtype C is found primarily in Africa; and subtype B, in Europe and North America. The other groups of HIV-1 (N, O, and P) are limited to Cameroon and thus classified as non-pandemic [12]. References to “HIV” that follow in this dissertation will refer to HIV-1.

**HIV ENTRY AND REPLICATION**

HIV enters target cells via binding to its primary receptor CD4, followed by binding to one of two potential co-receptors, CCR5 or CXCR4. CD4 is a glycoprotein found on the surface of immune cells, including T helper cells, monocytes/macrophages, and dendritic cells. CCR5 and CXCR4 are both chemokine receptors from the superfamily of G-protein coupled receptors [13]. The HIV envelope glycoprotein, gp120, first engages CD4, resulting in a conformational change that
exposes the domains which bind to CCR5 or CXCR4. Binding to a co-receptor triggers another conformational change, allowing the HIV fusion peptide, gp41, to insert into the membrane of the host target cell and initiate fusion between the virus and host membranes, allowing the virus to enter the cell [14].

After HIV enters the host cell, the virion capsid is removed [15], and HIV reverse transcriptase synthesizes a complementary DNA (cDNA) strand from the single-stranded HIV RNA genome, followed by production of a second DNA strand to yield a double-stranded HIV DNA genome. HIV integrase then joins with the HIV DNA genome, forming the pre-integration complex, which is transported into the nucleus of the host cell by HIV Vpr (viral protein R) [16]. HIV integrase incorporates the HIV DNA genome into the genome of the host cell, and this integrated HIV DNA genome is referred to as a provirus. The proviral DNA is transcribed into RNA under a promoter present in the HIV long terminal repeat (LTR) region. This transcription is enhanced by HIV Tat (transactivator of transcription) which increases transcription processivity [17, 18].

The full-length HIV RNA transcripts are exported from the nucleus into the cytoplasm by HIV Rev (regulator of expression of the virion) [19], which also increases stability and translation of the transcripts [20, 21]. The transcripts either remain intact as full-length HIV RNA genomes, or they are processed for HIV protein translation. Following assembly of the RNA genomes with HIV proteins, the virions bud from the host cell membrane. At the cell surface, HIV Vpu (viral protein U) and Nef (negative regulatory factor) enhance virion release through the degradation or downregulation of CD4 receptors [22-24] and through recruitment of cholesterol to
the site of viral budding [25]. Finally, the HIV protease cleaves the Gag-Pol polyprotein into the functional forms of Gag and Pol, yielding mature infectious virions [26].

During the replication process, HIV is able to evade some of the host defense mechanisms, including APOBEC3G hypermutation and antigen presentation. APOBEC3G is a host restriction factor that deaminates deoxycytidines in the HIV cDNA, resulting in hypermutation and generation of replication-incompetent provirus [27, 28]. To suppress this antiviral activity, HIV Vif (viral infectivity factor) binds to APOBEC3G and promotes its degradation [29, 30]. Also, HIV Nef downregulates major histocompatibility complex (MHC) class I molecules [31], thus preventing the recognition and killing of HIV-infected cells by CD8+ T cells (also known as cytotoxic T lymphocytes, CTLs) [32].

**HIV PATHOGENESIS**

If untreated, HIV infection results in profound host immune suppression. CD4+ T helper cells are the major targets for infection [33]; and during the acute stage of infection, both activated and memory CD4+ T cells are rapidly depleted from the gut-associated lymphoid tissues [34]. Chronic infection may remain asymptomatic for years, but eventually the combined effects of chronic inflammation and systemic depletion of CD4+ T cells result in a deterioration of the host immune system [35, 36]. A clinical diagnosis of AIDS is made after a person with HIV presents with a CD4+ T cell count of less than 200 CD4+ T cells per microliter of peripheral blood, with a CD4+ T cell percentage of less than 14 percent of total lymphocytes, or with
an AIDS-defining condition, including candidiasis, cryptococcosis, *Pneumocystis jirovecii* pneumonia, Kaposi sarcoma, lymphoma, etc. [37].

**ANTIRETROVIRAL THERAPY**

The opportunistic infections and cancers associated with untreated HIV infection can be prevented if the host immune system and host CD4+ T cells are preserved through the use of antiretroviral therapy (ART) to suppress viral replication [38]. The first pharmaceutical approved for the treatment of HIV was zidovudine in 1987. Since then, the number of drugs approved to treat HIV infection has expanded to include thirty single antiretroviral agents and eight fixed-dose combination tablets [39]. These antiretroviral drugs fall into four major classes, categorized by their target enzyme in the HIV replication cycle: reverse transcriptase inhibitors, entry inhibitors, integrase inhibitors, and protease inhibitors.

The reverse transcriptase inhibitors include seven nucleoside/nucleotide analogs (NRTIs) and five non-nucleoside inhibitors (NNRTIs). NRTIs, such as emtricitabine (FTC) or tenofovir (TDF), are DNA nucleoside or nucleotide analogs. They compete with the naturally occurring nucleoside/nucleotide and become incorporated into the cDNA strand, but then they terminate the strand elongation due to a chemical feature preventing 5’-3’ phosphodiester linkages [40]. NNRTIs, such as nevirapine or efavirenz, bind reverse transcriptase at an allosteric site, inducing conformational changes that block substrate binding and polymerization [41].

The two approved entry inhibitors, enfuvirtide and maraviroc, prevent entry of HIV into the host cell. Enfuvirtide is a peptide inhibitor that binds to the fusion peptide, gp41, and blocks it from forming a fusion pore for viral entry [42]. Maraviroc
is a CCR5 antagonist that binds to CCR5 and blocks its utilization as a co-receptor for entry [43].

Integrase inhibitors include raltegravir, elvitegravir, and dolutegravir. These drugs inhibit the integration of HIV DNA into the host genome by blocking the action of HIV integrase [44].

Protease inhibitors, like ritonavir or darunavir, block the action of HIV protease to cleave precursor polyproteins into their active forms, which are needed for the maturation of virions into infectious particles. In the presence of protease inhibitors, only non-infectious and immature viral particles are produced [45, 46].

In the early days of the epidemic, options for antiretroviral therapy were limited, and the goals clinically were to improve the survival of already ill AIDS patients [47, 48]. With the advent of new drugs and multiple classes of inhibitors, the standard of care has shifted from sequential monotherapy to combination therapy, or highly active antiretroviral therapy, the advantages of which include more sustained virological response (suppression of viremia) and reduced emergence of resistant strains. The objectives of ART have shifted accordingly: (1) to achieve durable suppression of viral replication, as measured by plasma viral loads below the limit of detection, and immune reconstitution, as measured by CD4+ T cell counts greater than 500 cells per microliter peripheral blood; (2) to reduce morbidity and mortality due to opportunistic infections and cancer, as well as chronic inflammation and its complications; and (3) to prevent transmission of HIV in “test and treat” or “treatment as prevention” strategies [49, 50].
The benefits of ART have been demonstrated in improvements in life expectancy and clinical outcomes in those receiving treatment [51-53]. Life expectancy has increased among treated HIV-positive individuals in the United States and Canada to 51.4 years after ART initiation, approaching that of the general population [54]. And while previous recommendations were to delay ART initiation in HIV patients until CD4+ T cell counts were less than 500 per microliter peripheral blood, these have been replaced with recommendations to initiate ART immediately for all HIV-diagnosed individuals, regardless of CD4+ T cell count [55, 56]. This shift arises from compelling evidence that early ART initiation reduces morbidity and mortality [57, 58] and prevents further transmission [49].

**HIV PERSISTENCE**

Despite the apparent efficacy and benefit of ART, it is not curative. Even though viral replication may be suppressed to the point of undetectable plasma viral load, the virus continues to persist in treated individuals. Because of this persistent infection, virtually all treated individuals experience a rapid rebound of viral replication within weeks after ART interruption, even if their plasma viremia has been suppressed for years [59]. Therefore, ART must be continued indefinitely in HIV-positive individuals, presenting challenges of cost, adherence, toxicity, and potential for resistance [60]. Persistent HIV infection under ART resides in the body within viral reservoirs, and these reservoirs can be broadly characterized as active or latent [61].
ACTIVE HIV RESERVOIRS

Patients on ART can maintain plasma viral loads below the limit of detection using standard clinical assays. However, steady-state levels of very low residual viremia (>1 HIV RNA copy per ml) have been observed using ultra-sensitive assays in patients who have been on treatment for years [62, 63], suggesting the presence of residual active HIV reservoirs that continue to release virus despite effective ART.

It is not yet clear if this residual viremia arises from the release of trapped virus, the ongoing production of virus in long-lived infected cells, and/or new cycles of infection/replication. The genetic stability of sequences obtained from residual viremia [64-66] and the futility of ART intensification (adding an additional drug to an ART regimen) to reduce residual viremia [67-69] suggest that new cycles of infection/replication are not a major source of persistent viremia during ART.

However, the persistence of low-level viremia over the span of several years [63] implies that HIV is being continually produced from an active reservoir. Two randomized clinical trials showed that the addition of an integrase inhibitor (raltegravir) resulted in a temporary increase of 2-LTR circles [70, 71]. These 2-LTR circles form from reverse-transcribed HIV DNA episomes before their integration into the host genome during the replication cycle, so the increase in 2-LTR circles means that the addition of raltegravir is blocking integration steps during ongoing replication. Also, addition of raltegravir results in lower levels of CD8+ T cell activation [72], which could have been from a low level of replication before ART intensification. Recent phylogenetic analyses by Lorenzo-Redondo et al., obtained from the blood and inguinal lymph nodes of patients beginning treatment, suggest
that drug concentrations are not fully suppressive in lymphoid tissues, thus allowing
HIV replication to continue without development of resistance [73].

Persistent low-level viremia during ART could be explained by cell-to-cell
spread, anatomical sanctuaries, or long-lived cellular reservoirs. *In vitro* experiments
have shown that cell-to-cell transmission is less sensitive to antiretroviral drugs than
infection by cell-free virus [74]. Cell-to-cell transmission has been observed between
lymphocytes and astrocytes in culture [75], but it has not yet been demonstrated *in vivo*. Reports of pyroptotic death of CD4+ T cells after cell-to-cell transmission would
predict progressive CD4+ T cell depletion in treated patients, but this is typically not
the case. The virus may persist in anatomical sanctuaries in the lymphoid tissues or
in the central nervous system due to poorer penetration of antiretroviral drugs into
these anatomical locations [73, 76, 77]. Lower drug concentration was correlated
with slower decay of follicular dendritic cell-associated virus and with detection of
viral RNA in productively infected cells, though it is not clear why drug penetration is
not as robust [76]. A recent study in elite controller rhesus macaques [78] suggests
that HIV may persist in the B cell follicles of lymph nodes because of the exclusion of
CD8+ T cells, which would typically control infection. Finally, tissue macrophages
and microglia in the central nervous system are long-lived and highly resistant to
viral cytopathic effects and apoptosis [79, 80], so infected macrophages could serve
as long-lived cellular reservoirs [81, 82].

**LATENT HIV RESERVOIRS**

In addition to active HIV reservoirs, there are latent HIV reservoirs made up of
latently infected cells, as defined by a reversibly non-productive state of infection in
which HIV resides as transcriptionally silent provirus in the host genome [83]. The presence of an inducible latent HIV reservoir was first demonstrated in 1997 as viral outgrowth from peripheral blood mononuclear cells (PBMCs) of treated patients [84-86]. The most well-characterized latently infected cells are resting central memory CD4+ T cells. While some studies have suggested a potential for latent infection in other cell types (naïve CD4+ T cells, stem memory T cells, transitional memory CD4+ T cells, gamma-delta T cells, hematopoietic progenitor cells, and macrophages), there is so far limited evidence to support these claims [87]. The long-lived durability of these potential cellular reservoirs has yet to be demonstrated in patients or animal models as rigorously as has been shown in resting central memory CD4+ T cells according to the above criteria [61].

Latently infected cells could be infected directly while in the resting state [88] or while they are transitioning toward a resting memory state [89]. In this quiescent state, they are transcriptionally silent, producing very low levels of HIV RNA (<50 copies per 10^6 cells) [90]. Without HIV RNA for protein translation, latently infected cells are invisible to antiretroviral therapies and to the host immune responses that recognize HIV antigen. Longitudinal studies have shown that these latently infected cells are long-lived with an extremely slow decay rate (half-life of 44 months) which extrapolates to an estimate of >70 years of treatment needed to eradicate the latent reservoir with ART alone [91].

Due to the persistence of HIV infection in active and latent reservoirs during ART, the virus rebounds after cessation of antiretroviral therapy. Currently available
therapies are not able to clear these reservoirs, and so there is still a need for an HIV cure.

**A NEED FOR AN HIV CURE**

A cure for HIV would no doubt be beneficial to patients by eliminating the effects of chronic HIV infection. Even in patients adherent to ART, there is evidence of increased chronic immune activation and inflammation [92] in comparison to uninfected persons, and chronic inflammation has been associated with increased risk of cardiovascular disease, cancer, and osteoporosis [92-100].

A cure would eliminate the need for lifelong drug treatment and potential drug toxicities. Earlier drugs required a high pill burden and were associated with a long list of adverse side effects: nausea, diarrhea, rash, myopathy, pancreatitis, lipodystrophy, hypercholesterolemia, mitochondrial toxicity, nephrotoxicity, and loss of bone mineral density [39]. Newer ART drugs avoid many but not all of the toxicities of first-generation regimens. For example, long-term tenofovir disoproxil fumarate is associated with nephrotoxicity in patients who have pre-existing renal insufficiency or are taking other nephrotoxic medications [101], and ritonavir is associated with hypertriglyceridemia [102, 103]. NRTIs are generally associated with mitochondrial toxicity due to the structural similarities between reverse transcriptase and mitochondrial DNA polymerase. Mitochondrial toxicity can manifest as myopathy, neuropathy, lipoatrophy, and lactic acidosis [104-106], though these adverse events were more commonly associated with zidovudine, stavudine, and didanosine which are now rarely used [107].
Curing a patient of HIV would also reduce the social and financial costs of infection. The status of being HIV-positive carries social stigma from intrapersonal/interpersonal levels up to larger institutional/structural levels [108, 109], and the cost of lifelong ART has been estimated at $379,668 in 2010 [110]. The financial cost of lifelong ART not only affects individual access but also global access, particularly in low-income countries where the majority of HIV-infected people reside; therefore a cure would be particularly beneficial in these areas where ART can be cost-prohibitive.

A cure for HIV would allow a patient to discontinue ART and not experience viral rebound or progression to AIDS, and there are several potential strategies to achieve this state [111]. A sterilizing or eradicative cure means a complete removal of all infectious forms of HIV from a patient; no replication-competent provirus would be present. A functional cure describes a state of post-treatment control where infection is controlled without ART; this might be achieved through a reduction but not necessarily elimination of the reservoir, or through a modification of the immune system. Reductions in the HIV reservoir might also make it possible to achieve temporary ART-free remission or delay in rebound after ART interruption.

Several recent reports of HIV cure, delayed rebound, and control of infection have introduced the possibility of cure as well as potential approaches for cure [111].

**THE BERLIN PATIENT**

The only example of a possible eradicative HIV cure is the case of Timothy Ray Brown, also known as the “Berlin patient.” Brown received an allogeneic hematopoietic stem cell transplant (HSCT) which was indicated for treatment of
acute myeloid leukemia. His donor was specifically chosen because the donor was homozygous for the CCR5delta32 mutation. People who are CCR5delta32-homozygous do not express CCR5 protein on the surface of their cells; and, as CCR5 is a co-receptor for HIV entry, they are highly resistant to infection [112, 113]. By 61 days after his transplantation, Brown’s entire immune system had been replaced by HIV-resistant CCR5delta32-homozygous donor cells; and, although he discontinued ART at the time of transplant, he did not experience any viral rebound [114, 115]. This case has been considered a possible sterilizing cure, as replication-competent HIV has still not been detected years later [116].

THE BOSTON PATIENTS

Following the Berlin patient, two more cases were described in 2012, the “Boston patients” [117]. Like the Berlin patient, the Boston patients received allogeneic HSCTs, but with two major differences: their donors were CCR5-wildtype, and they continued ART during and after the transplant. This approach bypassed the need for a CCR5delta32 donor, and it tested the hypothesis that allogeneic HSCT alone could be curative for HIV as long as ART was present to protect the HIV-susceptible donor cells from becoming infected. The patients were followed for two to four years after transplant during which ART was continued; and neither of them had detectable HIV DNA or detectable replication-competent virus in their peripheral blood cells. This evidence suggested that their reservoirs might have been eliminated, and so the patients discontinued ART but experienced viral rebound 3 and 8 months later respectively [118]. While these cases did not result in long-term remission, they demonstrated the possibility that the peripheral blood reservoir can
be reduced after allogeneic HSCT, and that rebound can be delayed later than the typical rebound of 2 to 3 weeks [119].

**THE MISSISSIPPI BABY**

Another case of delayed rebound was reported in the “Mississippi baby” in 2013 [120]. A newborn that had been infected perinatally was started on ART immediately after birth. At 18 months old, the infant was lost from care and returned at 23 months, when it was discovered that the infant had not been taking ART since 15 months. Despite having not taken ART from 15 to 23 months, the infant had no detectable plasma viremia or HIV DNA in the peripheral blood. It was hypothesized that early treatment might have been able to prevent the establishment of a reservoir. During follow-up, rebound was eventually detected at 26 months after ART discontinuation [121].

**THE VISCONTI COHORT**

Early treatment has also been implicated in cases of post-treatment control in fourteen patients from the VISCONTI cohort [122]. These patients began ART early during primary infection (a majority at Fiebig stage V) and continued treatment for a median of 36.5 months before ART interruption. At the time of the report, these patients had not been taking ART for a median of 89 months and were not experiencing viral rebound. Their levels of plasma viremia were detectable but very low (median 5.0 copies per ml), and replication-competent virus was still present in their resting cells; so these cases were considered “post-treatment controllers,” possibly mediated by a preservation of the immune response after early treatment [123].
ELITE HIV CONTROLLERS

The possibility of immune control of infection is also implied in cases of “elite controllers” (ECs). These patients are able to control the virus on their own, usually for at least 10 years, without ever initiating ART [124]; and they are very rare, representing only 0.15% of HIV-infected patients [125]. Genotypic and phenotypic analyses have suggested that viral isolates from ECs are fully virulent [126, 127], and there was even one case of HIV transmission from a patient who progressed to AIDS to a patient who remained an elite controller [128]. Therefore, EC status is likely due to features of the host. Indeed, certain MHC class I alleles are overrepresented in ECs, including HLA-B27, -B57, -B14, and -B51 [129, 130]; and strong CTL responses to HIV Gag antigen have been reported in ECs, including characteristics of polyfunctional memory T cells [131], CD27 expression for long-term survival [132], and high functional avidity [133]. However, not all ECs exhibit intense CTL responses [134], so there are likely other factors contributing to control of the virus.

Though these reports are important for generating hypotheses toward strategies, only the Berlin patient has ever been possibly cured of infection. The challenge in curing HIV lies in the long-term persistence of HIV infection even with antiretroviral therapy, and several approaches are being investigated to clear this persistent infection.

APPROACHES TO HIV CURE OR CONTROL

The case of the Berlin patient demonstrates that allogeneic HSCT with CCR5delta32 cells can reduce the HIV reservoir toward eradication. However, only
1% of the Caucasian population carries the CCR5delta32 mutation [135]. Another way to replicate the CCR5delta32-homozygous HSCT is through gene therapy or gene-editing technology. Using zinc-finger nucleases, CD4+ T cells from twelve HIV patients were modified *ex vivo* to remove the *CCR5* gene, thus mimicking CCR5delta32-homozygous cells [136]. While the procedure was safe within the parameters of the study, the *CCR5*-deleted cells only represented a minority of the reinfused cells, and ART discontinuation resulted in viral rebound. Similar strategies are currently being investigated to delete *CCR5* from hematopoietic stem cells in preclinical models as well [137].

Allogeneic HSCT itself might be effective in reducing the reservoir because (1) pre-transplant conditioning regimens designed to reduce tumor burden and to prevent graft rejection may also reduce the number of HIV-infected host cells, and (2) graft-versus-host effects may result in post-transplant clearance of HIV-infected cells. However, allogeneic transplant alone was not sufficient for cure in the Boston patients. Also, allogeneic HSCT is not generalizable due to the need for HLA-matched donors and the risks inherent to the procedure, so it would be inappropriate for healthy, HIV patients without an oncological indication for allogeneic HSCT.

Early initiation of ART is another approach that resulted in delayed rebound with the Mississippi baby or post-treatment control in the VISCONTI patients. This approach is supported by other studies which have shown that early treatment could result in smaller reservoirs [138-140]. However, early treatment is not sufficient to prevent the establishment of a latent reservoir. In fact, a study of early ART in SIV-infected macaques showed rebound even when treatment was started just three
days after exposure [141]. Also, early treatment requires a diagnosis during acute or primary HIV infection. This can be challenging because the symptoms of acute infection are vague and nonspecific [142], and intensive resources are needed to detect HIV RNA in blood before seroconversion. The development and use of new technologies to detect HIV RNA or p24 antigen should increase diagnoses of acute HIV infection and identification of candidates for early treatment [143].

The evidence for immune control in elite controllers suggests the possibility of recapitulating this control in other patients through immunization. Passive immunization could be accomplished by injection of broadly neutralizing antibodies that would block new infections and target infected cells for destruction by antibody-dependent cellular cytotoxicity [144-147]. Vaccination may also confer the ability to either resist or control infection. The bivalent RV144 vaccine was designed to induce humoral and cellular immune responses, and it demonstrated a 31.2% reduction in transmission in a phase III clinical trial in Thailand [148]. Hansen et al. vaccinated rhesus macaques with a cytomegalovirus vector containing SIV genes, before challenging them with SIV. This vaccine induced the generation of SIV-specific effector memory CD8+ T cells, and 13 out of 24 animals were able to control and clear infection [149, 150]. These results suggest that preexisting robust CTL responses may be able to clear the latent reservoir or prevent the formation of a latent reservoir.

CTLs could be enhanced to mount better anti-HIV immune responses, through expansion of endogenous polyclonal HIV-specific CTLs or through gene therapy to introduce anti-HIV T cell receptors. Polyclonal CTL therapy can be
produced by isolating CTLs from a patient, then selecting and expanding the clones with robust anti-HIV responses for reinfusion. This approach was safe in a phase 1 clinical trial, but its effect on viremia was transient and not statistically significant [151]. High-affinity TCRs are also being investigated [152], but this approach would be restricted to HLA-matched patients.

To bypass HLA restriction, chimeric antigen receptor (CAR) gene therapy is being developed. CARs are engineered by joining an extracellular targeting domain derived from CD4 and single-chain variable fragments specific for HIV gp120, with an intracellular activating domain to trigger CTL killing activity. Clinical trials using this technology have thus far shown safety and long-term persistence of CAR-transduced T cells, but no significant change in reservoir size [153-155].

Finally, the “shock-and-kill” or “kick-and-kill” approach seeks to target the latent HIV reservoir by reversing latency and inducing HIV gene expression, thereby allowing the clearance of infected cells through viral cytopathic effects, immune responses, or targeted cytotoxic agents [156]. Strategies to disrupt the latent reservoir are currently being developed to target the mechanisms of HIV latency.

MECHANISMS OF HIV LATENCY

There are three broad mechanisms identified so far by which HIV establishes latent infection and maintains latency: (1) transcriptional interference, (2) epigenetic silencing, and (3) unavailability of transcription factors.

Transcriptional interference occurs when HIV integrates within an actively transcribed host gene in the sense orientation, 5’LTR-to-3’LTR. As RNA polymerase II transcribes and elongates mRNA from the host gene upstream, it terminates
elongation at the polyA site in the HIV 5’LTR, thus preventing transcription of HIV RNA [157-159]. If transcription of the upstream host gene were to be silenced, then transcriptional interference would no longer occur, and HIV could be reactivated. Alternatively, transcriptional interference could be overcome by cellular activation, elevated NF-kB levels, and binding of NF-kB to the 5’LTR [160].

Integrated proviruses can also be silenced by epigenetic changes which promote the formation of condensed heterochromatin that is inaccessible to transcription factors or transcription machinery. After histone deacetylases (HDACs) are recruited to the HIV LTR by host factors, the HDACs remove acetyl groups from lysine residues on histone proteins [161, 162]. This removal of histone acetyl groups increases the ionic interactions between positively charged histones and negatively charged DNA, yielding a more compact chromatin structure and inducing transcriptional silencing of HIV [163, 164]. Several studies also report the association of histone methylation with chromatin condensation and silenced proviral DNA, specifically methylation of histone H3 lysines at positions 9 and 27 [165-170].

Certain transcription factors are needed for HIV expression, such as HIV Tat protein, as well as host NF-kB and P-TEFb (positive transcription elongation factor b). P-TEFb consists of cyclin CycT1 with cyclin-dependent kinase Cdk9 [171]. P-TEFb is recruited by Tat to the trans-activation response (TAR) element [172], where Cdk9 phosphorylates the C-terminal domain of RNA polymerase II and promotes elongation of the viral transcript [173]. P-TEFb also phosphorylates SPT5 (suppressor of Ty homolog-5) in order to convert the associated DSIF (DRB sensitivity-inducing factor) complex into a positive elongation factor; and it
phosphorylates the RD (named for Arg-Asp dipeptide repeat sequence) subunit of the NELF (negative elongation factor) complex, causing it to disengage [174]. P-TEFb levels are regulated through microRNA-mediated inhibition of translation of the CycT1 subunit, and CycT1 is present at very low levels in resting cells [175]. This inhibition can be counteracted by cellular activation which raises P-TEFb levels [176], but P-TEFb is also sequestered and inactivated by HEXIM (hexamethylene bisacetamide-inducible protein) within the 7SK snRNP (small nuclear ribonucleoprotein) complex [177, 178]. P-TEFb is released and activated in response to cellular stress or to molecules that change chromatin or DNA methylation [177-179].

These mechanisms of HIV latency — transcriptional interference, epigenetic silencing, and unavailability of host transcription factors — represent potential targets for interventions to reverse HIV latency and induce HIV expression.

**LATENCY-REVERSING AGENTS**

Multiple classes of latency-reversing agents (LRAs) have been proposed and are currently being investigated for their ability to induce HIV expression from latently infected cells [180]. Latency reversal could be accomplished by (1) disrupting epigenetic silencing, (2) activating T cells, or (3) increasing availability of host transcription factors [181].

Histone deacetylase (HDAC) inhibitors are a class of latency-reversing agent designed to disrupt latency by inhibiting epigenetic silencing mechanisms. As discussed above, histone deacetylases remove acetyl groups from histone lysine residues, allowing the positively charged histones to tightly bind proviral DNA
thereby preventing transcription [182]. HDAC inhibitors block the activity of HDACs, thus allowing histone acetyltransferases to re-acetylate histone proteins, neutralizing the lysine positive charge and relaxing the histone-DNA binding, making proviral DNA accessible to transcription factors and RNA polymerase II. HDAC inhibitors such as vorinostat, panobinostat, and romidepsin are already being tested for their ability to induce HIV expression from latently infected cells [183-185]. Methylation of histones, specifically methylation of histone 3 at lysine 9 and 27, has also been associated with condensed heterochromatin at the HIV LTR DNA [167]. Several histone methyltransferase inhibitors (BIX01294, chaetocin, DZNep) have been shown to reactivate latent HIV from cell lines transfected with LTR-driven luciferase reporter and from resting CD4+ cells of ART-treated patients [186, 187], with a notable synergistic effect in combination with HDAC inhibitors.

Protein kinase C (PKC) enzymes have been identified as a central factor in the signaling cascade of activated T cells [188]. After T cell activation, PKC localizes to the immunological synapse and is activated by diacylglycerol. Alternatively, PKC can also be activated by the administration of phorbol ester compounds [189-191]. PKC then initiates multiple signaling cascades that result in the activation of transcription factors NF-kB, AP-1, or NFAT, which then contribute to the reversal of HIV latency. Several PKC agonists or activators have been investigated for their ability to induce HIV expression from latency. For example, prostratin has been shown to induce HIV expression from a latent cell line model [192] as well as from PBMCs and resting CD4+ T cells of ART-treated patients [193, 194]. Bryostatin
treatment induced reporter expression in latently infected monocytic and lymphocytic cell lines [195].

Transcription factor P-TEFb can contribute to reversal of HIV latency by promoting the elongation of HIV transcription, therefore agents that release P-TEFb are of interest in latency reversal. HMBA is a molecule which activates the PI3K/Akt pathway, leading to the phosphorylation of HEXIM and release of P-TEFb from 7SK snRNP complex [196]. HMBA has been found to have a weak effect in a primary cell latency model [197], possibly due to the negative feedback loop by which HMBA increases the expression of new HEXIM [198]. Bromodomain inhibitors are another set of candidate agents that can disrupt the binding of bromodomain Brd4 with P-TEFb and allow Tat-mediated recruitment of P-TEFb. The molecule JQ1(S) inhibits Brd4 and has been shown to reactivate HIV in cell line models and to induce HIV outgrowth in resting cells from one out of three ART-treated patients [199].

**TARGETED IMMUNOLOGIC AGENTS**

The next step in the “kick-and-kill” strategy is clearance of latently infected cells that have been reactivated to induce HIV expression. Immunologic agents that effect the clearance of cells expressing HIV proteins could enhance the “kill” step in “kick-and-kill” of the latent reservoir. On their own, they could also serve to reduce active HIV reservoirs.

Broadly neutralizing antibodies against HIV have been characterized for their ability to block viral entry and prevent acquisition in animal models [200-204] and to suppress viral replication in CD4+ T cells isolated from ART-treated patients [205]. Some studies have also demonstrated the ability of anti-HIV antibodies to induce
killing of infected cells \textit{in vitro} through antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis [206] and to accelerate the clearance of infected cells \textit{in vivo} [144].

The targeting ability of anti-HIV antibodies can be linked to a cytotoxic agent in the form of recombinant immunotoxins. Recombinant immunotoxins are fusion proteins with an antibody-derived targeting arm that binds to a target protein and a cytotoxic arm that mediates cell-killing [207]. One example is 3B3-PE38, a fusion protein formed from the 3B3 single-chain variable fragment specific for HIV gp120 and \textit{Pseudomonas aeruginosa} exotoxin A [208]. The 3B3 targeting arm recognizes HIV gp120 expressed on the surface of actively infected cells; and exotoxin A inhibits elongation factor 2, thus shutting down protein translation and leading to cell death. This immunotoxin has demonstrated cytotoxic effects against several HIV isolates as well as synergy with RT inhibitor drugs \textit{in vitro} [209], and it prevented viral rebound in HIV-infected thy/liv SCID-hu humanized mice when combined with ART during an acute infection [210]. However, ART was not able to fully suppress cell-associated HIV RNA in the thymocytes of these mice during chronic infection, and the immunotoxin reduced but did not eradicate cell-associated HIV RNA in this model. Another study of 3B3-PE38 was carried out in BLT humanized mice by Denton \textit{et al.} [211]. This report demonstrated that antiretroviral therapy reduced the frequency of infected cells and the levels of cell-associated HIV RNA systemically in the tissues of infected BLT mice, in comparison to untreated mice. The addition of the 3B3-PE38 to ART further enhanced this reduction of infected cells and cell-
associated HIV RNA in tissues, thus demonstrating a depletion of productively infected cells \textit{in vivo}.

There is evidence that HIV-specific CTLs develop in response to HIV infection in patients and are associated with initial control of viremia [212-214]. This control may be mediated in part by the ability of CTLs to recognize and kill HIV-infected cells [215]. However, in most patients, the presence of HIV-specific CTLs is not sufficient to control or clear infection completely, possibly due to the emergence of escape mutants [216] or due to CTL exhaustion [217, 218]. Therefore, strategies are being investigated to enhance the ability of CTLs to recognize HIV-infected cells for killing.

One way to enhance CTL recognition of HIV-infected cells is through the use of bispecific antibody-derived molecules that engage CTLs with target cells and redirect the CTLs to kill the target cells. This strategy is called redirected lysis. Bispecific T cell Engagers (BiTEs), initially developed for cancer therapy [219-224], are fusion proteins containing an anti-tumor targeting arm that would bind to the surface protein marker of a tumor cell, and an anti-CD3 effector arm that would bind to CD3 on T cells [225]. It was shown that when the BiTE binds to the target tumor cell, it can be presented to T cells in a multivalent fashion and trigger activation of the T cell through CD3 engagement [226]. BiTEs demonstrated high potency to recruit CD8\(^+\) T cells to kill tumor cells \textit{in vitro} and in tumor-xenograft animal models [220]. As of 2015, BiTEs that have entered clinical trials include those targeting EpCAM for gastrointestinal and lung tumors, CD19 for non-Hodgkin's lymphoma and
acute lymphoblastic leukemia, and carcinoembryonic antigen for gastrointestinal tumors [227].

An anti-HIV BiTE, VRC07-antiCD3, has been developed by inserting the variable region from the VRC07 broadly neutralizing antibody into the targeting arm, in order to target the CD4 binding site of gp120. Addition of VRC07-antiCD3 BiTE resulted in cell lysis of latently infected cell lines co-cultured with purified human T cells, and it was shown to be well-tolerated in infected rhesus macaques [228].

A newer class of bispecific antibody-derived molecules for redirected lysis are dual affinity retargeting (DART) molecules. The mechanism of action is the same for DART molecules as for BiTEs, but DART molecules differ from BiTEs in two ways. There is no intervening linker sequence between the V regions of the DART molecules, and there are two cysteine residues at the C-terminus of each chain which form a disulfide bridge [229]. These features were engineered to improve the potency of DART molecules. One comparison study showed that DART molecules demonstrated increased affinity for CD3 and CD19, increased maximum cytotoxicity, and decreased EC\textsubscript{50} against target Raji or Daudi cell lines \textit{in vitro} [230]. A DART molecule targeting CD123 for acute myeloid leukemia (CD3xCD123) has been shown to deplete CD123\textsuperscript{+} cells \textit{in vivo} in cynomolgus monkeys and to prevent tumor growth in PBMC-engrafted mice that received an intradermal injection of KG-1a cells (AML cell line) [231]. Similarly, a DART molecule targeting CD19 (CD19xTCR) also slowed tumor growth in mice injected subcutaneously with Raji tumor cells and PBMCs [230].
Anti-HIV DART molecules have been developed using variable regions that target gp120: PGT121, PGT145, VRC01, and 10E8 from broadly neutralizing antibodies, and A32 and 7B2 from non-neutralizing antibodies. Two studies of anti-HIV DART molecules demonstrated clearance of HIV-infected cells in vitro [232, 233], but efficacy still has yet to be determined in vivo.

IN VIVO PLATFORMS FOR HIV CURE RESEARCH

Although an HIV cure will ultimately need to be proven in humans, testing in human subjects carries ethical considerations of creating risk for HIV patients who are otherwise healthy while taking ART, as well as practical difficulties, including variable host/infection parameters, medication compliance, and limited access to tissue material [234, 235]. Animal models that faithfully replicate key aspects of HIV/AIDS are therefore critical for the preclinical investigation of candidate HIV cure strategies for safety and efficacy. The two most commonly used animal models for HIV cure research are non-human primates (NHPs) and humanized mice [236-240].

NON-HUMAN PRIMATES FOR HIV CURE RESEARCH

Non-human primates (NHPs) are useful for HIV cure research because they are anatomically similar to humans, and because they provide opportunities to evaluate infection in tissues with large numbers of cells for analysis [241]. However, because of the species-specific tropism of HIV, NHPs are not susceptible to HIV infection [242]. Instead, NHP are susceptible to SIV infection, which resembles HIV infection in humans in some aspects [243]. The three commonly used species include rhesus (Indian or Chinese origin), pigtailed, and cynomolgus macaques [240]. These species are all susceptible to SIV infection with varying disease
outcomes depending on the specific strain and host [243, 244]. NHPs such as African green monkeys, sooty mangabeys, and mandrills naturally control SIV infection and do not progress to AIDS; comparative studies are useful in these particular species to identify mechanisms that prevent SIV-mediated disease [245-248].

SIV infection in macaques shares some important features with HIV that are key to cure research. Like HIV, SIV DNA is integrated in the target cell genome [249, 250], and latently infected cells can be induced to express SIV through costimulatory signals [251]. SIV-infected cells are distributed similarly in the peripheral blood, lymph nodes, and mucosal sites, as are HIV-infected cells in humans [252, 253]. Also, as with HIV, CTLs can lose the ability to clear SIV-infected cells due to the emergence of CTL-escape mutations [254, 255].

There are some limitations to the use of SIV and non-human primates as an animal model for HIV research. First, there are differences between SIV and HIV with respect to their genome. SIV contains the gene Vpx, not found in HIV, which encodes the Vpx protein that counteracts the activity of SAMHD1 (Sterile Alpha Motif-domain and Histidine Aspartic-domain containing protein 1) in macrophages thus enhancing infectivity in macrophages [256-258]. Conversely, HIV has the gene Vpu, not found in SIV, which encodes the Vpu protein that enhances virion release from the cell surface. Second, SIV proteins are less sensitive to antiretroviral drugs, in particular, protease, reverse transcriptase, and integrase inhibitors. Therefore, highly intensified ART regimens are required to suppress viral replication [259, 260], or chimeric RT-SHIV strains with HIV reverse transcriptase must be used [261, 262].
Third, some SIV isolates are able to utilize alternative co-receptors (GPR1, GPR15, STRL33, CXCR6) in addition to CCR5 [263-265], but they rarely utilize CXCR4 [266, 267].

As mentioned above, a recent study in rhesus macaques was key in characterizing the timeline for establishment of the latent reservoir. Rhesus macaques were infected intrarectally with SIVmac251, and ART was initiated at days 3, 7, 10, and 14 after infection [141]. Even though the macaques were treated for 24 weeks, virus rebounded in all animals after ART interruption, demonstrating that the latent reservoir was seeded by day 3.

Rhesus macaques have also been used to test several cure strategies. The HDAC inhibitor SAHA has been shown to increase histone acetylation in SIV-infected ART-suppressed rhesus macaques and to induce a small amount of virus reactivation [268, 269]. However, SAHA has also been shown to inhibit \textit{ex vivo} proliferation of effector CD8\(^+\) T cells isolated from rhesus macaques [270], suggesting that HDAC inhibitors may negatively affect the ability of CD8\(^+\) T cells to kill infected cells. There are no published studies using protein kinase C activators in SIV-infected NHP, but a narrow therapeutic window with substantial toxicity in NHPs has been described [240].

To test the effect of pre-transplant conditioning and HSCT, three rhesus macaques were infected with SHIV, treated with ART, preconditioned with irradiation (3x3.6 Gy), and administered an autologous HSCT. ART was discontinued 40-75 days after the transplant. Two out of three of the animals rebounded, showing that
the conditioning and autologous HSCT were insufficient to eliminate the reservoir [271].

Non-human primates may be useful as preclinical models for testing candidate HIV cure strategies because of the similar features between SIV and HIV infection (CD4+ depletion, viral rebound) and because of the ability to sample large numbers of cells at successive time points. However, suppressive antiretroviral therapies will need to be optimized in this model, and differences between SIV and HIV will need to be considered in the interpretation of NHP experiments.

**HUMANIZED MICE FOR HIV CURE RESEARCH**

Humanized mice are another animal model for HIV cure research in which HIV and human immune cells can be studied *in vivo*. Humanized mice are bioengineered by implantation of human thymus/liver tissue and/or transplantation of human hematopoietic stem cells [272, 273]. The mice then become repopulated with human immune cells and are susceptible to infection with HIV; and they have been utilized for *in vivo* studies of HIV replication, transmission, and prevention, as well as evaluation of therapeutic interventions [211, 274-281].

Humanized mice are useful for cure research because they provide an *in vivo* system with access to tissue analysis, they serve as a platform for the study of HIV clones or isolates with human immune cells, they generate HLA-restricted T cell responses if human thymus is implanted, and they are able to suppress viremia with the same antiretroviral drugs used in humans. However, humanized mice are chimeras in which human cells co-exist with mouse cells. This presents challenges
that must be considered in the interpretation of results. In addition, the cell numbers and sample volumes that can be collected from a single animal are limited.

Initial HIV cure studies in humanized mice were conducted in SCID-hu mice. SCID-hu mice are SCID (severe combined immunodeficiency) mice with a thymus/liver implant; they generate human T cells only in the implant and can be infected with HIV by direct injection into the implant. This model was used to demonstrate HIV latency [282], reactivation of latently infected cells using prostratin or interleukin-7 [283, 284], and clearance with an immunotoxin [285].

NRG (NOD-Rag2−/−-gammachain−/−) mice can support human cell engraftment after sublethal irradiation and intrahepatic injection of newborn pups with human hematopoietic stem cells [286-288], to generate NRG-Hu mice. These mice engraft with T cells, B cells, macrophages, and dendritic cells systemically for about 6 months. NRG-Hu mice have been shown to generate latent infection [289]; and a combination of LRAs (vorinostat, I-BET151, and anti-CTLA4) has been used with broadly neutralizing antibodies to prevent rebound in ART-suppressed NRG-Hu mice [145].

Bone marrow/liver/thymus (BLT) humanized mice combine a human thymus/liver transplant with an autologous human hematopoietic stem cell transplant for reconstitution in NSG (NOD/SCID-gammachain−/−) mice [290]. They reconstitute with T cells, B cells, monocyte/macrophages, NK cells, and dendritic cells systemically in both tissues and mucosal sites; and they are able to mount primary human HLA-restricted immune responses, thus representing the most complete humanized mouse model to date for HIV research [291]. BLT mice have been
utilized to demonstrate infection, ART suppression, and rebound, as well as generation of latency [292, 293]. As mentioned above, 3B3-PE38 immunotoxin [211] has been tested in the BLT model, with evidence of an enhanced reduction of cell-associated HIV RNA in tissues when immunotoxin was added to ART.

Humanized mice are an excellent platform for evaluation of HIV cure strategies and acceleration of progress towards an HIV cure. Toward this goal, I have evaluated several HIV cure interventions in BLT humanized mice. First, to reproduce the conditions of the Berlin patient, I tested the effect of a CCR5delta32 allogeneic transplant in infected, suppressed BLT mice (Chapter 2). Next, as a “kick” candidate, I evaluated the effects of a latency-reversing agent, HDAC inhibitor panobinostat, on HIV infection in vivo (Chapter 3). For the “kill” strategy, I studied CD8+ T cells in the control of nef-deficient HIV in BLT mice with protective HLA alleles (Chapter 4). Finally, I showed efficacy of DART molecules to deplete target cells in BLT mice (Chapter 5).
CHAPTER 2: INVESTIGATING THE EFFECT OF CCR5DELTA32
TRANSPLANTATION ON HIV INFECTION IN VIVO

SUMMARY

The Berlin patient is the only case of possible HIV cure in a human. This patient underwent intense myeloablative and lymphoablative conditioning, followed by an allogeneic hematopoietic stem cell transplant using cells from a donor who was homozygous for CCR5delta32. We sought to investigate the hypothesis that CCR5delta32 allogeneic transplant is able to cure HIV in vivo in HIV-infected, ART-suppressed BLT humanized mice. Toward this goal, we transplanted NSG mice with stem cells obtained from a CCR5WT and a CCR5delta32 donor. We found that the CCR5delta32-derived mice engrafted with higher levels of human cells in the peripheral blood, and they were completely resistant to infection with R5-tropic HIVJRCSF but still susceptible to infection with X4-tropic and dual-tropic virus. To determine parameters for conditioning, we determined that omitting radiation during the humanization procedure of BLT mice resulted in lower engraftment levels only in the first few months of reconstitution, but afterwards there was no difference in levels of human cells in the peripheral blood between irradiated and non-irradiated mice. We also found that administration of busulfan and antithymocyte globulin resulted in depletion of human cells in BLT mice. We infected non-irradiated BLT mice with HIVJRCSF and treated with antiretroviral therapy for three weeks, then conditioned with busulfan/antithymocyte globulin and transplanted either CCR5WT or
CCR5delta32 stem cells intravenously. ART was discontinued five and a half weeks later, and all mice rebounded with viremia. Using a CCR5 genotyping PCR/digest assay, we determined that the CCR5delta32 stem cells did not engraft in the transplanted BLT mice. In these studies, we have demonstrated that CCR5delta32 cells are resistant to HIV infection in a humanized mouse model, and we have identified opportunities for improving conditioning/transplant procedures for allogeneic transplant in BLT humanized mice.

INTRODUCTION

The human immunodeficiency virus (HIV) remains a major source of morbidity, mortality, and healthcare cost worldwide. While antiretroviral therapy (ART) can drastically reduce viremia in HIV patients, ART must be continued throughout life, or the infection rebounds. Without a cure, the necessity of lifelong adherence to ART presents challenges of cost, long-term toxicity, adverse interactions, and potential resistance.

The primary target cell for HIV infection is the CD4\(^+\) T cell. The envelope glycoprotein of HIV first binds CD4 as a primary receptor, then binds CCR5 or CXCR4 as a co-receptor before membrane fusion and viral entry. Viruses that use CCR5 as a co-receptor are termed “R5-tropic” or “R5,” and those that use CXCR4, “X4-tropic” or “X4.” R5 viruses are the dominant type detected during HIV transmission and early infection, while X4 viruses are typically detected during later stages of disease [294-297]. CCR5delta32 is a mutant version of the CCR5 allele that contains a 32-base-pair deletion in the CCR5 open reading frame, resulting in a frameshift and a truncated protein. This truncated CCR5 protein is then not
expressed on the cell surface for HIV binding/entry [112]. Without CCR5 available on the surface to serve as a co-receptor for HIV entry, individuals who are homozygous for the CCR5delta32 allele are highly resistant to HIV infection [112, 113]. Heterozygous individuals may have a reduced susceptibility to infection and delayed disease progression [113]. The allele frequency is 0.092 in Caucasian populations, but it is rare or absent in Western/Central Africa and Japanese populations [113].

In 2009, Hutter et al. [114] first reported the case of “the Berlin patient,” a 40-year-old man with HIV who had undergone an allogeneic hematopoietic stem cell transplant (HSCT) using cells from a CCR5delta32-homozygous donor. After having been diagnosed with HIV more than ten years earlier and taking ART for four years, the patient was newly diagnosed with acute myeloid leukemia. For initial treatment of the leukemia, he underwent two courses of induction chemotherapy and one course of consolidation chemotherapy. During this treatment, ART was discontinued due to liver and kidney toxicity, resulting in viral rebound, after which ART was resumed and viremia returned to below detection.

Seven months after the initial treatment, the leukemia relapsed, and so the patient underwent allogeneic HSCT using CD34+ peripheral blood stem cells from an HLA-matched, unrelated donor who was homozygous for the CCR5delta32 allele. As conditioning for the transplant, the patient was treated with antithymocyte globulin, cyclosporine, and mycophenolate mofetil; then he received a graft containing $2.3 \times 10^6$ CD34+ cells per kilogram body weight. ART was discontinued the day before the procedure, and complete chimerism (replacement of his own immune cells with the donor immune cells) was achieved by 61 days after the procedure. The
leukemia relapsed again 332 days after the first transplant; and so on day 391, he received a single dose of whole-body irradiation (200cGy) and a second graft from the same donor. The second transplant resulted in complete remission of the leukemia.

Eight years after these transplants and discontinuation of ART, the patient has maintained undetectable viremia [115, 116, 298]. HIV-1 DNA could not be detected in a rectal biopsy 159 days after transplant, and HIV-specific CD8⁺ T cell responses became undetectable [114]. At follow-up, his CD4⁺ T cells reconstituted in the peripheral blood to normal ranges after 2 years, and in the gut after 29 months [115]. No CCR5-expressing T cells or macrophages were detected in liver, brain, and colon biopsies, taken at 12, 17, and 24 months post transplant, respectively [115]. In a collaborative report among several laboratories, HIV RNA, HIV DNA, and replication-competent viral outgrowth could not be detected in peripheral blood cells collected three years after the transplant [116].

The Berlin patient is a single case, and it has not been replicated in another patient or in an animal model. Questions remain as to how the cure was achieved. The Berlin patient underwent two rounds of pre-transplant conditioning including antithymocyte globulin treatment and total body irradiation, which may have resulted in the destruction of infected cells [299, 300]. The allogeneic donor cells may have provided a graft-versus-host effect where donor cells eliminated host cells, as has been observed after allogeneic transplants for leukemia [301]. The CCR5delta32 genotype of the donor cells may have been critical, as these engrafting cells would
be resistant to viral entry and unable to sustain infection. It is not yet clear to what extent each of these factors contributed to the cure of the Berlin patient.

Previous studies have reported the persistence of HIV after conditioning and autologous HSCT in humans [302-304] and persistence of SHIV after conditioning and autologous HSCT in macaques [271] despite continued ART, demonstrating that pre-transplant conditioning for autologous BMT is not sufficient for a cure. The two “Boston patients” described by Henrich et al. received CCR5-wildtype allogeneic HSCTs while continuing ART, thus providing an opportunity to examine the allogeneic graft-versus-host effect on the HIV reservoir. Over two to four years following their transplants, the HIV reservoir in their peripheral blood cells had been reduced below detection, as measured by HIV DNA and viral outgrowth [117]. However, after the patients discontinued ART, they experienced viral rebound three and eight months later respectively [118]. In this case, allogeneic transplant may have reduced the HIV reservoir, at least in the peripheral blood compartment, and it may have delayed rebound; but the procedure was not sufficient for long-term remission or cure of HIV.

In order to test the hypothesis that a CCR5delta32 allogeneic transplant is sufficient for HIV cure, we have acquired adult hematopoietic stem cells from a CCR5WT donor and a CCR5delta32 donor. In the following studies, we investigated susceptibility of CCR5WT and CCR5delta32 NSG-Hu mice to HIV infection, validated a CCR5 genotyping PCR/digest assay, tested conditioning regimens for allogeneic transplant of BLT humanized mice, and performed a CCR5delta32 allogeneic transplant experiment in infected, ART-treated BLT mice.
METHODS

ETHICS STATEMENT

All animal experiments were conducted following guidelines for housing and care of laboratory animals in accordance with University of North Carolina at Chapel Hill (UNC Chapel Hill) regulations after review and approval by the UNC Chapel Hill Institutional Animal Care and Use Committee.

GENERATION OF CCR5WT and CCR5DELTA32 NSG-HU MICE

Hematopoietic stem cells were mobilized in a CCR5WT donor (M001) and a CCR5delta32 donor (M004) after five daily doses with G-CSF (Neupogen, Amgen, Thousand Oaks, CA), enriched from leukapheresis product using CliniMACS CD34 Reagent System (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were live frozen and stored in cryovials in liquid nitrogen. Prior to transplant, cells were thawed and counted by trypan blue exclusion, then 1-2x10^6 CCR5WT (M001) cells or 4x10^6 CCR5delta32 (M004) cells were injected intrahepatically into neonatal NSG pups that had been sublethally irradiated with 200 rad.

CCR5WT AND CCR5DELTA32 GENOTYPING PCR

Genotyping PCRs were prepared with 5' CTCCCAGGAATCATCTTTACC 3' forward primer and 5' TCATTTCGACACCGAAGCAG 3' reverse primer [114] (200nM each primer), 200µM dNTPs, 1x Crimson Taq Reaction Buffer, and Crimson Taq DNA polymerase (1.25U/50µl) (M0324, New England Biolabs, Ipswich, MA). PCR products was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and incubated with 1x NEBuffer 3 and 5U Apol restriction enzyme
(R0566, New England Biolabs) at 50°C for one hour. PCR products and digests were separated by gel electrophoresis on a 2% agarose gel.

**GENERATION OF BLT HUMANIZED MICE**

BLT mice were generated as described previously [305]. NOD/SCID-gamma chain⁻/⁻ mice (NSG, Stock #5557, The Jackson Laboratories, Harbor, ME) were implanted with human thymus and liver tissue, then transplanted with autologous human liver CD34⁺ cells (Advanced Bioscience Resources, Alameda, CA); then they were monitored for human reconstitution in peripheral blood by flow cytometry as previously described [275, 305, 306].

**HIV INFECTION AND ANTIRETROVIRAL TREATMENT OF BLT MICE**

Mice were infected by intravenous exposure to 30,000 tissue culture infectious units of HIV-1JRCSF, HIV-1LAI, or HIV-189.6. Infection was monitored in peripheral blood by measuring plasma levels of vRNA as described (limit of detection = 750 copies per ml plasma from 40µl plasma sample volume) using one-step reverse transcriptase real-time PCR (Applied Biosystems custom TaqMan Assays-by-Design, Thermo Fisher, Waltham, MA) [277, 278, 292]. Antiretroviral therapy in infected BLT mice was administered by daily intraperitoneal injection (tenofovir disoproxil fumarate 208mg/kg, emtricitabine 240mg/kg, raltegravir 56mg/kg) as previously described [292]. Tissues were harvested, and cells were isolated for DNA extraction as previously described [46, 47].

**CONDITIONING AND ALLOGENEIC TRANSPLANT OF BLT MICE**

Busulfan powder (Sigma-Aldrich B2635, St. Louis, MA) was dissolved in DMSO at a stock concentration of 15mg/ml, and then diluted in PBS before
administration. Mice were injected intraperitoneally with two doses of 25mg/kg, 24 hours apart. Lyophilized antithymocyte globulin (Genzyme, Cambridge, MA) was reconstituted in sterile water, filtered through a 0.22 micron filter, then injected intraperitoneally with two doses of 0.3mg per mouse, four days apart. Hematopoietic stem cells were isolated and frozen as described above from a CCR5WT patient (M002) and a CCR5delta32 patient (M004). Prior to transplant, cells were thawed and counted by trypan blue exclusion, and then 3.5x10^6 cells were injected intravenously into conditioned BLT mice.

**METHYLCELLULOSE CULTURE**

Cells were isolated from the bone marrow of mice as previously described [275, 306]. Bone marrow cells (2x10^6) were incubated overnight in IMDM with 1% bovine serum albumin and penicillin/streptomycin for adherence depletion. The following day, non-adherent cells were harvested and counted by trypan blue exclusion. 100,000 cells were plated with 3ml Methocult H4434 Classic (04444, Stemcell Technologies, Vancouver, Canada) and incubated at 37°C for three weeks. Colonies were aspirated, pelleted, and frozen at -80°C before DNA extraction by digest with Proteinase K (03115887001, Roche, Basel, Switzerland).

**STATISTICAL TESTS**

All statistical tests were performed using an alpha level of 0.05. Graphs were generated in Graphpad Prism (v. 6). Unpaired t test was utilized in Figures 2.1 and 2.4 with correction for multiple comparisons by Holm-Sidak method.
RESULTS

TRANSPLANTATION OF MICE WITH CCR5WT OR CCR5DELTA32 STEM CELLS

First, we confirmed the ability of the CCR5WT and CCR5delta32 stem cells to engraft in NSG mice. Neonatal NSG mice were irradiated and injected intrahepatically with either CCR5WT or CCR5delta32 stem cells to generate NSG-Hu mice, and then they were monitored for human immune reconstitution in the peripheral blood by flow cytometry. Specifically, peripheral blood cells were stained for surface markers human CD45 (hematopoietic cells), CD3 (T cells), and CD4 (CD4+ T cells). At week 23 post transplant, the cohort of mice that received CCR5delta32 cells had an average 51.6% human (CD45+) cells in peripheral blood. The CCR5WT cohort had an average of 10.6% human cells in peripheral blood (Fig. 2.1a). This difference was statistically significant, and the percentage of human cells in the peripheral blood remained higher in the CCR5delta32 cohort through week 39. The percentage CD3+ T cells was also higher in the CCR5delta32 cohort at week 23, but the differences were not significant at other time points (Fig. 2.1b). By week 31, the percentage of CD3+ T cells was greater than 90% in both cohorts. The percentage of CD4+ T cells was significantly higher in the CCR5delta32 mice (80%) than in the CCR5WT mice (60%) at weeks 31 and 35 (Fig. 2.1c).

These data demonstrate that the mobilized stem cells were able to engraft and reconstitute human hematopoietic cells in the NSG mice, including CD4+ T cells. The mice generated from the CCR5delta32 donor had greater reconstitution of human hematopoietic cells in the peripheral blood than recipient mice transplanted
with the CCR5WT donor. Therefore, it is unlikely that any difference in susceptibility of the CCR5delta32 mice to HIV infection could be explained by lower levels of human CD4+ T cells, which are the target cells for infection.

**SUSCEPTIBILITY OF CCR5WT AND CCR5DELTA32 MICE TO HIV INFECTION**

To determine their susceptibility to R5-tropic HIV infection, CCR5WT and CCR5delta32 NSG-Hu mice were challenged by intravenous exposure to R5-tropic HIVJRCSF. After exposure, all three CCR5WT mice had detectable viral loads by week 3, while none of the four CCR5delta32 mice had detectable viral loads over 8 weeks (Fig. 2.2a). To determine their susceptibility to X4-tropic HIV infection, mice from both cohorts were exposed to X4-tropic HIVLAI. CCR5WT mice (3/3) were viremic by week 2 after exposure, and CCR5delta32 mice (2/2) were viremic as early as week 1 after exposure (Fig. 2.2b). To determine the susceptibility of CCR5delta32 mice to dual-tropic virus, three CCR5delta32 mice were exposed to HIV89.6, and 2 out of 3 mice were viremic by week 1, with the third mouse becoming viremic at week 2 (Fig. 2.2c). These results show that both R5-tropic and X4-tropic HIV replicated *in vivo* in the CCR5WT mice, and that only X4- or dual-tropic HIV replicated *in vivo* in the CCR5delta32 mice, while R5-tropic virus did not.

**ANALYSIS OF CCR5WT AND CCR5DELTA32 GENOTYPE BY PCR/DIGEST**

In order to distinguish reconstitution with CCR5WT or CCR5delta32 cells, we tested a genotyping PCR assay with confirmation by restriction digest. Primers flanking the region of the human CCR5 gene containing the CCR5delta32 32-base pair deletion [114] were used for PCR, followed by digest with Apol restriction enzyme that recognizes a unique restriction site within the 32-base pair region (Fig.
2.3a). The expected PCR products would have sizes of 200-bp from the CCR5WT allele and 168-bp from the CCRd32 allele. After Apol restriction digest, the CCR5WT PCR product would be cleaved into 95-bp and 105-bp fragments, while the CCR5delta32 PCR product would not be cleaved. Using cloned plasmids of the CCR5WT and CCR5delta32 open reading frames as templates, we performed PCR and separated the products by agarose gel electrophoresis. The CCR5WT template yielded the expected 200-bp PCR product and Apol-digested 95-bp/105-bp fragments (Fig. 2.3b, lanes 1 and 3). The CCR5delta32 template yielded the expected 168-bp fragment PCR product and the uncleaved 168-bp fragment after Apol digest (Fig. 2.3b, lanes 2 and 4). These results validate a conventional PCR/digest assay for determination of CCR5WT or CCR5delta32 reconstitution.

**CONDITIONING OF BLT MICE FOR ALLOGENEIC TRANSPLANT**

Allogeneic transplant of BLT mice would require a conditioning regimen to deplete the existing human cells in the mice and prevent rejection of allogeneic donor cells, as is done in humans [307]. Previously, we have conditioned NSG mice with irradiation prior to the initial humanization procedure in order to facilitate engraftment of human cells. To reduce the potential for toxicity of multiple conditioning regimens, we tested human reconstitution in BLT mice that have not been irradiated versus those that have been irradiated. We generated two cohorts of BLT mice (n=10 each) from the same human donor, one cohort irradiated with 300 rad prior to humanization procedure and the other cohort not irradiated. Both cohorts were monitored for human reconstitution in the peripheral blood by flow cytometry. At weeks 6 through 13 post humanization, the irradiated mice had higher levels of
CD45\(^+\) cells in the peripheral blood compared to the non-irradiated mice (p<0.0001), but these differences were no longer significant after week 13 (Fig. 2.4a). There was a slight but significant difference in percent CD19\(^+\) at week 6 (86% in irradiated mice and 78% in non-irradiated mice, p<0.01), but there were no significant differences at later time points (Fig. 2.4b). The cohorts were not different with respect to their levels of human CD3\(^+\) cells (Fig. 2.4c), and the CD4\(^+\) T cell levels were lower in the irradiated mice only at weeks 11 and 13 post-transplant (Fig.2.4d). The kinetics of human reconstitution in irradiated and non-irradiated BLT mice show that irradiation results in higher levels of CD45\(^+\) cell engraftment in peripheral blood over the first 13 weeks after humanization; but by week 15, irradiated and non-irradiated mice reach the same level of engraftment.

Next, we tested busulfan as a myeloablative agent and antithymocyte globulin (ATG) as a lymphoablative agent for use in a conditioning regimen prior to allogeneic transplant of BLT mice. We injected two doses of 25mg/kg busulfan intraperitoneally into a non-irradiated BLT mouse, 24 hours apart. By week 6 after the injection, the percentage CD45\(^+\) cells in the peripheral blood decreased from 79% to 2.6% and continued to decrease to 0.8% at week 10 (Fig. 2.5a). In contrast, a cohort-matched BLT mouse that was not treated with busulfan maintained peripheral blood percentage CD45\(^+\) cells at or above 63%. Bone marrow cells from the busulfan-treated mouse were cultured in methylcellulose-based media for a human hematopoietic colony-forming assay, and they did not yield any erythroid or myeloid cells (data not shown). We also injected a non-irradiated BLT mouse with two doses of 0.3mg ATG intraperitoneally, four days apart. At day 4 after the first
injection, the percentage CD3+ T cells decreased from 54.8% to 18.7%; then, two more days after the second injection, this percentage dropped to 1.74% (Fig. 2.5b). In contrast, a cohort-matched BLT mouse that was not treated with ATG retained peripheral blood percentage CD3+ T cells at ~50%. These preliminary data suggest that busulfan and ATG could be effective myeloablative and lymphoablative agents, respectively, in BLT mice; and so they could be used in a conditioning regimen prior to allogeneic transplant.

**CCR5DELTA32 ALLOGENEIC TRANSPLANT IN INFECTED, SUPPRESSED BLT MICE**

Finally, we tested the effect of a CCR5delta32 allogeneic transplant in infected, suppressed BLT mice. We exposed three non-irradiated BLT mice to 90,000 tissue culture infectious units of HIV_{JRCSF} intravenously. After three weeks of infection, all three mice were started on ART (tenofovir, emtricitabine, raltegravir). After three weeks of ART, all three mice were administered busulfan (2x25mg/kg, 2 days and 1 day prior) and ATG (0.3mg, 5 days and 1 day prior) as conditioning before allogeneic transplant. For transplant, one mouse (5610, Fig. 2.6a) was administered 3.5x10^6 CCR5WT stem cells intravenously, and the other two mice (5611, Fig. 2.6b; 5612, Fig. 2.6c) were each administered 3.5x10^6 CCR5delta32 stem cells intravenously. ART was discontinued five and a half weeks after the transplant. Over the course of the experiment, peripheral blood was monitored for plasma viral load and levels of human CD45, CD3, and CD4 cells.

All three mice showed a decrease in percentage CD45+ cells in the peripheral blood immediately after the conditioning and transplant, followed by a return in
percentage CD45+ cells over time. After initiating ART, mice 5610 and 5611 reached undetectable viremia almost 6 weeks after initiation, and mouse 5612 reached undetectable viremia after 8 weeks. After discontinuing ART, mice 5610 and 5611 rebounded with detectable viremia 11 days after discontinuation, and viremia was immediately detectable in mouse 5612. These data demonstrate that the busulfan/ATG conditioning and the transplant with either CCR5WT or CCR5delta32 stem cells did not prevent rebound in infected BLT mice on ART.

**CCR5 GENOTYPING OF BLT MICE AFTER ALLOGENEIC TRANSPLANT**

One possible explanation for the lack of effect from the allogeneic transplant in this experiment is that the stem cells did not engraft in the BLT mice. To determine engraftment of the CCR5delta32 stem cells, we extracted genomic DNA from cells isolated from the tissues of 5611 and 5612, the two mice that received CCR5delta32 stem cells. The extracted DNA samples were then used as template in the CCR5-genotyping PCR described above. Each tissue from 5611 (peripheral blood, lymph node, spleen, organoid, bone marrow, liver, lung, and gut) yielded a 200-bp PCR product, similar to the band obtained with CCR5WT control plasmid (Fig. 2.7a). After ApoI digest, the PCR products were cleaved to produce the same 95-bp/105-bp band as observed with the CCR5WT control plasmid (Fig. 2.7b). These results are also representative of the tissues from mouse 5612 (data not shown). Bone marrow cells from these mice were cultured in methylcellulose-based media for a human hematopoietic colony-forming assay, and DNA was extracted from 24 myeloid colonies from mouse 5611 and 6 myeloid colonies from mouse 5612. All these samples yielded the same CCR5WT results as in Figure 2.7 (data
not shown). These results demonstrate that CCR5delta32 cells could not be detected in tissues or in hematopoietic colonies from 5611 and 5612 by conventional PCR/restriction digest, thus indicating a failure of allogeneic engraftment.

**DISCUSSION**

In these studies, we sought to investigate the hypothesis that a CCR5delta32 allogeneic transplant is sufficient for HIV cure. First, we needed to confirm that CCR5delta32 cells are resistant to HIV infection \textit{in vivo} in humanized mice. As expected, NSG-Hu mice engrafted with CCR5delta32 cells were resistant to HIV infection by an R5-tropic virus, HIV\textsubscript{JRCSF} (Fig. 2.2), and this was not due to lower levels of human cells (Fig. 2.1). This resistance was specific to the tropism of the virus because CCR5delta32 NSG-Hu mice were still susceptible to infection by an X4-tropic virus, HIV\textsubscript{LAI}, and by a dual-tropic virus, HIV\textsubscript{89.6}.

In order to monitor CCR5WT versus CCR5delta32 chimerism, we would need an assay to distinguish the CCR5 genotypes. Using primers from the Hutter \textit{et al.} report, we validated a PCR/ApoI digest assay using CCR5WT and CCR5delta32 plasmid control templates (Fig. 2.3).

Allogeneic transplant in patients requires conditioning regimens to remove host immune cells and allow the engraftment of donor immune cells. First, to avoid the potential toxicity of combining radiation before the initial humanization procedure with later conditioning regimens, we compared the human reconstitution of BLT mice that were irradiated versus those that were not irradiated. In the peripheral blood, irradiated BLT mice engrafted with higher levels of CD45\(^+\) cells in the peripheral blood in the first few months. Later on, these differences were no longer significant
between irradiated and non-irradiated BLT mice with respect to CD45+, CD3+, or CD4+ levels in the peripheral blood. These results demonstrate that non-irradiated BLT mice are non-inferior in supporting human immune reconstitution. Avoiding this initial irradiation could be preferable for an allogeneic transplant model that requires additional conditioning, as conditioning toxicity may be cumulative [308-310].

We evaluated the effect of two conditioning reagents in BLT mice, busulfan and antithymocyte globulin. Busulfan is an alkylating agent used for myeloablation prior to transplant, and it has been observed to be less toxic than radiation for humanized mice [311, 312]. Antithymocyte globulin is a polyclonal antibody directed against human T cells. Busulfan treatment resulted in a loss of human CD45+ cells in the peripheral blood of a BLT mouse by 6 weeks post treatment, as well as lack of human hematopoietic colony-forming cells from the bone marrow at harvest. ATG treatment of a BLT mouse resulted in loss of peripheral blood CD3+ cells within a week. These data are encouraging for the further investigation of busulfan and ATG in a conditioning regimen for allogeneic transplant in humanized mice.

Finally, we conducted a CCR5delta32 allogeneic transplant in BLT mice infected with HIVJRCSF and treated with antiretroviral therapy. All three mice were conditioned with busulfan and ATG, and one of the mice received CCR5WT stem cells, while the other two received CCR5delta32 stem cells. ART was administered daily from 3 weeks before transplant through 5.5 weeks after transplant, then discontinued. All three mice reached viremia below detection while on ART, but rebounded after discontinuation. Using the CCR5 genotyping PCR/digest assay, we determined that CCR5delta32 engraftment was not successful in the mice.
The results of this experiment show that the conditioning regimen used, busulfan and ATG administration, was not sufficient to result in an *in vivo* cure in this setting. However, we are not able to reach a conclusion regarding the ability of allogeneic transplant or of CCR5delta32 transplant to cure HIV in this model until we can demonstrate that allogeneic or CCR5delta32 engraftment has occurred. There are several opportunities to optimize the parameters for conditioning and allogeneic transplant. The effects of busulfan and ATG on BLT mice can be further characterized with regard to maximum tolerated dose, kinetics of myeloablation/lymphoablation, and systemic effects on tissue-resident immune cells. Alternative conditioning regimens can also be tested. Anti-human CD117 (c-Kit) antibody has been previously used for hematopoietic stem cell depletion in humanized mice [313], and an anti-CD45-saporin immunotoxin has been shown to effectively condition immunocompetent mice for transplant and induce Jurkat cell death *in vitro* [314].

Another barrier to the engraftment of the CCR5delta32 stem cells could have been HLA mismatches between the CCR5delta32 stem cells and the existing human cells in the BLT mice, resulting in a recognition and killing of the allogeneic stem cells during the transplant by the existing human immune cells. In patient settings, the gold standard is to identify a fully HLA-matched sibling or unrelated donor in order to reduce the likelihood of rejection of the donor cells [315]. This could theoretically be overcome by an optimized conditioning regimen that completely removes or suppresses the existing human cells. The goal of HLA matching in patients also is to reduce the likelihood of graft-versus-host disease in patients. However, an advantage of allogeneic transplant as compared to autologous
transplant is that it may reduce the incidence of relapse due to a graft-versus-leukemia effect in which the administration of donor lymphocytes results in recognition and killing of host leukemic cells [301, 316]. We may be able to more directly assess a possible graft-versus-HIV effect through infusion of allogeneic donor lymphocytes.

**CONTRIBUTIONS**

Michael Swanson, J. Victor Garcia, and Perry Tsai conceived the study and designed the experiments. Michael Swanson performed experiments for Figures 2.1 and 2.2. Cole Thompson performed experiment for Figure 2.4. Perry Tsai performed experiments for Figures 2.3, 2.5, 2.6, and 2.7. Perry Tsai compiled data for figures and wrote the chapter. J. Victor Garcia provided comments for the chapter.
Figure 2.1. Reconstitution in CCR5WT and CCR5delta32 NSG-Hu mice. Neonatal NSG pups were sublethally irradiated then injected intrahepatically with either CCR5WT or CCR5delta32 stem cells. Peripheral blood was collected and analyzed by flow cytometry. (a) Percentage CD45$^+$ cells out of live cells. (b) Percentage CD3$^+$ cells out of CD45$^+$ cells. (c) Percentage CD4$^+$ cells out of CD3$^+$ cells. Mean±SEM plotted. P values were calculated by unpaired t-test with correction for multiple comparisons by Holm-Sidak method, comparing CCR5WT mice in blue to CCR5delta32 mice in red. ** p<0.01, **** p<0.0001. For CCR5WT mice, n=10, 10, 9, 9, 6, 5, 4 for successive time points; for CCR5delta32 mice, n=6 for all time points.
Figure 2.2. Susceptibility of CCR5WT and CCR5delta32 mice to HIV infection. CCR5WT and CCR5delta32 NSG-Hu mice were challenged to intravenous exposure with (a) HIVJRCSF, (b) HIVLAI, or (c) HIV89.6. Plasma viral loads plotted.
Figure 2.3. CCR5 genotyping by PCR and restriction digest. (a) Segment of CCR5 open reading frame amplified by genotyping PCR primers. Annealing sites highlighted in yellow; 32-base pair region bolded and underlined; Apol restriction site highlighted in red. (b) Gel electrophoresis separation of PCR/digest products. Lane 1, PCR product from CCR5WT plasmid template; lane 2, PCR product from CCR5delta32 plasmid template; lane 3, Apol digest of CCR5WT PCR product; lane 4, Apol digest of CCR5delta32 PCR product.
Figure 2.4. Reconstitution of irradiated and non-irradiated BLT mice. BLT mice were generated after irradiation with 300 rad or after no irradiation. Peripheral blood was collected and analyzed by flow cytometry. (a) Percentage CD45$^+$ cells out of live cells. (b) Percentage CD19$^+$ cells out of CD45$^+$ cells. (c) Percentage CD3$^+$ cells out of CD45$^+$ cells. (d) Percentage CD4$^+$ cells out of CD3$^+$ cells. Mean±SEM plotted. P values were calculated by unpaired t-test with correction for multiple comparisons by Holm-Sidak method, comparing irradiated mice in red to non-irradiated mice in gray. ** p<0.01, *** p<0.001 **** p<0.0001. For irradiated mice, n=10 at week 0, 9 at week 6, 8 at week 8, and 7 at week 18. For non-irradiated mice, n=10 at all time points.
Figure 2.5. Conditioning of BLT mice by busulfan and antithymocyte globulin. A BLT mouse was treated with two intraperitoneal injections of 25mg/kg busulfan, 24 hours apart. Peripheral blood was collected and analyzed for percentage CD45$^+$ cells out of live cells in comparison to an untreated BLT mouse (a). A BLT mouse was treated with two intraperitoneal injections of 0.3mg antithymocyte globulin, four days apart. Peripheral blood was collected and analyzed for percentage CD3$^+$ cells out of CD45$^+$ cells in comparison to an untreated BLT mouse (b).
Figure 2.6. CCR5WT or CCR5delta32 allogeneic transplant in infected, ART-treated BLT mice. Three BLT mice were exposed intravenously to 90,000 tissue culture infectious units HIVJRCSF. ART was initiated after three weeks of infection. Busulfan and ATG were administered after three weeks ART, followed by intravenous injection with $3.5 \times 10^6$ CCR5WT stem cells (a, mouse 5610), or with $3.5 \times 10^6$ CCR532 stem cells (b, mouse 5611; c, mouse 5612). ART was discontinued 5.5 weeks after the transplant. Over the course of the experiment, peripheral blood was monitored for plasma viral load (red) and levels of human CD45$^+$ (grey), CD3$^+$, and CD4$^+$ (green) cells.
Figure 2.7. CCR5 genotyping of tissues from infected, ART-treated BLT mouse transplanted with CCR5delta32 stem cells. Genomic DNA was extracted from tissues of mouse 5611 and analyzed by CCR5 genotyping PCR and digest. (a) Gel electrophoresis of CCR5 genotyping PCR products. Lane 1, 100bp ladder; 2, CCR5WT plasmid; 3, CCR5delta32 plasmid; 4-11, DNA from peripheral blood, lymph nodes, spleen, thymic organoid, bone marrow, liver, lung, and gut. (b) Gel electrophoresis of Apol restriction digests. Lane 1, CCR5WT plasmid; 2, 100bp ladder; 3, CCR5delta32 plasmid; 4-11, DNA from peripheral blood, lymph nodes, spleen, thymic organoid, bone marrow, liver, lung, and gut.
CHAPTER 3:  *IN VIVO* ANALYSIS OF THE EFFECT OF PANOBINOSTAT ON CELL-ASSOCIATED HIV RNA AND DNA LEVELS AND LATENT HIV INFECTION

**SUMMARY**

The latent reservoir in resting CD4\(^+\) T cells presents a major barrier to HIV cure. Latency-reversing agents are therefore being developed with the ultimate goal of disrupting the latent state, resulting in induction of HIV expression and clearance of infected cells. In this chapter, we have investigated the *in vitro* and systemic *in vivo* effect of panobinostat, a clinically relevant pan-HDACi, on HIV latency. We showed that panobinostat induced histone acetylation in human PBMCs as well as HIV RNA expression and outgrowth of replication-competent virus *ex vivo* from resting CD4\(^+\) T cells of HIV-infected patients on suppressive antiretroviral therapy (ART). Next, we demonstrated that panobinostat induced systemic histone acetylation *in vivo* in the tissues of BLT humanized mice. Finally, in HIV-infected, ART-suppressed BLT mice, we evaluated the effect of panobinostat on systemic cell-associated HIV RNA and DNA levels and the total frequency of latently infected resting CD4\(^+\) T cells. Our data indicate that panobinostat treatment resulted in systemic increases in cellular levels of histone acetylation, a key biomarker for *in vivo* analysis of the effect of panobinostat on cell-associated HIV RNA and DNA levels and latent HIV infection. Retrovirology. 2016;13:36. doi:10.1186/s12977-016-0268-7.
vivo activity. However, panobinostat did not affect the levels of cell-associated HIV RNA, HIV DNA, or latently infected resting CD4+ T cells.

INTRODUCTION

Antiretroviral therapy (ART) is able to suppress plasma viral load in HIV-infected patients to undetectable levels, resulting in a reduction in morbidity and mortality. However, these drugs are not able to cure HIV infection, so most patients must remain on treatment indefinitely. The major barrier to cure is that persistent infection leads to viral rebound after ART is interrupted [59]. In addition to ongoing replication in lymphoid tissue sanctuary sites [73], this persistent infection resides as integrated and transcriptionally silent provirus in the genomes of resting CD4+ T cells [84-86, 91, 317, 318], creating a latent reservoir defined as a "reversibly nonproductive state of infection" with the "capacity to produce infectious virus particles" [83].

One strategy to cure HIV is to purge the latent reservoir through latency reversal followed by the clearance of infected cells [156, 185, 319]. Specifically, latency-reversing agents (LRAs) would induce HIV RNA transcription and viral protein synthesis, followed by death of infected cells mediated by viral cytopathic effects, the host immune system, or a targeted cytotoxic agent. HIV latency is maintained partly by the action of histone deacetylase (HDAC) enzymes. Specifically, deacetylation of histones contributes to a restricted chromatin environment and to transcriptional repression of HIV [320, 321]. To reverse this mechanism, HDAC inhibitors (HDACi) have been extensively investigated as potential LRAs [322-326].
In particular, panobinostat (pan-HDAC inhibitor, LBH589) has been studied for its ability to disrupt HIV latency. Previous studies have shown that panobinostat induces HIV expression in latently infected cell line models like U1 and ACH2 [327], in primary CD4+ T cell models of latency [327, 328], and in resting CD4+ T cells isolated from chronically infected, ART-suppressed patients [329, 330]. Clinical trials are currently underway in humans [184, 331]; and initial results indicate that, although in vivo administration of panobinostat resulted in a modest (3.5-fold) increase of cell-associated HIV RNA in peripheral blood, it did not reduce the size of the latent reservoir in the panobinostat-treated patients [184].

While HIV cure interventions will ultimately need to be proven effective in humans, animal models such as rhesus macaques or humanized mice are advantageous for preclinical investigation of candidate latency-reversing agents, particularly because of the opportunity to analyze multiple tissue samples other than peripheral blood. In vivo analysis of HDAC inhibitors in non-human primates have provided conflicting results regarding their ability to induce HIV expression. In one study, SIVmac239-infected ART-suppressed Indian rhesus macaques showed increases in peripheral blood cell-associated HIV RNA after administration of the HDAC inhibitor vorinostat [269]. In contrast, in another study, vorinostat did not significantly change viral RNA levels in another study of SIVmac251-infected ART-suppressed Chinese rhesus macaques [268].

We used bone marrow–liver–thymus (BLT) humanized mice to investigate the systemic in vivo effect of panobinostat on histone acetylation, cell-associated HIV RNA, HIV DNA, and latently infected resting CD4+ T cells. BLT mice have been
shown to recapitulate key features of HIV transmission, infection, pathogenesis, and treatment [211, 272, 274, 278-281, 291, 332-336]. Furthermore, the frequency of latently infected resting CD4+ T cells from tissues isolated from HIV-infected, ART-suppressed BLT mice has been previously measured using a quantitative viral outgrowth assay (QVOA) [292]. In this current study, we demonstrated that, despite robust levels of systemic histone acetylation after panobinostat treatment, there were no detectable changes in the levels of cell-associated HIV RNA, HIV DNA, or latently infected resting CD4+ T cells. These results are largely consistent with those previously reported in human studies.

METHODS

ETHICS STATEMENT

All animal experiments were conducted following guidelines for housing and care of laboratory animals in accordance with University of North Carolina at Chapel Hill (UNC Chapel Hill) regulations after review and approval by the UNC Chapel Hill Institutional Animal Care and Use Committee. HIV-infected patients receiving stable, standard-of-care ART with plasma HIV-1 RNA <50 copies/ml and a CD4 count of >300/µl for at least 6 months were enrolled following informed consent. Studies were approved by the UNC Chapel Hill institutional biomedical review board and the Food and Drug Administration.

ISOLATION OF RESTING HUMAN CD4+ T CELLS FOR RNA INDUCTION AND QUANTITATIVE VIRAL OUTGROWTH ASSAY

Mononuclear cells were isolated from patient leukapheresis products or were pooled from the peripheral blood, lymph nodes, bone marrow, spleen, liver, lung,
and thymic organoid of each mouse (one mouse = one pooled sample). Samples from humanized mice were first enriched for human cells using an EasySep Mouse/Human Chimera Isolation Kit (#19849, Stemcell Technologies, Vancouver, Canada). Resting human CD4+ T cells were negatively selected by magnetic separation from each sample essentially as described from human leukapheresis product [337] and from BLT mice [292]. Briefly, cells were incubated with antibodies against murine CD45 and TER119 and against human CD8, CD14, CD16, CD19, CD56, glycophorin A, CD41, HLA-DR, CD25 (35 µl/ml) (Stemcell Technologies), CD31, and CD105 (0.5 µg/ml) (eBiosciences, San Diego, CA). Cells bound to antibody were removed by magnetic separation with EasySep (mice) or StemSep (human) custom isolation kit (Stemcell Technologies), and the flowthrough containing purified resting human CD4+ T cells was collected.

**MEASUREMENT OF RNA INDUCTION AND QUANTITATIVE VIRAL OUTGROWTH FROM RESTING CELLS**

Purified resting CD4+ T cells (10–12x10^6) were cultured in media alone (untreated) or panobinostat (20 nM) overnight (18–20 h) for RNA induction, then aliquoted into wells containing 1x10^6 cells each. Total RNA was isolated from each well using the Magmax 96 Total RNA isolation kit (Ambion, Austin, TX). Duplicate pools of cDNA were synthesized from DNase-treated RNA using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA). Two additional wells from each treatment condition did not include reverse transcriptase and served as control for DNA contamination. PCR amplification of cDNA was performed in triplicate using the Biorad CFX 96 Real-Time PCR detection system (Biorad,
Hercules, CA) with previously published primers and probe [338]. A standard curve was generated for each PCR reaction using cDNA synthesized from in vitro-transcribed RNA where the p5'HIV plasmid served as template [339]. Results of the triplicate PCR replicates were averaged.

For QVOA, purified resting cells were cultured in the presence of 15 nM efavirenz or 4 µM abacavir, and 1 µM raltegravir, for 24 hours. Cells where then stimulated in limiting dilution cultures containing panobinostat (20 nM), phytohemagglutinin (PHA, 1 µg/ml) or vehicle (DMSO, 0.0002 %) [337]. The frequency of infectious units per million or per billion resting human CD4⁺ T cells was calculated as a maximum likelihood estimate (or median posterior estimate if all wells were negative) using an IUPM calculator and IUPMStats v.1.0 as previously described [340].

**GENERATION OF BLT HUMANIZED MICE**

BLT mice were generated as described previously [306]. NOD/SCID-gamma chain⁻/⁻ mice (NSG, stock #5557, The Jackson Laboratory, Bar Harbor, ME) mice were sublethally irradiated, implanted with human thymus and liver tissue, and transplanted with autologous human liver CD34⁺ cells (Advanced Bioscience Resources, Alameda, CA). Transplanted/implanted mice were monitored longitudinally for human reconstitution in peripheral blood by flow cytometry [275, 292, 306]. BLT mice (n = 21) used for these experiments contained an average of 51.6 % ± 17.7 SD human CD45⁺ cells in the peripheral blood, of which 60.3 % ± 25.1 SD expressed CD3 on their cell surface. The percentage of human CD3⁺ cells expressing human CD4 was 74.7 % ± 9.3 SD.
ANALYSIS OF HISTONE ACETYLATION

Human PBMCs were exposed in vitro for 6 hours to panobinostat, and H3 acetylation was measured by flow cytometry as previously described [341]. H4 acetylation in cells from BLT tissues was assessed by ELISA. Mononuclear cells from each tissue were isolated as previously described [275, 306]. Total cell numbers harvested from each tissue are summarized as follows: average 1.4x10^7 ± 5.3x10^6 SD bone marrow, 1.5x10^7 ± 5.9x10^6 SD liver, 4.3x10^6 ± 2.0x10^6 SD lung, 1.5x10^6 ± 1.1x10^6 SD lymph node, 1.4x10^7 ± 7.3x10^6 SD spleen, 9.7x10^7 ± 1.0x10^8 SD thymic organoid. Cell aliquots were pelleted and resuspended in 200 µl 1% Triton X-100 (#X100, Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS), then further diluted in PBS with 3% bovine serum albumin (BSA). ELISA plates (Corning Costar, Corning, NY) were coated with 2 µg/ml of anti-H4 monoclonal antibody (MBL, Woburn, MA) in 100 µl coating buffer (Sigma-Aldrich, St. Louis, MO) and incubated overnight at 4 °C. Plates were washed for 5 minutes in 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) in PBS followed by blocking with 3% BSA/PBS for 2 hours to minimize non-specific binding. Cell lysates (100 µl) were added to 50 µl 1:500 anti-H4K5/8/12/16 monoclonal antibody (Millipore, Billerica, MA) conjugated to alkaline phosphatase. Plates were incubated overnight at 4 °C with gentle shaking followed by five washes with 0.05% Tween 20/PBS and gentle shaking. 100 µl of Tropix CDP-Star Sapphire II substrate (Applied Biosystems, Carlsbad, CA) was added to each well, and plates were incubated at room temperature for 20 min, followed by measurement of luminescence counts using an EnVision plate reader (Perkin Elmer, Waltham, MA).
HIV INFECTION AND TREATMENT OF BLT MICE

BLT mice were infected by intravenous exposure to $3 \times 10^4$ tissue culture infectious units HIV-1JR-CSF. Infection was monitored in peripheral blood by measuring plasma levels of HIV RNA as previously described using one-step reverse transcriptase real-time PCR (limit of detection = 750 copies per ml from 40 µl plasma sample volume) (custom TaqMan Assays-by-Design, Applied Biosystems, Grand Island, NY) [278, 292].

Antiretroviral therapy was administered to BLT mice via 1/2” pellets of irradiated Teklad chow 2020X containing 1500 mg emtricitabine, 1,560 mg tenofovir disoproxil fumarate, and 600 mg raltegravir per kg (Research Diets, New Brunswick, NJ). Panobinostat (LBH589, #S1030, Selleckchem, Houston, TX) was dissolved in DMSO then diluted in 10% (2-hydroxypropyl)-beta-cyclodextrin (#H107, Sigma-Aldrich, St. Louis, MO) to a final concentration 0.4% DMSO for intraperitoneal administration at a dose of 2 mg/kg. This dose was chosen after a higher dose of 5 mg/kg over 2 weeks (four doses, 3–4 days apart) resulted in 60% (3/5) mortality.

Tissues were harvested and cells isolated as previously described [46, 47]. Total cell numbers harvested from each tissue are summarized as follows: average $9.2 \times 10^7 \pm 3.1 \times 10^7$ SD bone marrow, $1.6 \times 10^7 \pm 7.6 \times 10^6$ SD liver, $5.9 \times 10^6 \pm 4.1 \times 10^6$ SD lung, $1.3 \times 10^6 \pm 1.6 \times 10^6$ SD lymph node, $1.5 \times 10^6 \pm 1.3 \times 10^6$ SD peripheral blood, $1.8 \times 10^7 \pm 2.1 \times 10^7$ SD spleen, $1.0 \times 10^8 \pm 9.5 \times 10^7$ SD thymic organoid. Cells were aliquoted for HIV DNA quantification (limit of detection = 4.5 copies), HIV RNA quantification (limit of detection = 4.5 copies), and flow cytometric analysis [275, 306]. Due to the use of carrier RNA during RNA extraction, HIV RNA
measurements were normalized to the number of human CD4\(^+\) T cells. Flow cytometry data were collected using a BD FACSCanto cytometer and analyzed using BD FACSDiva software v. 6.1.3 (BD Biosciences, San Jose, CA).

**STATISTICAL TESTS**

All statistical tests were performed using an alpha level of 0.05. Unpaired t test was utilized in Fig. 3.1a. Mann–Whitney test was utilized in Figs. 3.1b, 3.2, 3.4, 3.5 and 3.6b. Wilcoxon matched-pairs signed rank test was utilized in Fig. 3.1c. Graphs were generated in Graphpad Prism (v. 6).

**RESULTS**

**INDUCTION OF HIV EXPRESSION WITH PANOBINOSTAT FROM RESTING CD4\(^+\) T CELLS ISOLATED FROM HIV-INFECTED PATIENTS ON SUPPRESSIVE ANTIRETROVIRAL THERAPY**

First, we assessed the effect of panobinostat on histone H3 acetylation in uninfected human PBMCs by flow cytometry. After 6 hours incubation with panobinostat, there was a 3.0-fold increase in the level (mean fluorescence intensity) of histone acetylation in PBMCs with 10 nM panobinostat and a 3.5-fold increase with 20 nM panobinostat relative to DMSO control (p < 0.0001) (Fig. 3.1a).

In order to measure the effect of panobinostat on latently infected cells, we isolated resting CD4\(^+\) T cells from HIV-infected patients who were durably suppressed on ART for at least 6 months (Table 3.1). We incubated resting cells from three patients with 20 nM panobinostat overnight and measured levels of cell-associated HIV RNA. The levels of HIV RNA increased by 6.2-fold (p < 0.001), 3.7-fold (p < 0.01), and 3.6-fold (p < 0.0001) in cells from patients 5, 6, and 8,
respectively, in comparison to untreated cells (Fig. 3.1b). Resting CD4\(^+\) T cells from seven patients were also plated by limiting dilution and incubated with or without panobinostat (20 nM) to determine quantitative viral outgrowth. The mean infectious units per billion cells detected with untreated cells was 389, and the mean with panobinostat-treated cells was 630 (p<0.05) (Fig. 3.1c). Together, these results demonstrate that panobinostat induced histone acetylation in primary human lymphocytes and allowed the recovery of HIV transcription and viral outgrowth from the resting CD4\(^+\) T cells obtained from HIV-infected aviremic patients. Based on these encouraging in vitro results, we proceeded to evaluate the effect of panobinostat in vivo.

**IN VIVO HISTONE ACETYLATION IN TISSUES AFTER TREATMENT WITH PANOBINOSTAT**

Having characterized the ex vivo activity of panobinostat in primary human cells, we proceeded to perform a systemic in vivo evaluation using BLT humanized mice. BLT mice were administered either vehicle (n = 5) or a single 2 mg/kg dose of panobinostat (n = 3) by intraperitoneal injection. After 24 h, tissues were harvested, and cells were isolated for determination of histone acetylation by ELISA. The levels of histone acetylation were significantly higher in five of the six tissues analyzed (p < 0.05 for liver, lung, lymph node, spleen, and thymic organoid); the difference was also higher in the bone marrow of panobinostat-treated mice with a trend toward significance (p = 0.07) (Fig. 3.2a). Taken together, a single 2 mg/kg dose of panobinostat resulted in increased histone acetylation 24 h later in all tissues analyzed, with a median RLU of 92,806 per million cells versus 5946 in the vehicle...
group, a 15.6-fold difference that was highly statistically significant (p < 0.0001) (Fig. 3.2b). These results indicate that the administration of panobinostat resulted in systemic histone acetylation in vivo in the tissues of BLT mice at this dose and time point, and that the determination of levels of histone acetylation can be used as a biomarker for histone deacetylase inhibition in vivo.

**ANALYSIS OF THE EFFECT OF PANOBINOSTAT TREATMENT IN HIV-INFECTED, ART-SUPPRESSED BLT MICE**

In order to evaluate the in vivo effect of panobinostat on cell-associated HIV RNA, BLT mice (n = 13) were infected with HIV-1JRCSF (3x10^4 tissue culture infectious units) administered via intravenous inoculation (Fig. 3.3a). Three weeks after infection all animals had plasma viral loads greater than 1x10^6 HIV RNA copies/ml (Fig. 3.3b). All infected mice were then administered ART consisting of raltegravir, emtricitabine, and tenofovir as indicated in the Methods section. As early as 2 weeks after initiation of ART, plasma viral loads were below the limit of detection (750 copies/ml) and remained undetectable for the duration of the experiment (Fig. 3.3b). Six weeks after therapy initiation, suppressed mice were treated twice a week for 2 weeks with panobinostat (2 mg/kg intraperitoneally, n = 9 mice) or vehicle (control group, n = 4 mice) or (total four doses 3–4 days apart), in addition to the ART. Four days after last panobinostat administration, tissues from panobinostat- and vehicle-treated mice were harvested. Single cell suspensions were prepared from bone marrow, liver, lung, lymph node, spleen, thymic organoid, and peripheral blood, then analyzed by flow cytometry or used to isolate nucleic acids to quantitate HIV RNA and DNA levels by real-time PCR. There were no
significant differences (p > 0.05) in the median levels of cell-associated HIV RNA in individual tissues between the control group and panobinostat group (Fig. 3.4a). The median level of cell-associated HIV RNA of all tissues from the control group was 377 copies per 100,000 CD4+ T cells, compared to 193 from the panobinostat group. However, this difference was not statistically significant (p > 0.05) (Fig. 3.4b). Similar to the RNA results, there were no significant differences in median levels of HIV DNA in individual tissues between the groups (Fig. 3.5a). The median level of HIV DNA of all tissues from the control group was 287 copies per 100,000 CD4+ T cells, compared to 145 from the panobinostat group; but this difference was not statistically significant (p > 0.05) (Fig. 3.5b). These results indicate that panobinostat administration did not have an observable effect on the levels of cell-associated HIV RNA or HIV DNA in the tissues of infected, suppressed BLT mice.

**ANALYSIS OF THE EFFECT OF PANOBINOSTAT ON THE LEVELS OF LATENTLY INFECTED RESTING HUMAN CD4+ T CELLS**

In order to evaluate the effect of panobinostat on the latent HIV reservoir, we pooled cells from all the different tissues obtained from each HIV-infected ART-suppressed mouse and isolated resting human CD4+ T cells. The resting phenotype of the isolated cells was assessed by flow cytometry. Isolated resting cells were characterized by their lack of CD25 and HLA-DR cell surface expression (Fig. 3.6a). Consistent with their resting state, the levels of HIV RNA in cells obtained from these well suppressed animals were below the limit of detection in two out of four samples from vehicle-treated mice; the levels of HIV RNA in the other two samples had an average of 512 copies per 100,000 resting CD4+ T cells. The levels of HIV RNA
were below the limit of detection in eight out of nine samples from panobinostat-treated mice. In the single sample where we were able to quantitate HIV RNA in resting CD4\(^+\) T cells, it was 2100 copies per 100,000 resting cells. These low levels of HIV RNA in resting cells are consistent with results from human samples [90] and further illustrate the fact that, as we have previously published [292], in our QVOA analysis we are indeed evaluating latently infected resting cells.

To determine the levels of latently infected cells containing replication-competent proviruses, resting cells were first incubated with antiretroviral drugs overnight, then washed and plated for QVOA using PHA for maximal stimulation, essentially as we have previously described [292]. Under our experimental conditions, the levels of latently infected cells per million resting CD4\(^+\) T cells (IUPM) between the control and the panobinostat-treated groups were not statistically significantly different (\(p > 0.05\)) (Fig. 3.6b). Together, these results demonstrate that panobinostat administration at this dosing did not result in statistically significant differences in levels of cell-associated HIV RNA, HIV DNA, or latently infected cells in the tissues of HIV-infected, ART-suppressed BLT mice.

**DISCUSSION**

Even with effective antiretroviral therapies, a cure for HIV remains elusive, due to persistent replication in lymphoid tissue sanctuaries and latent infection of resting CD4\(^+\) T cells. Strategies to reverse latency and clear persistent infection may bring us closer to a cure by (1) inducing HIV expression in latently infected cells through latency-reversing agents, followed by (2) clearance of those cells mediated by viral cytopathic effects, immune clearance, or targeted cytotoxic agents. In this
study, we evaluated the effect of histone deacetylase inhibitor panobinostat to modulate levels of cell-associated HIV RNA and HIV DNA as well as the size of the latent reservoir in BLT humanized mice. First, we observed that panobinostat treatment in vitro increased histone acetylation in uninfected human PBMCs and modestly induced HIV RNA expression in primary resting CD4+ T cells isolated from HIV-infected, ART-suppressed human subjects (Fig. 3.1). We also showed that ex vivo treatment with panobinostat induced outgrowth of replication-competent HIV from latently infected resting cells of several HIV-infected, ART-suppressed patients. Our results confirm and support previous observations that panobinostat can reverse HIV latency in vitro [184, 327]. Next, panobinostat administration resulted in significantly higher histone acetylation levels 24 hours post dosing in tissues obtained from panobinostat-treated BLT mice compared to vehicle-treated animals (Fig. 3.2).

Following demonstration of panobinostat bioactivity in BLT mice, we investigated the effect of panobinostat administration on HIV RNA, DNA, and latency in infected BLT mice suppressed with ART. We have previously shown that, in infected BLT mice, systemic levels of HIV RNA plateau 4 weeks after ART initiation [36]. In the current experiments, BLT mice received two additional weeks of ART to ensure that steady state levels had been reached. When we administered a 2-week course of panobinostat (four doses of 2 mg/kg, 3–4 days apart) to BLT mice that were infected with HIV and suppressed on ART, we did not observe a significant difference in levels of cell-associated HIV RNA between panobinostat-treated and vehicle-treated BLT mice, 4 days after the last panobinostat administration (Fig. 3.4).
Given previous evidence that panobinostat activates transcription of HIV \textit{in vitro} \cite{184, 327, 330}, we would have predicted an increase in cell-associated HIV RNA in panobinostat-treated BLT mice. However, we have shown that, while ART dramatically reduces levels of cell-associated HIV RNA in BLT mice, actively infected cells persist and continue to express HIV RNA \cite{211}. If panobinostat did produce a specific effect of HIV activation in a rare population of latently infected cells, the increase in HIV transcription from these cells appeared to be unobservable in the context of persistent HIV RNA expression within tissues at the time point they were harvested.

If latency-reversing agents induce HIV expression in latently infected cells, then infected cells could be cleared by viral cytopathic effects or immune mechanisms; and if this effect were sufficiently profound, a reduction in cell-associated HIV DNA might be measurable. However, we did not observe a significant difference in the levels of cell-associated HIV DNA at harvest between panobinostat-treated compared to vehicle-treated BLT mice (Fig. 3.5). This result may be explained by the predominance of persisting actively infected cells \cite{211} or of cells infected with defective proviruses \cite{84, 342, 343} that do not encode viral antigens to allow clearance, thereby masking the potential reduction in cells carrying HIV DNA. Also, the quantification of HIV DNA in this study did not include measurement of integrated HIV DNA specifically. Some investigators have suggested that integrated DNA might correlate more significantly with the frequency of latently infected resting CD4$^+$ T cells \cite{343}. The adaptation and validation of
integrated HIV DNA quantification assays in fully suppressed BLT mice might be useful for an additional measure of the HIV reservoir in these models.

In order to more directly measure the frequency of cells containing latent, replication-competent HIV, we isolated resting human CD4+ T cells from the mice and utilized a QVOA, and a significant difference was not observed between the panobinostat- and vehicle-treated groups (Fig. 3.6) (average 3.5 and 5.5 IUPM, respectively). These values from BLT mouse samples reflect total latent infection measured after maximal stimulation by PHA treatment, and so it is not surprising that these values are about one order of magnitude higher than the results from resting CD4+ T cells of aviremic patients (average 500 IUPB = 0.5 IUPM) (Fig. 3.1c) which were stimulated only with panobinostat.

It should be noted that, while humanized mice are advantageous for studying HIV persistence in tissues, the limited amount of peripheral blood that can be collected makes it impractical to study the peripheral blood latent reservoir. Therefore, our data reflect tissue reservoirs, in contrast to the peripheral blood reservoirs typically measured in patients. Also, although we did not measure histone acetylation levels directly in BLT ART-suppressed mice, an increase in histone acetylation was demonstrated in uninfected mice in Fig. 3.2, and the ability of panobinostat to induce histone acetylation in humans undergoing ART has been previously demonstrated [184]. Notwithstanding, these results are similar to and complement those in previously reported clinical trials of panobinostat in HIV patients [184]; and they could be explained by insufficient induction of latent proviral genomes, insufficient clearance of latently infected cells, or a combination of the two.
One important aspect of the kick and kill approach to eradication is the implementation of effective killing strategies. Recent evidence suggests that panobinostat might alter cytotoxic T lymphocyte activity. Some groups have published observations that HDAC inhibitors may enhance the effector responses of CD8+ T cells [344-347], while others have observed that HDAC inhibitors may reduce the ability of CD8+ T cells to clear HIV-infected cells [348]. It will be of great interest in future studies to investigate the functional ability of CD8+ T cells to clear HIV-1 infected cells in vivo and the possible confounding effect of latency-reversing agents.

Nevertheless, the lack of a quantifiable response to panobinostat in this model is consistent with the limited increases observed in vitro, specifically in the induction of HIV replication from latently infected resting CD4+ T cells as demonstrated in the QVOA utilizing highly relevant patient samples. It is possible that a modified dosing or sampling regimen may reveal efficacy in our system, and this will require further study. Targeted cytotoxic agents [211, 233, 349] may be necessary as well to enable clearance. Additionally, alternative HDACis, such as romidepsin [183, 328, 350-352], or combinations of HDACis with other latency-reversing agents may further enhance HIV reactivation [330, 353] and should be tested in future experiments.

For example, NOD-Rag1−/−-gammachain−/− mice injected intrahepatically with human liver-derived CD34+ cells (NRG-Hu mice) have been used to test the effect of a combination of inducers — vorinostat, I-BET151, αCTLA4 — and broadly neutralizing antibodies. A significant number (57%) of mice treated with the
combination of inducers and antibodies failed to rebound upon therapy interruption. Cell-associated HIV RNA and DNA were largely undetectable (11/13 and 5/13, respectively) in the spleens of non-rebounders, but the frequency of latently infected cells was not quantified [145].

The data presented herein demonstrate one clear parallel between what is observed in humans and BLT humanized mice. In humans, panobinostat treatment did not result in a measurable decrease in the levels of latently infected cells. Similarly, in BLT mice, we did not observe a difference in the levels of latently infected cells between panobinostat-treated and vehicle-treated animals. The fact that treatment with a single latency-reversing agent did not result in measurable reductions in the levels of latently infected cells in humans or BLT humanized mice serves to highlight the need for the evaluation of latency-reversing agent combinations. The implementation of therapeutic interventions that couple suppressive ART with combinations of latency-reversing agents requires a rational and comprehensive approach where the individual contribution of each agent alone is evaluated, and animal models that reflect the human condition will be useful for accelerating this progress towards an HIV cure [354]

We have performed a systemic in vivo analysis in BLT humanized mice of the effect of panobinostat on histone acetylation and on cell-associated HIV RNA, DNA, and latent infection. After panobinostat treatment, we observed robust levels of systemic histone acetylation in BLT mice. However, we did not observe a statistically significant difference in the levels of cell-associated HIV RNA or HIV DNA in HIV-infected, ART-suppressed BLT mice. These results are consistent with the modest
effects of panobinostat noted *in vitro* and in HIV-infected patients. Consistent with
the results obtained in humans, in BLT mice panobinostat administration did not
result in a decrease in the levels of latently infected resting CD4⁺ T cells. Together,
the results obtained in both systems suggest that combination therapies may be
necessary to reverse latency and enable clearance.

**CONTRIBUTIONS**

Perry Tsai designed and performed the experiments, and wrote the
manuscript. Guoxin Wu developed and performed the tissue histone acetylation
analysis. Caroline E. Baker and Rae Ann Spagnuolo performed molecular analyses
of HIV RNA and DNA. William O. Thayer assisted with *in vivo* experiments. David
Margolis and Nancie Archin performed analysis of patient samples. Rosa Sanchez,
Stephanie Barrett, Bonnie Howell, and Daria Hazuda assisted with design,
implementation, and analysis of the study. J. Victor Garcia conceived the study,
designed and coordinated the experiments, and wrote the manuscript.
Figure 3.1. Effect of panobinostat on histone acetylation, HIV RNA, and viral outgrowth from patient cells. (a) Human PBMCs (n = 3) were incubated with panobinostat or DMSO control for 6 h, and histone H3 acetylation was measured by flow cytometry. (b) Resting CD4+ T cells were isolated from leukapheresis product obtained from three HIV-infected patients on suppressive antiretroviral therapy (outlined in Table 1), then pulsed with panobinostat (20 nM) or untreated. HIV RNA levels were measured from 10 to 12 individual wells (1 × 10^6 cells each) by quantitative real-time PCR. (c) Resting CD4+ T cells were isolated from leukapheresis product obtained from seven HIV-infected patients on suppressive antiretroviral therapy (outlined in Table 1), then incubated with panobinostat (20 nM) or untreated. Viral outgrowth was measured by QVOA. Mean±SEM plotted with comparison by unpaired t test in a; Mann–Whitney test in b; Wilcoxon matched-pairs signed rank test in c: ns p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Blue bars/symbols denote control; pink and red bars/symbols denote panobinostat-treated.
Figure 3.2. Panobinostat administration induces systemic histone acetylation.
NSG/BLT mice were administered panobinostat (2 mg/kg, n = 3) or vehicle (n = 5) intraperitoneally. After 24 h, tissues were harvested, and cells were isolated and resuspended in 1% Triton-X/PBS. Cell lysates were analyzed for histone acetylation by ELISA. (a) Data from individual tissues. (b) Data from all tissues. Median±interquartile range plotted with comparisons by Mann–Whitney test: ns p > 0.05, *p < 0.05, ****p < 0.0001. Green circles denote control; mustard squares denote panobinostat-treated.
Figure 3.3. Outline of panobinostat treatment of HIV-infected, ART-suppressed BLT mice. (a) Experimental outline. BLT mice were infected intravenously with HIV-1JR-CSF. Starting at 2 weeks post exposure, mice were bled weekly for plasma viral load and flow cytometry analysis. Starting at 3 weeks post exposure, mice were administered antiretroviral therapy (ART) consisting of tenofovir disoproxil fumarate, emtricitabine, and raltegravir (light blue shading). After 6 weeks of ART, one group of mice was administered panobinostat at a dose of 2 mg/kg intraperitoneally (red circles, n = 9) or vehicle (blue squares, n = 4), twice a week for 2 weeks, in addition to ART (light red shading). At the end of the experiment, mice were harvested, and cells were isolated for real-time PCR analysis and flow cytometry analysis. (b) Plasma viral load was measured by quantitative real-time PCR (limit of detection = 750 copies/ml, dashed line), and (c) %CD4+ T cells was measured by flow cytometry. Mean±SEM plotted.
Figure 3.4. Analysis of cell-associated HIV RNA levels in the tissues of infected, suppressed, panobinostat-treated BLT mice. BLT mice were infected with HIV-1JR-CSF, suppressed by antiretroviral therapy, and treated with panobinostat as described in Fig. 3.3. Cells were then isolated from peripheral blood, bone marrow, liver, lung, lymph nodes, spleen, and thymic organoid for real-time quantitative PCR analysis of cell-associated HIV RNA levels. (a) Data from individual tissues. (b) Data from all tissues. Median±interquartile range plotted with comparisons by Mann–Whitney test: ns p > 0.05. Blue circles denote control; red squares denote panobinostat-treated.
Figure 3.5. Analysis of HIV DNA levels in the tissues of infected, suppressed, panobinostat-treated BLT mice. BLT mice were infected with HIV-1JR-CSF, suppressed by antiretroviral therapy, and treated with panobinostat as described in Fig. 3.3. Cells were then isolated from peripheral blood, bone marrow, liver, lung, lymph nodes, spleen, and thymic organoid for real-time quantitative PCR analysis of HIV DNA levels. (a) Data from individual tissues. (b) Data from all tissues. Median±interquartile range plotted with comparisons by Mann–Whitney test: ns p > 0.05. Blue circles denote control; red squares denote panobinostat-treated.
Figure 3.6. Analysis of panobinostat treatment on HIV latency in infected, suppressed BLT mice. BLT mice were infected with HIV-1JR-CSF, suppressed by antiretroviral therapy, and treated with panobinostat as described in Fig. 3.3. Resting human CD4+ T cells were isolated from the pooled tissues of each mouse, and analyzed by flow cytometry (a). (b) Numbers of latently infected cells per million resting CD4+ T cells were determined by quantitative viral outgrowth assay. Median±interquartile range plotted with comparisons by Mann–Whitney test: ns p > 0.05. Blue circles denote control; red squares denote panobinostat-treated.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Time on ART (months)</th>
<th>Time undetectable (months)</th>
<th>Current viral load (copies/ml)</th>
<th>Current CD4 count (cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>Male</td>
<td>White</td>
<td>74</td>
<td>70</td>
<td>&lt;40</td>
<td>977</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>Male</td>
<td>White</td>
<td>76</td>
<td>&gt;6</td>
<td>&lt;50</td>
<td>720</td>
</tr>
<tr>
<td>3</td>
<td>N/A</td>
<td>Male</td>
<td>White</td>
<td>38</td>
<td>32</td>
<td>&lt;40</td>
<td>1145</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
<td>Male</td>
<td>White</td>
<td>121</td>
<td>83</td>
<td>&lt;40</td>
<td>487</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>Male</td>
<td>Black/African American</td>
<td>53</td>
<td>52</td>
<td>&lt;40</td>
<td>798</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>Male</td>
<td>Black/African American</td>
<td>235</td>
<td>110</td>
<td>&lt;20</td>
<td>1302</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
<td>Male</td>
<td>White</td>
<td>193</td>
<td>76</td>
<td>&lt;40</td>
<td>746</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>Male</td>
<td>White</td>
<td>267</td>
<td>75</td>
<td>&lt;40</td>
<td>439</td>
</tr>
</tbody>
</table>

**Table 3.1. Patient characteristics.**
CHAPTER 4: CD8-MEDIATED CONTROL OF INFECTION IN BLT MICE

SUMMARY

CD8+ T cells, or CTLs, may be able contribute to clearance of infected cells toward HIV cure. We sought to investigate the ability of CD8+ T cells to control HIV infection in three cohorts of BLT humanized mice, generated from donors with protective HLA alleles HLAB*2705 and HLAB*4402. These mice did not control wildtype HIV JRCSF (JRCSF) infection. In contrast, several mice infected with a nef-deleted mutant of HIV JRCSF (JRCSF Nef dd) were able to control viremia to below detection without antiretroviral therapy (2/6 and 3/6 in cohorts C19 and X19, respectively). Overall, the peak viral loads and average viral loads were lower in JRCSF Nef dd-infected mice than in JRCSF-infected mice in cohorts C19 and X19. We were also able to detect HLA-restricted, HIV-specific CD8+ T cells in the mice by pentamer staining, as well as functional IFN-gamma secretion by ELISPOT and intracellular cytokine staining. However, frequencies of pentamer-positive CD8+ T cells were not significantly different between JRCSF- and JRCSF Nef dd-infected mice. Finally, we performed CD8+ T cell depletion in several mice that were controlling infection and observed rapid increases in viral load. These studies demonstrate that functional, HIV-specific CD8+ T cells are generated in HIV-infected BLT humanized mice, but these cells do not control wildtype HIV infection. CD8+ T cells can mediate control of infection with JRCSF Nef dd, possibly because nef-deleted virus does not downmodulate MHC class I on the surface of infected cells.
INTRODUCTION

A cure for HIV would require clearance of persistent reservoirs of replication-competent HIV, including actively infected cells and latently infected cells that have been reactivated with a latency-reversing agent. CD8+ T cells, or cytotoxic T lymphocytes (CTLs), kill infected cells by recognizing viral peptide antigens bound to MHC class I on the surface. Therefore, CTLs could play an important role in the clearance of HIV-infected cells.

There is evidence that CTLs are involved in the control of HIV infection. Virus-specific CTL responses arise during primary infection, prior to the production of neutralizing antibodies [213]; and the level of CTL activity in a patient correlates with the efficiency with which they control primary viremia [212]. CTLs also exert selective pressure on HIV, as demonstrated by the generation of mutations to escape CTL recognition [355].

The association of certain MHC class I alleles with a lack of disease progression or with viral control without ART implicates CTL immunity in the control of infection [356]. Specifically, HLA-B57 and HLA-B27 have both been linked to controller status [357]. High levels of CTL activity have been associated with ongoing control [358-360], while the failure of CTLs to respond to escape mutants is associated with disease progression [361-365].

In addition to CTL escape, HIV is able to evade the CTL response by downmodulating surface expression of MHC class I through the viral protein Nef [31]. This downmodulation decreases recognition of infected cells by CTLs, resulting in evasion of CTL recognition and killing [32, 366]. Nef clones with deletions or with
deficiencies in CD4/MHC class I downregulation have been observed in case reports of long term non-progressors [367-370] and elite controllers [371]. Notably, a group of six patients, known as the Sydney Blood Bank Cohort, acquired HIV through blood transfusions from a common donor [372]. They were all infected with HIV containing deletions in the nef and nef-LTR regions [370]. Three of the six, in addition to the donor, exhibited slow disease progression over 10 years [373]; and three remained asymptomatic and undetectable for viremia for 26, 28, and 29 years [374, 375]. Four of the patients also exhibited strong, durable CTL responses to HIV Gag and Pol [376].

Nef-deleted virus has also been utilized as a candidate live attenuated vaccine (LAV) in rhesus macaque studies. Infection using SIVmac239/nef-deletion resulted in low viral burden in macaques [377], as well as complete or partial protection (2 log reduction or more in peak viral load and viral setpoint) from a subsequent challenge with wildtype virus [378-380]. Viral control was mediated at least in part by CD8\(^+\) T cells, based on evidence that depletion of CD8\(^+\) T cells resulted in increases in viremia [381, 382].

There is little data so far regarding the generation of CTL responses in BLT humanized mice, particularly in response to HIV infection. In the initial characterization of the BLT mouse model, functional CTL responses were demonstrated by increases in Vbeta2+ T cells and plasma cytokine levels after administration of toxic shock syndrome toxin-1 [306]. Increases in CD45RA\(^-\)CD27\(^+\) central memory T cells and \textit{ex vivo} production of IFN-gamma were also documented in response to infection with Epstein-Barr virus [306].
Regarding HIV, increases in viral replication have been reported after CD8+ T cell depletion in HIV-infected NSG-Hu mice [383], but some groups have not been able to detect CTL responses to HIV in humanized mice [384, 385]. A limitation to these studies is that they utilized humanized mouse models that did not include a human thymus needed for HLA-restricted T cell education. The BLT humanized mouse model features a human thymus implant, and HLA-restricted HIV-specific CTL responses have been observed from BLT mice, using IFN-gamma ELISPOT and intracellular cytokine staining [332, 386]. Dudek et al. also demonstrated viral load reduction with an HLAB*57 cohort in addition to viral sequence evolution in vivo in BLT mice and the generation of escape mutations within CTL epitopes [386].

We hypothesize that CTLs respond to HIV and contribute to the control of HIV infection in BLT humanized mice. To investigate this hypothesis, we identified cohorts of BLT mice generated from donors with known protective HLA alleles. In these mice, we assessed the CTL response to HIV through infection with wildtype HIVJRCSF (JRCSF) and nef-deleted HIVJRCSF (JRCSFNeft), HLA-restricted pentamer staining, ex vivo ELISPOT and intracellular cytokine staining, and in vivo CD8+ T cell depletion.

METHODS

ETHICS STATEMENT

All animal experiments were conducted following guidelines for housing and care of laboratory animals in accordance with University of North Carolina at Chapel Hill (UNC Chapel Hill) regulations after review and approval by the UNC Chapel Hill Institutional Animal Care and Use Committee.
GENERATION AND INFECTION OF BLT MICE

BLT mice were generated as described previously [306]. NOD/SCID-gamma chain−/− mice (NSG, stock #5557, The Jackson Laboratory, Bar Harbor, ME) mice were sublethally irradiated, implanted with human thymus and liver tissue, and transplanted with autologous human liver CD34+ cells (Advanced Bioscience Resources, Alameda, CA); then they were monitored for human reconstitution in peripheral blood by flow cytometry [275, 292, 306]. Percentages of CD45+, CD3+, and CD4+ cells from each cohort of mice are described in Table 4.1.

Stocks of JRCSF [387] and JRCSF Nef dd [336] were prepared as previously described [388, 389]. BLT mice were exposed intravenously to 90,000 tissue culture infectious units of JRCSF or JRCSF Nef dd. Infection was monitored in peripheral blood by measuring plasma levels of HIV RNA as previously described (limit of detection = 1500 copies per ml from 20µl plasma sample volume) using one-step reverse transcriptase real-time PCR (custom TaqMan Assays-by-Design, Applied Biosystems, Grand Island, NY) [278, 292]. Average viral loads were calculated as the total area under the curve of viral load divided by the total number of weeks of infection. Tissues were harvested and cells isolated as previously described [275, 306] for ELISPOT and flow cytometry described below.

For CD8+ T cell depletion experiments, BLT mice were injected intravenously with one 3mg/kg dose of anti-CD8α antibody MT807R (a gift from Dr. Guido Silvestri, Emory University School of Medicine, Atlanta, GA).
HLA HAPLOTYPING

Cells from the human fetal livers used for the generation of BLT mice were initially screened for HLA haplotype by flow cytometry staining with the following antibodies: anti-CD45 APC (clone HI30 #555485) (BD Biosciences, San Jose, CA), B7/27 FITC (FH1453), B27 FITC (B27F50X) (One Lambda, Canoga Park, CA), A2/A28 PE (clone REA143 #130-099-582), B12 PerCP-Vio700 (clone REA138 #130-099-873), A9 PE-Vio770 (clone REA127 #130-099-548), and B8 APC-Vio770 (clone REA145 #130-099-590) (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry data were acquired using a BD FACSCanto Cytometer and analyzed using BD FACSDiva software (v. 6.1.3).

To confirm HLA haplotype, genomic DNA was extracted using Promega Maxwell automated DNA extractor and kits (Madison, WI), and sequences for HLA haplotyping were acquired using SeCore HLA typing reagents on an ABI3500 capillary sequencer and analyzed using uTYPE software (Life Technologies, Carlsbad, CA). Ambiguous allele combinations were resolved by sequence-specific oligonucleotide probe hybridization (Thermo Fisher, Waltham, MA).

PENTAMER STAINING

To detect HLAB*2705/KK10-specific T cells, HLAB*2705/KK10 (KRWIILGLNK, Gag peptide 263-272) R-PE-labeled Pro5 MHC Class I Pentamer was acquired from ProlImmune (Oxford, United Kingdom). Adapted from the manufacturer’s protocol, cells were washed in 0.1%BSA/PBS wash buffer, incubated with 10µl pentamer for 10 minutes at room temperature shielded from light, washed, then incubated for 30 minutes at 4°C with anti-human CD45 APC (clone HI30
Flow cytometry data were acquired using a BD FACSCanto Cytometer and analyzed using BD FACSDiva software (v. 6.1.3) with the following gating strategy: live $\rightarrow$ 7AAD neg $\rightarrow$ CD45$^+$ $\rightarrow$ CD3$^+$ $\rightarrow$ CD8$^+$ $\rightarrow$ pentamer-positive.

**ELISPOT ASSAY**

Autologous B cell lines (BCLs) were produced by infecting donor cells with EBV supernatant from B95-8 cells [390, 391], then maintained in R10 culture media (RPMI1640 with 10%heat-inactivated fetal calf serum, 10mM HEPES, 2mM L-glutamine, 1mM sodium pyruvate, 1x penicillin/streptomycin).

BCLs were irradiated (5000cGy) then pulsed for one hour in 100µl R10 containing 10µg KK10 peptide or 1µg Gag peptide pool per 5x10$^6$ cells at 37°C, in a 5% CO$_2$ humidified incubator. Mononuclear cells from mouse tissues (2-8x10$^5$) were incubated with peptide-pulsed BCLs (ratio of 1:2 in 200µl) in triplicate on a 96-well Immobilon-P filter plate (Millipore, Billerica, MA) pre-coated with anti-human IFN-gamma antibody (clone 1-D1K) (Mabtech, Nacka Strand, Sweden). The cells were incubated with 1:100 Fastimmune CD28/49d costimulatory reagent (347690 BD Biosciences) for 18-20 hours at 37°C, then the cells were removed. The plates washed with 0.05% Tween/PBS, then incubated with biotinylated mouse anti-human IFN-gamma antibody (clone 7-B6-1, Mabtech). Wells were developed using Vectastain Elite ABC horseradish peroxidase kit (PK-6100, Vector Laboratories, Burlingame, CA). Negative control wells were set up using non-pulsed BCLs, and positive control wells were set up using PMA (1ng/ml) and ionomycin (50ng/ml).
ELISPOT data was collected using an AID MultiSpot reader and analyzed using AID EliSpot software (version 7.0) (Autoimmun Diagnostika, Strasburg, Germany).

**INTRACELLULAR CYTOKINE STAINING**

Cells isolated from liver were incubated with peptide-pulsed BCLs (ratio of 1:2 in 200µl) with monensin (420701) (Biolegend, San Diego, CA) for 6 hours at 37°C in a 5% CO₂ humidified incubator. After stimulation, cells were washed and stained for 20 minutes at room temperature with surface antibodies: anti-CD56 PerCP (clone HCD56 #318342), CD19 (clone HIB19 #302228), CD16 PerCP (clone 3G8 #302030), CD14 PerCP (clone HCD14 #435632), CD8 BV510 (clone RPA-T8 #301048), CD4 AF488 (clone OKT4 #317420) (Biolegend, San Diego, CA), CD3 BV421 (clone UCHT1 #562426) (BD Biosciences, San Jose, CA). Cells were fixed in Fixation Buffer (420801), permeabilized in Permeabilization wash buffer (421002), and stained with anti-IFN-gamma PE (clone 4S.B3 #502509) (Biolegend, San Diego, CA). Flow cytometry data were acquired using a BD LSR II Fortessa Cytometer and analyzed using BD FACSDiva software (v. 6.1.3) with the following gating strategy: live → CD14/CD16/CD19/CD56 negative → CD3⁺ → CD8⁺ → IFN-gamma.

**STATISTICAL TESTS**

All statistical tests were performed using an alpha level < 0.05. Graphs were generated in Graphpad Prism (v. 6). Mann-Whitney test was used in Figure 4.3, 4.4f,g.
RESULTS

IDENTIFICATION OF BLT COHORTS WITH HIV-PROTECTIVE HLA ALLELES

To identify cohorts of BLT mice with HIV-protective HLA alleles, we initially screened cells from the fetal livers used to generate the BLT mice by flow cytometry with HLA allele-specific antibodies (Fig. 4.1). This was then followed by confirmation by HLA haplotype sequencing. Out of 64 cohorts, we identified three cohorts with protective HLA alleles: cohort C19 with HLAB*2705/B*4402, cohort X19 with HLAB*3501/B*4402, and cohort J22 with HLAB*2705/B*4601 (Table 4.1). HLAB*2705 [392-394] and HLAB*4402 [395-397] have been associated with lower viral loads in HIV patients.

REPLICATION OF JRCSF AND JRCSFNefdd in BLT MICE

To examine the potential for control of HIV infection, we exposed mice from these three cohorts intravenously to 90,000 tissue culture infectious units JRCSF or JRCSFNefdd [336]. Viral loads are plotted from mice in the C19 cohort (Fig. 4.2a-b), X19 cohort (Fig. 4.2c-d), and J22 cohort (Fig. 4.2e-f). Notably, viral loads in two out of six JRCSFNefdd-infected C19 mice and in three out of six JRCSFNefdd-infected X19 mice declined to levels at or below the limit of detection (1500 copies/ml), without antiretroviral therapy.

Comparing the peak viral loads (Fig. 4.3a-c), the JRCSF-infected C19 mice exhibited significantly higher peak viral loads than JRCSFNefdd-infected (p=0.0173) (Fig 4.3a). JRCSF-infected X19 mice also had higher peak viral loads than JRCSFNefdd-infected, with a trend toward significance (p=0.0649) (Fig. 4.3b). Comparing average viral loads as time-weighted area under the curve, JRCSF-
infected mice had higher average viral loads than JRCSFNefdd-infected mice in both C19 and X19 cohorts (Fig. 4.3d-e). Among the J22 mice, neither the peak nor the average viral loads were significantly different between JRCSF- and JRCSFNefdd-infected mice (Fig. 4.3c,f). Together, these data suggest that JRCSFNefdd replication may be blunted and even controlled in some mice with protective HLA alleles.

**DETECTION OF FUNCTIONAL HIV-SPECIFIC CTL RESPONSES**

To measure HIV-specific CTL responses in BLT humanized mice, we performed pentamer staining on mononuclear cells isolated from the tissues of infected mice. HLAB*2705 patients have been characterized by an HLAB*2705-restricted immunodominant response to a conserved epitope in HIV Gag known as KK10 (amino acids 263-272, KRWIIILGLNK) [398], therefore we used fluorescently-tagged pentamers derived from the HLAB*2705/KK10 peptide/MHC complex to detect T cells with T cell receptors specific for KK10 (Fig. 4.4a).

We detected HLAB*2705/KK10-specific CD8+ T cells by pentamer staining in the bone marrow, liver, lung, thymic organoid, and spleen from two C19/JRCSF mice and two C19/JRCSFNefdd mice (Fig. 4.4b-c). The frequencies of pentamer-positive CD8+ T cells ranged from 0.1% to 2.3% in tissues of JRCSF-infected C19 mice, and from 0.1% to 3.1% in tissues of JRCSFNefdd-infected C19 mice. HLAB*2705/KK10-specific CD8+ T cells were also detected in the same tissues obtained from the mice in cohort J22, three infected with JRCSF and four infected with JRCSFNefdd (Fig. 4.4d-e). The frequencies of pentamer-positive CD8+ T cells
ranged from 0.01% to 1.3% in tissues of JRCSF-infected J22 mice, and from 0.02% to 15.6% in tissues of JRCSF\textit{Nef}\textit{dd}-infected J22 mice. Mouse 1944 (J22/JRCSFNef\textit{dd}) in particular had high frequencies of pentamer-positive cells. Comparing all pentamer-positive frequencies from C19/JRCSF tissues with those from C19/JRCSF\textit{Nef}\textit{dd} tissues, there is no significant difference between the two groups (p=0.7959) (Fig. 4.4f). Comparing between J22/JRCSF and J22/JRCSF\textit{Nef}\textit{dd} tissues, the frequencies in J22/JRCSF\textit{Nef}\textit{dd} tissues were higher with a trend toward significance (p=0.0657) driven by the high values from mouse 1944 (Fig. 4.4g).

We performed ELISPOT assays for IFN-gamma to determine if the HIV-specific CTL responses are functional. After stimulation with autologous irradiated B cells pulsed with KK10 peptide, we detected IFN-gamma secretion by ELISPOT from mononuclear cells of the bone marrow, liver, lung, thymic organoid, and spleen of two C19/JRCSF mice. With background subtracted from negative control wells (B cells with no peptide, Fig. 4.5a), the responses ranged from 100 to 370 spot forming units (SFU) per million cells in mouse 7573 (Fig. 4.5b), and from 20 to 1340 SFU/10^6 cells in mouse 7575 (Fig. 4.5c). The cells from these tissues also responded when we used a pool of JRCSF Gag-derived peptides: 430-1600 SFU/10^6 cells in mouse 7573 (Fig. 4.5d), 90-1520 SFU/10^6 cells in mouse 7575 (Fig. 4.5e).

We could also detect IFN-gamma production by intracellular cytokine staining, in cells from the livers of three J22/JRCSF mice. With negative control background subtracted, the percentages of CD8^+ T cells producing IFN-gamma in response to JRCSF Gag-peptide pool were 0.17%, 0.68%, and 0.30% in mice 1933, 1942, and
1950 respectively (Fig. 4.5f). The percentage of CD8$^+$ T cells producing IFN-gamma in response to KK10 peptide were 0.33%, 0.47%, and 0.36% (Fig. 4.5f).

Together, these data show that we are able to detect and measure functional HIV-specific CTLs from HIV-infected BLT mice in response to Gag peptide pool and to HLAB*2705-restricted KK10 peptide. Notably there was not a significant difference in the frequencies of KK10-specific CTLs in JRCSF- versus JRCSF\textit{Ne}f\textit{dd}-infected BLT mice.

**EFFECT OF CD8$^+$ T CELL DEPLETION ON HIV INFECTION IN BLT MICE**

To determine if CTLs are contributing to the control of infection \textit{in vivo}, we performed CD8$^+$ T cell depletion in JRCSF\textit{Ne}f\textit{dd}-infected mice (one C19, two X19) with low viremia. In mouse 7555 (C19/JRCSF\textit{Ne}f\textit{dd}) (Fig. 4.6a), the viral load was 4,013 HIV RNA copies/ml at week 10 after exposure. Four days after this measurement, a CD8-depleting antibody was administered, and the viral load increased to 46,424 copies/ml at week 11 (11.5-fold over week 10). At week 12, the viral load from this mouse again increased to 712,569 copies/ml (15.3-fold over week 11). When the CD8$^+$ T cells were again detectable in peripheral blood (weeks 13 and 14) viremia declined to 17,891 and 21,872 copies/ml. In contrast, mouse 8480 from a cohort with non-protective HLA alleles (I20, HLAB*0801/B*5401) infected with JRCSF showed only a 1.7-fold increase in viremia after CD8$^+$ depletion (Fig. 4.6b), and the viral load did not decrease when the CD8$^+$ T cells reconstituted in the peripheral blood.

Two X19/JRCSF\textit{Ne}f\textit{dd} mice were infected for 8 weeks, then administered CD8-depleting antibody. After CD8$^+$ depletion, the viremia in mouse 7922 increased
by 47.1-fold from 1,404 to 66,120 copies/ml at week 9 (Fig. 4.6c). Mouse 8059 had an increase in viremia from below detection (1500 copies/ml) to 54,121 copies/ml at week 9 and 914,722 copies/ml at week 10 (Fig. 4.6d).

These data suggest that CD8+ T cells are required for the control of viremia in JRCSF*Nef*dd-infected mice with protective HLA alleles.

**DISCUSSION**

CD8+ T cells, or CTLs, could play an important role in the clearance of infected cells in order to cure HIV. We investigated the hypothesis that CTLs respond to HIV and contribute to the control of HIV infection in BLT mice, and we focused our investigation on mice from three cohorts generated from donors with known protective HLA alleles, HLAB*2705 and/or HLAB*4402. Despite association of protective HLA alleles with control of HIV infection in humans, we did not observe control of wildtype JRCSF infection in these cohorts.

We hypothesized that HIV Nef may prevent the control of infection in BLT mice with protective HLA alleles by downmodulating MHC class I expression on the surface of infected cells. Our group has previously performed analyses of the role of HIV Nef during infection *in vivo*. Using an X4-tropic HIVLAI containing a *nef* deletion (LAINef*dd*), Zou *et al.* determined that Nef is necessary to enhance HIV replication and to deplete CD4+CD8+ thymocytes in BLT mice [334]. Later, Watkins *et al.* applied a similar strategy to study the role of Nef in an R5-tropic virus by generating JRCSF*Nef*dd. They found that the *nef* deletion did not affect viral replication *in vitro*, and that peak viral loads were not significantly different between JRCSF- and JRCSF*Nef*dd-infected BLT mice. The mean viral load was significantly different at 8
weeks of infection, but not at later time points. Interestingly, in one cohort of mice with a protective HLA allele (B*4201) [399], two mice infected with JRCSFNeFdd had lower viral loads at all time points compared to two JRCSF-infected mice of the same cohort, with as much as a 200-fold difference at week 10 [336].

In these studies, we compared JRCSF and JRCSFNeFdd infection in three more cohorts with protective HLA alleles. In two of the three protective-HLA cohorts, we found significant lower peak viral loads and average viral loads of mice infected with JRCSFNeFdd as compared to mice infected with JRCSF (Fig. 4.3). Several mice even controlled viremia to below detection (Fig. 4.2). Previously, Dudek et al. observed a reduction in JRCSF viral loads only in B*57 BLT mice, relative to all other haplotypes in the study including one with B*27 [386]. Similarly, Watkins et al. did not observe cohort-specific viral load reduction with JRCSF infection, and JRCSFNeFdd did not result in lower viral loads in five out of six cohorts. Our observations support the hypothesis that the effects of protective HLA alleles to control infection may be enhanced in BLT mice when nef-deleted virus is used; and, as Nef does not enhance JRCSF viral replication [336], this control of infection may be driven by host factors including the protective HLA alleles.

To investigate the generation of HIV-specific CTL responses in the mice, we performed pentamer staining, and we detected HLAB*2705-restricted KK10-specific CD8+ T cells in tissues from C19 mice and J22 mice infected with either JRCSF or JRCSFNeFdd (Fig. 4.4). However, there was not a significant difference in the frequencies of KK10-specific CD8+ T cells between JRCSF- and JRCSFNeFdd-infected mice. Although there could be differences in CD8+ T cells specific for other
epitopes, this observation suggests that Nef does not play an important role in the priming or frequency of HIV-specific CTLs. Rather, Nef-mediated downmodulation of MHC class I affects the ability of CTLs to recognize and kill infected cells [400]. This has also been observed in vivo for cowpox virus infection: MHC class I inhibition by cowpox does not affect CD8+ T cell priming but prevents infected cells from being recognized by virus-specific CD8+ T cells [401].

To assess the functionality of HIV-specific CD8+ T cells, we performed IFN-gamma ELISPOT. We detected IFN-gamma production in cells from tissues obtained from two C19 mice infected with JRCSF, in response both to KK10 specifically and to a pool of HIV Gag peptides (Fig. 4.5). Using IFN-gamma intracellular cytokine staining, cells from the livers of three J22 mice infected with JRCSF showed IFN-gamma production in response to KK10 and to Gag (Fig. 4.5). These data are similar in level to those previously published using JRCSF-infected BLT mice [386]. In the future, it will be important to assess the polyfunctionality of these CTL responses as well as to demonstrate direct killing of infected cells.

Finally, to determine whether or not CTLs contribute to control infection in vivo, we performed CD8+ T cell depletions in three JRCSFNetd-infected mice. Viremia increased 177-fold in mouse 7555, 47-fold in mouse 7922, and at least 600-fold in mouse 8059. In mouse 7555, viremia also decreased after CD8+ T cells returned in the peripheral blood. These spikes in viremia suggest that CTLs are contributing to the control of infection in vivo. It should be noted that the CD8-depleting antibody we used targets CD8alpha subunit which is also expressed on
natural killer cells. Therefore, NK cells cannot yet be ruled out for contributing to control of infection.

Here, we have reported detection and measurement of functional, HIV-specific CD8+ T cells in HIV-infected BLT mice. However, during infection with wildtype JRCSF, these immune responses are not sufficient to control infection. This lack of control could be due to several factors, such as CTL escape mutations, immune evasion, or CD8+ T cell dysfunction [402]. In two cohorts of mice with protective MHC class I HLA alleles, we have noted an ability to naturally control infection with a nef-deleted virus as well as the abrogation of this control with CD8+ T cell depletion. These observations support a hypothesis that CD8+ T cells can control infection by recognizing infected cells through MHC class I, and that this recognition is being evaded during wildtype HIV infection, likely due to Nef-mediated MHC class I downregulation.

If CD8+ T cells are to play a role in HIV cure strategies, barriers like immune evasion will need to be overcome. Several strategies are being studied to boost CD8+ T cell immune responses to HIV: ex vivo expansion [403, 404], therapeutic vaccines [405, 406], immunomodulation like PD-1 blockade [407, 408], chimeric antigen receptors [409, 410], or bispecific molecules [228, 233]. The BLT humanized mouse will be a valuable tool in the in vivo evaluation of these kill strategies towards a cure for HIV.

CONTRIBUTIONS

Richard Watkins, John Foster, J. Victor Garcia, and Perry Tsai conceived the study. Perry Tsai performed experiments in Figures 4.1, 4.4, and 4.5a-e. Richard
Watkins, Perry Tsai, Orrin Thayer, and Jessie Xiong collaborated on experiments in Figures 4.2 and 4.6. Perry Tsai performed analysis in Figure 4.3. Yinyan Xu and Nilu Goonetilleke performed experiment in Figure 4.5f. Perry Tsai compiled data for figures and wrote the chapter. J. Victor Garcia and Nilu Goonetilleke provided comments for the chapter.
FIGURES

Figure 4.1. Flow cytometric analysis of HLA alleles. Donor cells for each BLT cohort were stained with anti-CD45, A2/A28, A9, B8, B12, and B27 antibodies. Gating strategy was as follows: live → human CD45$^+$ → HLA allele. Flow cytometry results are shown here for cohort C19.
Figure 4.2. Viral loads of JRCSF and JRCSF\textit{Nef}dd in BLT mice with protective HLA alleles. BLT humanized mice were exposed intravenously to 90,000 tissue culture infectious units. Peripheral blood samples were collected and analyzed for plasma viral load (limit of detection = 1500 copies/ml, horizontal dotted line). Viral loads are plotted from C19 mice infected with JRCSF (a) or with JRCSF\textit{Nef}dd (b); X19 mice infected with JRCSF (c) or with JRCSF\textit{Nef}dd (d); J22 mice infected with JRCSF (e) or with JRCSF\textit{Nef}dd (f).
Figure 4.3. Analysis of peak viral loads and average viral loads in protective-HLA BLT mice infected with JRCSF or JRCSF\textit{Nefdd}. Peak viral loads were recorded, and average viral loads were calculated as time-weighted area under the curve, or total area under the curve divided by the total number of weeks infected. Peak viral loads are plotted with median±interquartile range, comparing JRCSF-infected with JRCSF\textit{Nefdd}-infected, in mice from the (a) C19, (b) X19, or (c) J22 cohort. Average viral loads are plotted as median±interquartile range, comparing JRCSF-infected with JRCSF\textit{Nefdd}-infected, in mice from the (d) C19, (e) X19, or (f) J22 cohort. P values were calculated by Mann-Whitney test.
Figure 4.4. Pentamer staining of HIV-specific CD8+ T cells from tissues of infected HLAB*2705 BLT mice. Cells isolated from tissues of infected B*2705 BLT mice were stained with HLAB*2705/KK10 pentamer and with a fluorescence-minus-one (FMO) stain without pentamer. Cells were gated on live (FSC/SSC) → 7AAD negative → CD45+ → CD3+ → CD8+ → pentamer. Frequency of pentamer-positive cells was calculated as percentage pentamer-positive cells out of CD8+ T cells, subtracting background from the FMO stain; representative flow cytometry plot shown in (a). Percentage pentamer-positive cells are plotted with mean from C19 mice infected with JRCSF (b) or JRCSFNefdd (c), and from J22 mice infected with JRCSF (d) or JRCSFNefdd (e). Data from JRCSF-infected vs. JRCSFNefdd-infected mice are plotted together with mean±SEM from C19 mice (f) and from J22 mice (g). P values were calculated using Mann Whitney test.
Figure 4.5. Functional assessment of HIV-specific CD8+ T cells from tissues of infected HLAB*2705 BLT mice. Cells isolated from tissues of two JRCSF-infected C19 BLT mice were stimulated with BCLs pulsed with KK10 peptide or Gag peptide pool, then IFNγ secretion was measured by ELISPOT assay. Spot forming units were averaged from triplicate wells, and background was subtracted from negative control wells using BCLs with no peptide (a). Spot forming units from each tissue are plotted in response to KK10 from mouse 7573 (b) and 7575 (c), and in response to Gag from mouse 7573 (d) and 7575 (e). Cells isolated from livers of three infected JRCSF-infected J22 mice were stimulated with BCLs pulsed with KK10 peptide or Gag peptide pool, then IFNγ production was measured by intracellular cytokine staining (f). Flow cytometry results are shown as follows: FMO (no anti-IFNγ), Negative control (BCL with no peptide), JRCSF Gag peptide pool, KK10 peptide, PMA/ionomycin positive control.
Figure 4.6. Effect of CD8+ T cell depletion in infected BLT mice. Three protective-HLA BLT mice infected with JRCSF/Nefdd and one non-protective-HLA BLT mouse infected with JRCSF were administered CD8-depleting antibody at time point indicated by vertical dotted line. Peripheral blood samples were collected and analyzed for plasma viral load (shown in red, limit of detection = 1500 copies/ml, horizontal dotted line) and percent CD8+ cells out of CD3+ (shown in blue). Data are shown from (a) mouse 7555, C19 infected with JRCSF/Nefdd, (b) 8480, I20, JRCSF, (c) 7922, X19, JRCSF/Nefdd, and (d) 8059, X19, JRCSF/Nefdd.
<table>
<thead>
<tr>
<th>Cohort</th>
<th>HLA haplotype</th>
<th>Infection (n)</th>
<th>%CD45 mean(SD)</th>
<th>%CD3 mean(SD)</th>
<th>%CD4 mean(SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C19</td>
<td>HLAB*2705</td>
<td>JRC (5)</td>
<td>67.2(9.6)</td>
<td>58.6(21.2)</td>
<td>88.2(1.3)</td>
</tr>
<tr>
<td></td>
<td>HLAB*4402</td>
<td>JRCNefdd (6)</td>
<td>72(5.4)</td>
<td>56.3(12.3)</td>
<td>87.5(3.8)</td>
</tr>
<tr>
<td>X19</td>
<td>HLAB*3501</td>
<td>JRC (6)</td>
<td>70.3(27.8)</td>
<td>73.7(15.0)</td>
<td>69.5(27.0)</td>
</tr>
<tr>
<td></td>
<td>HLAB*4402</td>
<td>JRCNefdd (6)</td>
<td>61.7(17.1)</td>
<td>48.5(10.7)</td>
<td>74.5(5.0)</td>
</tr>
<tr>
<td>J22</td>
<td>HLAB*2705</td>
<td>JRC (4)</td>
<td>53.3(5.5)</td>
<td>53.8(6.3)</td>
<td>85.3(5.5)</td>
</tr>
<tr>
<td></td>
<td>HLAB*4601</td>
<td>JRCNefdd (6)</td>
<td>54.5(8.9)</td>
<td>52.0(8.6)</td>
<td>85.8(3.9)</td>
</tr>
</tbody>
</table>

**Table 4.1. Cohort characteristics.** Cohort identifier, HLA haplotype, infection (n = number of mice), percentage CD45⁺ out of live, percentage CD3⁺ out of CD45⁺, and percentage CD4⁺ out of CD3⁺ cells in the peripheral blood as mean(standard deviation).
CHAPTER 5: CD19xCD3 DART PROTEIN MEDIATES HUMAN B CELL DEPLETION IN VIVO IN HUMANIZED BLT MICE

SUMMARY

Novel therapeutic strategies are needed for the treatment of hematologic malignancies. Bispecific antibody-derived molecules, such as Dual-Affinity Re-Targeting (DART®) proteins, are being developed to redirect T cells to kill specific target cells, such as tumor cells or infected cells. Here we present our findings of specific and systemic human B cell depletion by a CD19xCD3 DART protein in humanized BLT mice. Administration of the CD19xCD3 DART protein resulted in a dramatic sustained depletion of human CD19+ B cells from the peripheral blood, as well as a dramatic systemic reduction of human CD19+ B cell levels in all tissues (bone marrow, spleen, liver, and lung) analyzed. When human CD8+ T cells were depleted from the mice, no significant B cell depletion was observed in response to CD19xCD3 DART protein treatment, confirming that human CD8+ T cells are the primary effector cells in this in vivo model. These studies validate the use of BLT humanized mice for the in vivo evaluation and preclinical development of bispecific molecules that redirect human T cells to selectively deplete target cells.

---

INTRODUCTION

Therapies using targeted monoclonal antibodies have proven safe and effective against hematologic malignancies [411]. For example, rituximab, which targets the B cell marker CD20, produces overall response rates of 47-73% in untreated indolent non-Hodgkin’s lymphoma when used as a single-agent [412-414]. When added to another chemotherapy regimen, rituximab produced overall response rates of 64-81% in relapsed non-Hodgkin’s lymphoma [415-417] and 90-95% in untreated chronic lymphocytic leukemia [418-420]. However, not all patients respond to rituximab, and many of those who do eventually experience disease relapse [421-424]. Monoclonal antibody therapies directed against other B cell antigens, such as CD19, CD22, CD30, CD37, CD40, or CD52, are at different stages of preclinical/clinical testing [424-430]. B cell-targeted therapies with novel mechanisms of action are still necessary in order to improve cure rates, and innovative approaches could prove cost-effective in the treatment of hematologic malignancies [431].

Existing monoclonal antibody therapies rely on the action of complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity [432, 433], or utilize a conjugated toxin or radiolabeled isotope [432]. Other strategies harness the ability of cytotoxic T lymphocytes (CTLs) to kill target cells, relying on ex vivo manipulation to expand tumor-specific CTLs [434] or to express chimeric antigen receptors [435]; but these approaches are limited by major histocompatibility (MHC) restriction in tumor-specific CTLs, as well as scalability and risks such as cytokine release syndrome or macrophage activation syndrome [436].
Recently, the development of bispecific T cell-redirecting antibody-derived molecules has made possible treatment strategies that bypass the requirement for MHC matching or ex vivo manipulation and expansion of CTLs. These bispecific molecules bind simultaneously to a receptor on T cells and to a specific antigen on a target cell, thus redirecting T cells to kill the target cells. One example is blinatumomab, a Bispecific T Cell Engager (BiTE®) molecule targeting CD19, which demonstrated complete responses in 72% of patients with persistent or relapsed minimal residual disease and a median overall survival of 9 months [437].

To build upon this success, newer generations of bispecific molecules have been developed, like the Dual-Affinity Re-Targeting (DART®) molecules. DART molecules differ from BiTE molecules in two ways: there is no intervening linker sequence between the V regions of DART molecules, and there are two cysteine residues at the C-terminus of each chain which form a disulfide bridge [229, 230]. In a previous report comparing DART molecules with BiTE molecules, DART molecules seemed to perform better than BiTE molecules with respect to antigen binding, ability to crosslink target/effector cells, induction of T cell activation markers, EC50 for target cell lysis, and maximal target cell lysis [230, 438]. A CD123xCD3 DART protein (directed against human CD3 and human CD123) was active against human AML cell line engraftments in NSG/β2m-/- mice reconstituted with human PBMCs, and, due to its crossreactivity to both antigens from cynomolgus monkeys, depleted CD123+ cells when administered to the monkeys [231]. A CD19xTCR DART protein (directed against human CD19 and human T cell receptor αβ subunit) was active against human B cell lymphoma xenografts in NOD-SCID mice
reconstituted with human PBMCs [230]. A CD19xCD3 DART protein in an extended half-life format was active against B cell lymphoma xenografts in mice reconstituted with human PBMCs, and, due to its crossreactivity to both antigens from cynomolgus monkeys, depleted CD19+ B cells in peripheral blood and lymph nodes when administered to the monkeys [439]. However, there has yet to be a systemic in vivo evaluation of the effect of CD19xCD3 DART molecules on human immune cells generated de novo from hematopoietic stem cells.

Bone marrow-liver-thymus (BLT) humanized mice could serve as an excellent preclinical model for the in vivo evaluation of CD19xCD3 DART molecules. BLT mice are generated by implanting human thymus and liver tissue into sublethally irradiated NOD/SCID-gamma chain null mice, followed by transplanting autologous human CD34+ hematopoietic stem cells [306, 440]. BLT mice develop robust levels of human hematopoietic cells throughout the body, including T cells, B cells, monocytes/macrophages, and dendritic cells [290]; and this model has been utilized in the study of B cells, immune reconstitution, and HIV infection [274-279, 292, 305, 441-443].

Using BLT humanized mice, we evaluated the efficiency of human B cell depletion by a CD19xCD3 DART protein, in which the binding arms are equivalent to those utilized in blinatumomab [444]. Our results demonstrate that this DART molecule is effective at depleting human B cells in peripheral blood and tissues. We also show that this effect occurred on mature B cells and that progenitor cells remain functionally capable of generating new human B cells in the bone marrow. Finally, we demonstrate that B cell depletion by this CD19xCD3 DART molecule is
dependent on human CD8+ T cells. Together, our results provide in vivo evidence of the functional capacity of T cell-redirecting DART proteins and validate the utilization of the BLT humanized mouse model for the preclinical evaluation of these molecules.

METHODS

ETHICS STATEMENT

All animal experiments were conducted following NIH guidelines for housing and care of laboratory animals and in accordance with University of North Carolina at Chapel Hill (UNC Chapel Hill) regulations after review and approval by the UNC Chapel Hill Institutional Animal Care and Use Committee.

GENERATION OF BLT HUMANIZED MICE

BLT mice were generated as described previously [305]. NOD/SCID-gamma chain−/− mice (NSG, Stock #5557, The Jackson Laboratories) were sublethally irradiated, implanted with human thymus and liver tissue, and transplanted with autologous human liver CD34+ cells (Advanced Bioscience Resources); then they were monitored for human reconstitution in peripheral blood by flow cytometry[275, 305, 306]. All BLT mice (n=31) used for these experiments contained an average of 63.5% ± 13.6 SD human CD45+ cells in the peripheral blood, of which 39% ± 9.3 SD expressed human CD19, and 53.3% ± 10.7 SD expressed CD3 on their cell surface. The percentage of human CD3+ cells expressing human CD4 was 78.3% ± 6.5 SD.

DART MOLECULES

As described by Moore et al.[230], the CD19xCD3 DART molecule, in basic format, was constructed using anti-human CD19 Fv sequences from HD37 [445] and
anti-human CD3 Fv sequences from TR66 [444]; and the irrelevant arm of the control DART molecule (4420xCD3) was constructed from anti-fluorescein Fv sequences from 4-4-20 [446]. The binding arms of this CD19xCD3 DART protein do not cross-react with murine antigens. The DART proteins were produced in CHO-S cells and purified as described [230].

**TREATMENT OF BLT MICE**

BLT mice were injected intravenously with CD19xCD3 DART molecule or 4420xCD3 (anti-fluorescein) DART molecule (MacroGenics, Rockville, MD) at a dose of 1mg/kg either one time or two times 7 days apart. Vehicle-treated mice were injected with normal saline, 0.9% sodium chloride (#0409-4888-10, Hospira, Lake Forest, Illinois). For CD8+ T cell depletion, BLT mice were injected intravenously with anti-CD8α antibody (MT807R1, gift from Dr. Guido Silvestri, Emory University School of Medicine, Atlanta, GA) at a dose of 3mg/kg or with vehicle (normal saline).

**ANALYSIS OF BLT MICE**

Peripheral blood and tissues were collected and cells isolated as previously described for flow cytometric analysis [275, 306]. Antibodies used in these experiments included anti-human CD45 APC (clone HI30, catalog #555485), CD45 FITC (2D1, 347463), CD3 FITC (HIT3a, 555339), CD8 APC-Cy7 (SK1, 557834), CD8 PerCP (SK1, 347314), CD19 PE-Cy7 (SJ25C1, 557835), and CD10 APC (HI10a, 340923) (BD Biosciences, San Jose, CA). Flow cytometry data were acquired using a BD FACSCanto Cytometer and analyzed using BD FACSDiva software (v. 6.1.3) with the following gating strategy: live cells → human CD45+ → human CD19+ (→ human CD10+) or live → human CD45+ → human CD3+ → human
CD8$. The numbers of cells per microliter peripheral blood were calculated by dividing the number of positive events acquired by the number of microliters of peripheral blood stained. The numbers of cells per tissue were estimated by multiplying the total number of live cells isolated from the tissue by the fraction of positive events over live events. Cytokine analysis was performed on plasma samples using a Human Inflammatory Cytokine Kit (551811, BD Biosciences); these data were acquired using a BD LSR II Cytometer and analyzed using FCAP Array Infinite (Soft Flow, St. Louis Park, MN).

**STATISTICAL ANALYSIS**

All statistical tests were performed with an alpha level of 0.05. Repeated-measures two-way ANOVA with Sidak’s multiple comparisons test was used to generate the p values in Figures 5.1b-e, 5.6b-c, 5.7a-f, 5.8b-c, 5.9a-b. Unpaired t test was utilized to generate the p values in Figures 5.5, 5.6d-f, 5.7g-h, 5.8d-e, 5.9c-d. Graphs were generated in Graphpad Prism (v. 6).

**RESULTS**

**DEPLETION OF HUMAN CD19+ B CELLS IN PERIPHERAL BLOOD AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN**

In order to evaluate the efficacy of CD19xCD3 DART proteins to deplete human B cells in vivo, we utilized BLT humanized mice. BLT mice were constructed as previously described [305]. The mice used for all experiments were 16-20 weeks post humanization procedure, and they were well reconstituted with human CD45+ cells (63.5% ± 13.6 SD). Of the human CD45+ cells present in peripheral blood at the beginning of the experiments, 39% ± 9.3 SD expressed human CD19, and
53.3% ± 10.7 SD expressed CD3 on their cell surface. The percentage of human CD3+ cells expressing human CD4 was 78.3% ± 6.5 SD (data not shown).

For our experiments, we used a CD19xCD3 DART molecule in which the CD19 arm was derived from anti-human CD19 antibody HD37 and the CD3 arm was derived from anti-human CD3 antibody TR66, as described by Moore et al. [230]; these binding arms are equivalent to those of blinatumomab [444]. This bispecific molecule binds to human CD3 on T cells and to human CD19 on B cells, and it recruits the cytotoxic activity of T cells to kill target B cells. The CD19xCD3 DART molecule was administered to BLT mice (n=3) intravenously (1mg/kg) in two doses, 7 days apart. A similar group of BLT mice was administered vehicle (normal saline, n=3). Peripheral blood was collected for flow cytometric analysis on days 0, 1, 3, 7, and 11 after the first injection (Fig. 5.1a, gating scheme in Fig. 5.2a).

Initially after CD19xCD3 DART molecule administration (day 1), the levels of total human CD45+ cells detected from the peripheral blood dropped to 3.2% ± 1.3 SEM (or 38 ± 11 CD45+ cells per microliter of blood); but this decrease was transient, as the levels of CD45+ cells returned to approximately normal levels by day 3 (Fig. 5.1b-c). With regard to the target human CD19+ B cells, the pretreatment (day 0) percentage of CD19+ cells in peripheral blood was 27.8% ± 1.5. One day after CD19xCD3 administration, the percent CD19+ cells was not quantifiable due to a transient decline of CD45+ cells overall. The levels of CD19+ cells in the peripheral blood were lower than 0.1% on days 3, 7, and 11 after CD19xCD3 administration (Fig. 5.1d). This decrease in human CD19+ B cells was also reflected the absolute numbers of cells. Specifically, the number of CD19+ cells per microliter in the
peripheral blood of the CD19xCD3-treated animals was reduced from 413 ± 67 CD19+ cells per microliter to <1 CD19+ cell per microliter of blood (Fig. 5.1e). In contrast to the depletion of B cells in the CD19xCD3-treated mice, the levels of CD19+ cells declined only to 17% by day 11 in the peripheral blood of vehicle-treated mice. It should be noted that this decrease observed in percent human CD19+ is consistent with the increases in human T cell reconstitution that occur over time in this animal model (Fig. 5.3). However, the number of human CD19+ cells per microliter did not change significantly between day 0 and day 11 in the animals receiving vehicle (Fig. 5.1e). The differences in the levels of human B cells between the CD19xCD3-treated and vehicle-treated animals were statistically significant at days 3, 7, and 11.

To evaluate the specificity of the effect of the CD19xCD3 DART molecule, we administered a control DART protein (4420xCD3), with one arm directed against an irrelevant target (fluorescein), to BLT mice (n=4). We did not observe a decrease in the levels of human CD19+ B cells (Fig. 5.4).

We also measured the levels of human inflammatory cytokines (IL-1beta, IL-6, IL-8, IL-10, IL-12p70, TNF) in plasma samples from mice treated with vehicle, control DART, or CD19xCD3 DART (n=4 each group). Before treatment, the levels of all cytokines tested were below the limit of quantitation (LOQ = 20 pg/ml) in all mice. One day after treatment, the levels remained below LOQ in the mice treated with vehicle or control DART molecule. In contrast, one day after CD19xCD3 treatment, we noted a significant increase in the plasma levels of IL-6, IL-8, and IL-10: 337±122 pg/ml IL-6, 528±110 pg/ml IL-8, and 1800±622 pg/ml IL-10.
(mean±SEM, p<0.0001 with two-way repeated measures ANOVA and Tukey’s multiple comparisons test). Three days after treatment, the cytokine levels in the CD19xCD3-treated mice decreased back to basal levels. Together, these results show (1) a transient increase in cytokine levels in response to CD19xCD3 DART molecule treatment that resolves by 72 hours, and (2) the efficient and specific depletion of human CD19+ B cells from the peripheral blood of BLT humanized mice.

**DEPLETION OF HUMAN CD19+ B CELLS IN TISSUES AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN**

In order to determine the systemic effects of the CD19xCD3 DART molecule, we harvested and isolated cells from the bone marrow, spleen, liver, and lung of the animals in the experiment described in Figure 1 at day 11, and measured levels of human CD19+ B cells in each tissue. We observed a marked depletion of CD19+ cells in all the tissues analyzed of the CD19xCD3-treated animals, down to <0.1% (Fig. 5.5a). In contrast, the percentages of CD19+ cells in the tissues from animals that received vehicle remained normal (61.6% ± 0.9 in the bone marrow, 48.3%± 1.0 in the spleen, 14.2% ± 1.6 in the liver, and 12.1% ± 0.6 in the lung). The absolute numbers of CD19+ cells was reduced over 2100-fold in the bone marrow of the CD19xCD3-treated animals and over 7600-fold in the spleen, as compared to vehicle-treated animals. We could not detect CD19+ cells by flow cytometry in the livers and lungs of the mice that received the CD19xCD3 DART molecule (Fig. 5.5b). These differences in CD19+ cell levels were statistically significant between the vehicle group and the CD19xCD3-treated group across all tissues analyzed.
Together, these results demonstrate that the CD19xCD3 DART molecule can efficiently deplete human CD19+ B cells from tissues.

**REGENERATION OF HUMAN CD19+ B CELLS AFTER ADMINISTRATION OF CD19xCD3 DART PROTEIN**

Next, we sought to investigate the durability of depletion as well as potential for regeneration of human CD19+ B cells after administration of the CD19xCD3 DART molecule. We treated BLT mice with CD19xCD3 or vehicle (n=3 for each group). We monitored the levels of human CD19+ B cells in peripheral blood at days 1, 3, 7, 14, 21, and 28 after the first injection (Fig. 5.6a).

As in the previous experiment in Figure 1b-c, we observed a depletion of CD19+ cells in the peripheral blood of the CD19xCD3-treated animals over the first two weeks. At day 21 after the first injection, the levels of CD19+ cells in the peripheral blood began to increase in the CD19xCD3-treated group. By day 28, the differences in the levels of CD19+ cells were not statistically significant between the vehicle-treated mice and the CD19xD3-treated mice (Fig. 5.6b-c). Analysis of the levels of CD19+ cells in the tissues at this time point were not significantly different between the animals that received vehicle versus CD19xCD3, indicating that regeneration of the B cell population had occurred in the CD19xCD3-treated animals (Fig. 5.6d-e).

To confirm that the human CD19+ B cells that were present at day 28 in the CD19xCD3-treated animals were not residual from the pre-treatment period, we assessed the developmental stage of the CD19+ cells appearing after CD19xCD3 administration. Specifically, we measured the levels of immature B cells as
determined by human CD10 cell surface expression (gating scheme in Fig. 5.2b).
The percentage of CD10+ cells out of the CD19+ population was significantly higher in the CD19xCD3-treated mice across all tissues analyzed, reaching >90% in the bone marrow, spleen, and liver, and 80% in the lung (Fig. 5.6f). These results demonstrate the production of new B cells in mice previously treated with the CD19xCD3 DART protein.

EFFECT OF CD19xCD3 DART PROTEIN ADMINISTRATION ON THE LEVELS OF HUMAN T CELLS IN VIVO

To examine the effect of the CD19xCD3 DART molecule on human T cells in vivo, in particular CD8+ cytotoxic T cells, we measured the levels of human CD45+, CD3+, and CD8+ cells in peripheral blood after two administrations of vehicle or CD19xCD3 (1mg/kg) seven days apart, over a period of four weeks after the first administration.

Similar to the results presented Figure 1, one day after DART administration we observed a sharp decline in human CD45+ lymphocytes (both percentage and absolute number) in the peripheral blood of CD19xCD3-treated animals (Fig. 5.7a-b). However, this decline was only transient: by day 3, there was no longer a significant difference in the levels of human hematopoietic cells between the vehicle- and the CD19xCD3-treated mice. The levels of CD19+ cells were again significantly lower in the CD19xCD3-treated group at day 14 (7 days after the second administration), but there was not a significant difference at day 28.

With regard to T cells, we observed the same sharp decline at day 1 in the percentage and absolute number of human CD3+ and CD8+ T cells in the peripheral
blood. After day 1, there was an initial increase in the percent of CD3⁺ cells on day 3 and day 7 in the CD19xCD3-treated group over the vehicle-treated group, although the absolute numbers of CD3⁺ cells were not significantly different (Fig. 5.7c-d). The percent CD8⁺ T cells were also higher in the CD19xCD3-treated animals on days 3, 7, and 14 than in the vehicle-treated animals; but the absolute numbers of CD8⁺ cells were not significantly different at day 3 or day 7 (Fig. 5.7e-f). By day 28, there were no significant differences in the percentages or absolute numbers of peripheral blood human CD45⁺, CD3⁺, or CD8⁺ cells between the animals receiving CD19xCD3 and those receiving vehicle.

At harvest on day 28, there was a small but statistically significant increase in the percentage of CD8⁺ cells out of CD3⁺ cells in the bone marrow and spleen of the CD19xCD3-treated animals: 18.2% ± 1.5 (vehicle) vs. 28.6% ± 1.8 (CD19xCD3) (p<0.05) and 14.9% ± 1.3 (vehicle) vs. 19.7% ± 0.5 (CD19xCD3) (p<0.01), respectively (Fig. 5.7g). The percentages of CD8⁺ cells were not significantly different in the livers or the lungs of these mice. We observed no difference in the total number of CD8⁺ cells in any of the tissues analyzed (Fig. 5.7h). These results demonstrate that exposure to the CD19xCD3 DART molecule does not have a significant effect on the levels of human CD8⁺ T cells in peripheral blood or tissues.

**DEPENDENCE OF CD19xCD3 DART PROTEIN-MEDIATED DEPLETION ON THE PRESENCE OF HUMAN CD8⁺ T CELLS**

To investigate the mechanism of action *in vivo* of the CD19xCD3 DART molecule, we evaluated the necessity of human CD8⁺ T cells for target B cell depletion. One group of BLT mice was administered a CD8-depleting antibody (anti-
CD8α antibody MT807R1, kindly provided by Dr. Guido Silvestri) [447, 448] intravenously at 3mg/kg (CD8-depleted group, n=8), and one group was administered vehicle (normal saline, CD8-intact group, n=7). Four days later, both groups were administered CD19xCD3 once intravenously (1mg/kg). Peripheral blood was collected and analyzed at 4 days and 1 day prior to CD19xCD3 administration, and at days 3, 7, and 14 after administration. Tissues were harvested and analyzed at day 15 (Fig. 5.8a).

To confirm depletion of human CD8+ T cells, we measured the levels of CD8+ cells in the peripheral blood and tissues. By three days after administration of the CD8-depleting antibody (or one day prior to CD19xCD3 administration), the percentage CD8+ cells decreased to <0.3% ± 0.1, and the number of CD8+ cells decreased to 2 ± 0.7 CD8+ cells per microliter peripheral blood in the CD8-depleted group. The levels of CD8+ cells in the CD8-intact group were 19% CD8+ out of CD3+ and 528 CD8+ cells per microliter at this time point (Fig. 5.8b-c). In the tissues (harvested at day 15 after CD19xCD3 administration), the percentage CD8+ cells were <1% in the bone marrow and <0.5% in the spleen, liver, and lung of the CD8-depleted mice (Fig. 5.8d). The absolute numbers of CD8+ cells were 28-fold lower in the bone marrow, 89-fold lower in the spleen, 186-fold lower in the liver, and 110-fold lower in the lung from CD8-depleted animals as compared to the CD8-intact animals (Fig. 5.8e).

As in the experiment in Figure 1, the human CD19+ B cells were virtually completely depleted in the peripheral blood of the CD8-intact mice (Fig. 5.9a-b). In contrast, in the CD8-depleted mice, we observed only a transient decline in the
levels of CD19+ cells at day 3 followed by a recovery to near normal CD19+ levels by day 7. The differences in the levels of CD19+ cells between the CD8-intact animals and the CD8-depleted animals were statistically significant at days 7 and 14. Consistent with the depletion of the human CD19+ B cells from the peripheral blood, the levels of CD19+ cells in the tissues were also significantly reduced in the CD8-intact group as compared to the CD8-depleted group (Fig. 5.9c). Even though there was no difference between the groups in number of total bone marrow CD19+ cells, there was at least a 10-fold statistically significant reduction in the total number of CD19+ cells in the spleen, liver, and lung of the CD8-intact versus CD8-depleted animals (Fig. 5.9d). Together, these results demonstrate that the depletion of human CD19+ B cells by CD19xCD3 DART molecule is dependent on the presence of human CD8+ T cells.

**DISCUSSION**

Building upon the success of monoclonal antibody therapies in the treatment of hematologic malignancies, novel strategies can still be useful toward improving cure rates. Bispecific molecules like Dual-Affinity Re-Targeting (DART®) molecules are promising for their ability to recruit T cells to kill target cells without the need for ex vivo expansion. DART molecules also bypass the requirement for antigen processing or MHC-restricted antigen presentation because they bind directly to the target surface marker. The in vivo effects of T cell-redirecting DART molecules have been evaluated in xenograft-bearing mice reconstituted with human PBMCs and in nonhuman primates, and several DART candidates are currently being evaluated in human clinical trials for treatment of relapsed/refractory metastatic colorectal
carcinoma, acute myeloid leukemia, and B cell malignancies (NCT02152956, NCT02248805, NCT02454270).

Humanized BLT mice represent an excellent preclinical model for the in vivo evaluation of CD19xCD3 DART molecules, as they harbor HLA-matched target and effector cells (human CD19+ B cells and human CD3+ T cells, respectively). BLT mice also allow us to assess 1) the extent of target cell depletion systemically, 2) the effect on other relevant human hematopoietic cells, and 3) the role of cytotoxic T cells after DART protein administration in vivo.

In our study, we observed a profound depletion of target human CD19+ B cells in both peripheral blood and tissues (bone marrow, spleen, liver, and lung) after two intravenous administrations of 1mg/kg CD19xCD3 (Fig. 5.1d-e, 5.5a-b). This effect was specific to the CD19-targeting arm, as a fluorescein-directed 4420xCD3 DART molecule did not result in similar CD19+ cell depletion (Fig. 5.4). CD19+ cells began to return to the peripheral blood of CD19xCD3-treated BLT mice by day 21 after the first injection (or by day 14 after the second injection) (Fig. 5.6b-c); and by day 28, the levels of B cells in the tissues of CD19xCD3-treated mice as compared to vehicle-treated mice were not statistically different (Fig. 5.6d-e). A vast majority of the human CD19+ cells appearing in the tissues had an immature phenotype at day 28 after the first injection (Fig. 5.6f), as indicated by CD10 staining, suggesting that the B cells present are the result of de novo generation and development rather than of homeostatic proliferation.

A recent report evaluating an anti-CD20/CD3 T cell-dependent bispecific antibody reported initial increases in human CD8+ T cell counts followed by
decreases to baseline or lower in the peripheral blood and spleen of CD3ε/CD20-transgenic mice or NSG mice humanized by CD34+ cell transplant [449]. We investigated the effect of the CD19xCD3 DART molecules in humanized BLT mice. Interestingly, we observed a transient decrease in virtually all the human lymphocytes in the peripheral blood on day 1 after the first injection (Fig. 5.1b-c, 5.7a-f). This initial decline is followed by a recovery to near normal levels of the peripheral blood human lymphocytes by day 3, with the remarkable absence of B cells (Fig. 5.1d-e). A similar transient lymphopenia has been observed in chimpanzees following administration of a bispecific anti-CD19/anti-CD3 single-chain BiTE construct at 10 hours followed by recovery at 24-72 hours[450]. This transient lymphopenia could be explained by redistribution of lymphocytes that are adhering to blood vessel walls or migrating into tissues. Despite temporary differences, peripheral blood human CD45+, CD3+, and CD8+ cell levels in the CD19xCD3-treated animals returned to levels similar to those in the vehicle group by day 28 after the first injection of CD19xCD3 (Fig. 5.7a-f). Notably, the number of human CD8+ T cells were not significantly different between the vehicle- and CD19xCD3 DART treated animals in the bone marrow, spleen, liver, and lung (Fig. 5.7h).

Finally, we evaluated the dependence of target human CD19+ B cell depletion by CD19xCD3 DART molecules on the presence of human CD8+ T cells. BLT mice that were CD8-depleted prior to CD19xCD3 administration did not exhibit the reduction of CD19+ cells in the peripheral blood that was observed in CD8-intact animals (Fig. 5.9b-c), and the CD8-depleted group had significantly higher systemic
levels of CD19+ cells, except in the bone marrow (Fig. 5.9d-e). These data suggest that human CD8+ T cells are required for depletion of human CD19+ B cells by CD19xCD3 DART molecules.

This study validates the humanized BLT mouse as a preclinical model for the use and evaluation of bispecific reagents, in particular Dual-Affinity Re-Targeting (DART) molecules. The BLT mouse model provides opportunities to study the \textit{in vivo} effects of bispecific molecules on human target cells both in the peripheral blood and the tissues. Future directions could include the evaluation of DART molecules directed against other target immune cells or against viral antigens expressed on infected cells. A recent paper from Sung \textit{et al.} showed data on the efficacy \textit{ex vivo} of a DART molecule directed against HIV envelope protein [233], thus providing a rationale for further \textit{in vivo} studies in BLT humanized mice of DART molecules as a targeted cytotoxic therapy against HIV-infected cells, as we have previously demonstrated using an immunotoxin strategy [211].

\textbf{CONTRIBUTIONS}

Perry Tsai and J. Victor Garcia conceived the study. Perry Tsai performed all experiments with the assistance of William O. Thayer and Tia M. Morgan.
Figure 5.1. CD19xCD3 DART protein administration depletes human CD19+ B cells from the peripheral blood. (a) Experimental outline. NSG/BLT mice were administered CD19xCD3 (n=3, 1 mg/kg intravenously) or vehicle (n=3) at day 0 and day 7. Peripheral blood (PB) was collected and analyzed at days 0, 1, 3, 7, and 11. (b) Percent CD45+ cells out of live cells and (c) number CD45+ cells per microliter detected in PB by flow cytometry (vehicle group in blue, CD19xCD3 group in red). (d) Percent CD19+ cells out of CD45+ cells and (e) number CD19+ cells per microliter detected in PB by flow cytometry (open circle = below limit of quantitation). Mean±SEM plotted. p values were calculated by repeated-measures two-way ANOVA with Sidak’s multiple comparisons test, comparing vehicle vs. CD19xCD3. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Peripheral blood and tissues were stained for analysis by flow cytometry as described in the Methods. (a) Representative flow cytometry dot plots for analysis of human B cells and human T cells (peripheral blood shown). Gating scheme was as follows: live → human CD45$^+$ → human CD19$^+$ for human CD19$^+$ B cells; live → human CD45$^+$ → human CD3$^+$ → human CD8$^+$ for human T cells. (b) Representative flow cytometry dot plots for analysis of immature CD10$^+$ B cells (bone marrow shown). Gating scheme was as follows: live → human CD45$^+$ → human CD19$^+$ → human CD10$^+$. 

Figure 5.2. Gating scheme for flow cytometry analysis.
Figure 5.3. The percent human CD19$^+$ cells out of human CD45$^+$ cells decreases as percent human CD3$^+$ cells increases over time in the peripheral blood of NSG/BLT humanized mice. NOD/SCID-gamma chain null mice (n=7) were sublethally irradiated, implanted with human liver and thymus tissue from one donor, and transplanted with autologous human CD34$^+$ hematopoietic stem cells. Peripheral blood was collected at 8, 12, 16, and 20 weeks after humanization procedure, and stained for human CD45, CD19, and CD3 markers. Shown are percent CD19$^+$ cells out of CD45$^+$ cells (open diamond) and percent CD3$^+$ out of CD45$^+$ (open circle) cells detected in peripheral blood by flow cytometry over time.
Figure 5.4. 4420xCD3 DART protein administration does not deplete human CD19+ B cells from the peripheral blood. NSG/BLT mice were administered 4420xCD3 DART protein (1 mg/kg intravenously) at day 0 and day 7. Peripheral blood (PB) was collected and analyzed at days 0, 1, 3, 7, and 11. (a) Percent CD19+ cells out of CD45+ cells and (b) number CD19+ cells per microliter detected in PB by flow cytometry.
Figure 5.5. CD19xCD3 DART protein administration depletes human CD19+ B cells from the tissues. NSG/BLT mice were administered CD19xCD3 (n=3, 1 mg/kg intravenously) or vehicle (n=3) at day 0 and day 7. Tissues were harvested and analyzed at day 11. (a) Percent CD19+ cells out of CD45+ cells and (b) number CD19+ cells detected in tissues by flow cytometry (vehicle group in blue; CD19xCD3 group in red). Mean±SEM plotted. p values were calculated by unpaired t-test, comparing vehicle vs. CD19xCD3. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 5.6. Immature human CD19+ B cells regenerate in NSG/BLT mice after CD19xCD3 DART protein administration. (a) Experimental outline. NSG/BLT mice were administered CD19xCD3 (n=3, 1 mg/kg intravenously) or vehicle (n=3) at day 0 and day 7. Peripheral blood (PB) was collected and analyzed at days 0, 1, 3, 7, 14, 21, and 28; tissues were harvested and analyzed at day 28. (b) Percent CD19+ cells out of CD45+ cells and (c) number CD19+ cells per microliter detected in PB by flow cytometry (vehicle group in blue; CD19xCD3 group in red). (d) Percent CD19+ cells out of CD45+ cells and (e) number CD19+ cells detected in tissues by flow cytometry (vehicle group in blue, CD19xCD3 group in red). (f) Percent immature CD10+ cells out of CD19+ cells detected in tissues by flow cytometry. Mean±SEM plotted. p values were calculated in b-c by repeated-measures two-way ANOVA with Sidak’s multiple comparisons test, comparing vehicle vs. CD19xCD3. p values were calculated in d-f by unpaired t-test, comparing vehicle vs. CD19xCD3. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Gray diamond indicates data from age-matched NSG/BLT mice, n=4.
Figure 5.7. CD19xCD3 DART protein administration results in transient differences in the levels of human T cells in the peripheral blood of NSG/BLT mice, and over time there are no significant differences in absolute numbers of human CD8+ T cells in the peripheral blood or tissues as compared to vehicle-treated mice. NSG/BLT mice were administered CD19xCD3 (n=3, 1 mg/kg intravenously) or vehicle (n=3) at day 0 and day 7. Peripheral blood (PB) was collected and analyzed at days 0, 1, 3, 7, 14, 21, and 28; tissues were harvested and analyzed at day 28. (a) Percent CD45+ cells out of live cells and (b) number CD45+ cells per microliter detected in peripheral blood by flow cytometry (vehicle group in purple, CD19xCD3 group in brown). (c) Percent CD3+ cells out of CD45+ cells and (d) number CD3+ cells per microliter. (e) Percent CD8+ cells out of CD3+ cells and (f) number CD8+ cells per microliter. (g) Percent CD8+ cells out of CD3+ cells and (h) number CD8+ T cells detected in tissues by flow cytometry. Mean±SEM plotted. p values were calculated in a-f by repeated-measures two-way ANOVA with Sidak’s multiple comparisons test, comparing vehicle vs. CD19xCD3. p values were calculated in g-h by unpaired t-test, comparing vehicle vs. CD19xCD3. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Gray diamond indicates data from age-matched NSG/BLT mice, n=4.
**Figure 5.8. Human CD8+ T cells are depleted after administration of CD8-depleting antibody.** (a) Experimental outline. NSG/BLT mice were administered CD8-depleting antibody (CD8-depleted, n=8, 3mg/kg intravenously) or vehicle (CD8-intact, n=7) 4 days prior to administration of CD19xCD3 DART protein to all mice (1mg/kg intravenously). Peripheral blood (PB) was collected and analyzed at 4 days and 1 day prior to and at days 3, 7, and 14 after administration of CD19xCD3 DART protein; tissues were harvested and analyzed at day 15. (b) Percent CD8+ cells out of CD3+ cells and (c) number CD8+ cells per microliter detected in PB by flow cytometry (CD8-depleted in orange, CD8-intact in green). (d) Percent CD8+ cells out of CD3+ cells and (e) number CD8+ cells detected in tissues by flow cytometry. Mean±SEM plotted. p values were calculated in b-c by repeated-measures two-way ANOVA with Sidak’s multiple comparisons test, comparing CD8-depleted versus CD8-intact. p values were calculated in d-e by unpaired t-test, comparing CD8-depleted versus CD8-intact. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 5.9. Human CD19+ B cell depletion by CD19xCD3 DART protein is dependent on the presence of human CD8+ T cells. NSG/BLT mice were administered CD8-depleting antibody (CD8-depleted, n=8, 3mg/kg intravenously) or vehicle (CD8-intact, n=7) 4 days prior to administration of CD19xCD3 DART protein to all mice (1mg/kg intravenously). Peripheral blood (PB) was collected and analyzed at 4 days and 1 day prior to and at days 3, 7, and 14 after administration of CD19xCD3 DART protein; tissues were harvested and analyzed at day 15. (a) Percent CD19+ cells out of CD45+ cells and (b) number CD19+ cells per microliter detected in PB by flow cytometry (CD8-depleted in orange, CD8-intact in green). (c) Percent CD19+ cells out of CD45+ cells and (d) number CD19+ cells detected in tissues by flow cytometry. Mean±SEM plotted. p values were calculated in a-b by repeated-measures two-way ANOVA with Sidak’s multiple comparisons test, comparing CD8-depleted vs. CD8-intact. p values were calculated in c-d by unpaired t-test, comparing CD8-depleted vs. CD8-intact. ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

STUDY SUMMARY

Over 30 years after the discovery of HIV as the causative agent of AIDS, there is still no cure for this disease. Even with effective antiretroviral therapy (ART), the establishment of persistent HIV reservoirs presents both obstacles and opportunities towards a cure for HIV. As knowledge emerges regarding the nature of HIV persistence, strategies are also emerging to clear all HIV reservoirs [156]. Testing these strategies in human subjects will be necessary; but due to ethical and practical constraints, animal models will be needed to further our knowledge about persistent reservoirs in vivo and to investigate the safety and efficacy of candidate HIV cure strategies [354].

Our long-term goal is to develop a cure for patients infected with HIV. In the studies presented in this dissertation, we have examined several different strategies to achieve an HIV cure in vivo using humanized mice as an animal model of HIV. First, we demonstrated the complete resistance of humanized mice, constructed using CD34+ cells from a CCR5delta32 donor, to HIV infection. This established an opportunity for the investigation of CCR5delta32 allogeneic transplantation for cure in an animal model (Chapter 2). Then we demonstrated the bioactivity of the candidate latency-reversing agent panobinostat in BLT mice; but in accordance with its modest ability to reactivate HIV in vitro from resting cells obtained from ART-suppressed patients, we did not observe an effect on the HIV reservoir in vivo using
this single agent (Chapter 3). A cure by latency reversal will likely require a
clearance of infected cells by host immune response or a targeted immunotherapy,
and so we investigated the control of HIV infection by CTLs in BLT mice (Chapter 4)
and the systemic in vivo efficacy of a dual affinity retargeting molecule that redirects
CTLs to kill target cells (Chapter 5).

**SUMMARY OF STUDIES ON CCR5DELTA32 TRANSPLANTATION**

In order to investigate the factors that contributed to the possible cure of the
Berlin patient, we sought to study the use of CCR5delta32 allogeneic transplant in
humanized mice. First, we demonstrated that CCR5delta32-homozygous stem cells
are able to engraft in NSG mice, at even higher levels than in CCR5WT mice (Fig.
2.1). The CCR5delta32 humanized mice were then completely resistant to infection
with R5-tropic HIVJRCSF (Fig. 2.2), thus recapitulating the resistance observed in
CCR5delta32-homozygous humans and confirming that CCR5delta32 cells are
resistant to infection in vivo. Next, we tested parameters for performing a
CCR5delta32 allogeneic transplant in BLT mice. To determine CCR5delta32
engraftment, we validated a PCR/digest assay for distinguishing CCR5WT from
CCR5delta32 alleles (Fig. 2.3). Omitting irradiation during the initial humanization
procedure did not produce a long-term difference in human cell engraftment in BLT
mice (Fig. 2.4), and the administration of busulfan and antithymocyte globulin
resulted in loss of human cells in the peripheral blood over time (Fig. 2.5). However,
these conditions were not sufficient to allow engraftment of allogeneic CCR5delta32
stem cells (Fig. 2.7) or to prevent rebound in HIV-infected, ART-suppressed mice
(Fig. 2.6).
Having identified barriers and opportunities for developing an allogeneic transplant protocol in humanized mice, we sought to investigate a “kick-and-kill” strategy for HIV cure, starting with a candidate latency-reversing agent panobinostat.

**SUMMARY OF STUDIES ON PANOBINOSTAT**

The “kick-and-kill” approach toward HIV cure is to reverse HIV latency *in vivo*, such that latently infected cells begin to express HIV antigen and then are recognized and killed via the immune system or targeted cytotoxic therapies. We investigated the effects of a candidate latency-reversing agent, histone deacetylase inhibitor panobinostat, both *in vitro* and *in vivo*. Panobinostat treatment resulted in increased histone acetylation levels in human PBMCs and increased HIV RNA levels in resting cells isolated from ART-suppressed patients. Panobinostat also induced the outgrowth of replication-competent virus from latently infected resting cells, but this effect was modest (Fig. 3.1). In BLT humanized mice, panobinostat exhibited *in vivo* bioactivity, with significantly higher levels of histone acetylation observed in nearly all tissues from panobinostat-treated mice (Fig. 3.2). Next, we tested the effect of panobinostat on HIV infection in ART-suppressed BLT mice (Fig. 3.3). Across all tissues, there were no significant differences in the levels of cell-associated HIV RNA (Fig. 3.4) or HIV DNA (Fig. 3.5); and there was not a significant difference in the frequency of latently infected cells (Fig. 3.6) between panobinostat-treated and vehicle-treated mice.

The observed lack of efficacy of panobinostat on HIV infection in BLT mice could be due to several factors, including lack of clearance of reactivated cells by
CD8+ T cells (the "kill" step in "kick-and-kill"). Therefore, we evaluated whether or not CD8+ T cells are able to control HIV infection in BLT mice.

**SUMMARY OF STUDIES ON CD8-MEDIATED CONTROL OF HIV INFECTION**

We investigated the hypothesis that CD8+ T cells are able to control HIV infection in BLT humanized mice, and we approached this hypothesis by focusing our investigation on three cohorts of BLT mice derived from donors with protective HLA alleles (Fig. 4.1, Table 4.1) and infected with wildtype HIV\textsubscript{JRCSF} or \textit{nef}-deleted HIV\textsubscript{JRCSF} (Fig. 4.2). We found that in two of the three protective-HLA cohorts, mice infected with JRCSF\textit{Nefdd} had lower peak viral loads and average viral loads than mice infected with wildtype JRCSF (Fig. 4.3). Several mice from these cohorts even controlled their viremia to below the limit of detection without antiretroviral therapy. We were able to detect the presence of HLAB*2705-restricted KK10 peptide-specific CD8+ T cells in both of the cohorts derived from B*2705 donors (Fig. 4.4), and we were able to detect functional HIV-specific CTLs as assessed by IFN-gamma ELISPOT and intracellular cytokine staining (Fig. 4.5). However, the frequency of KK10-specific CD8+ T cells was not different overall between JRCSF- and JRCSF\textit{Nefdd}-infected mice (Fig. 4.4). Finally, we observed rapid increases in viremia after CD8+ T cell depletion in three mice infected with JRCSF\textit{Nefdd}; and in one of the three, viremia declined again as CD8+ T cells were returning in the peripheral blood (Fig. 4.6).

Even though functional HIV-specific CD8+ T cells are generated in infected BLT mice, these cells are not able to control wildtype HIV infection \textit{in vivo}, possibly due to the downmodulation of surface MHC class I molecule by HIV Nef. Additional
immunological agents may be necessary to restore or enhance the ability of CD8+ T cells to recognize infected cells for killing, and so we investigated the efficacy of a dual affinity retargeting (DART) molecule for redirected lysis.

**SUMMARY OF STUDIES ON CD19xCD3 DART MOLECULE**

We first needed proof-of-principle that a DART molecule would be able to effect target cell killing in BLT humanized mice before evaluating an anti-HIV DART in the context of infection and ART. Since BLT humanized mice reconstitute with human B cells as well as human T cells, we evaluated the efficacy of a CD19xCD3 dual affinity re-targeting (DART) molecule designed to redirect CD8+ T cells to kill target CD19+ B cells. Administration of the CD19xCD3 DART molecule resulted in significant depletion of CD19+ cells from the peripheral blood, bone marrow, spleen, liver, and lung (Fig. 5.1, 5.5). This depletion was temporary, as CD19+ cells began to return in the peripheral blood and tissues by day 28 after first injection (Fig. 5.3). Additionally, most of the returning CD19+ cells displayed an immature CD10+ phenotype (Fig. 5.6f), suggesting *de novo* regeneration rather than homeostatic proliferation. Over the same time period, CD19xCD3 DART molecule administration resulted in transient differences in the levels of T cells, but the absolute numbers of CD8+ T cells were not significantly different in the peripheral blood or tissues at day 28 after first injection (Fig. 5.7). Finally, we performed CD8+ T cell depletion (Fig. 5.8) before administering the DART molecule and found that DART-mediated depletion was dependent on the presence of CD8+ T cells (Fig. 5.9). With evidence that DART molecules can effect target cell killing in BLT humanized mice, the next step would be to test the effect of anti-HIV DART molecules on HIV infection in
infected, ART-suppressed BLT mice, possibly in combination with a latency-reversing agent.

**IMPLICATIONS OF CURRENT STUDIES**

Our experiments using CCR5delta32 stem cells have provided confirmatory evidence that CCRdelta32 cells are resistant to R5-tropic HIV infection in humanized mice, as they are in humans [112]. This observation supports the potential for a CCR5delta32 allogeneic transplant to result in an HIV cure in an infected humanized mouse, similar to the Berlin patient [114, 115].

While we did not achieve engraftment of the CCR5delta32 allogeneic transplant under our experimental conditions, we began to address several steps toward performing allogeneic transplants in BLT humanized mice that could be optimized for future experiments. These include omitting initial irradiation, administering busulfan for myeloablative conditioning, and administering antithymocyte globulin for depletion of existing human T cells. Our results suggest that humanized mice could serve as a preclinical platform to test current or new conditioning regimens for their effect on human cells in vivo, which would be informative toward the comparison of a variety of high-dose, reduced-intensity, and nonmyeloablative conditioning regimens [307]. If conditioning protocols can be optimized for allogeneic transplant and engraftment in BLT mice, then we can evaluate the effects of allogeneic transplant on HIV reservoirs and, specifically, whether CCR5delta32 cells would be necessary or sufficient for an HIV cure by allogeneic transplant.
The evidence that CCR5delta32 humanized mice are completely resistant to R5-tropic infection has implications for CCR5-editing gene therapy approaches for HIV cure. The rarity of the CCR5delta32 mutation (1% of the Caucasian population and rarer in other races) [135] makes the Berlin patient strategy impractical on a large scale, so researchers have focused on mimicking the CCR5delta32 genotype using gene therapy. Modification of CCR5 expression could be achieved by downregulation through RNA interference or by gene deletion using zinc-finger nucleases, TALENs, or the CRISPR/Cas9 system [451]; several of these approaches have been tested in humanized mouse models [452-457]. While some studies have demonstrated reduced viral loads after infection and selection for CCR5-modified cells in humanized mice, they are also not completely resistant to infection, likely due to the fact that only a minority of cells are successfully modified. Our results show that complete resistance to infection is in fact achievable in humanized mice when all of the human cells are CCR5-deficient. Also, the conditioning regimens that we have begun to develop may also be used for the depletion of existing human immune cells prior to the introduction of autologous CCR5-modified cells.

Our studies evaluating panobinostat in BLT mice represent a step forward in research in latency reversal. First, our observations of histone acetylation in BLT mice after panobinostat administration demonstrate both the bioactivity of panobinostat in a humanized mouse model and the utility of BLT mice to evaluate biomarkers for HDAC inhibitor activity. The lack of effect of panobinostat on the latent reservoir in BLT mice is similar to results found in human patients [184],
despite promising in vitro data [327]. Together, these results suggest that a single latency-reversing agent is not sufficient to reduce the latent reservoir in vivo, and so combinations of latency-reversing agents may be necessary [145, 330, 458].

Our studies establish the BLT mouse as a potential animal model platform in which multiple latency-reversing agents can be tested either alone or in combination for their bioactivity and for extensive tissue studies in the context of HIV infection and ART suppression. The use of animal models to evaluate safety and efficacy of combinations of latency-reversing agents will be critical to the translation of HIV cure research in the laboratory to clinical approaches for HIV eradication, as advocated by Ananworanich and Barre-Sinoussi [354]. They outline a possible roadmap for accelerated combination HIV cure research. First, candidate combinations would be evaluated for safety in animal studies. Next, approved combinations would be evaluated for efficacy in animal studies and for safety in phase 1 human clinical trials at the same time. In this step, the superior efficacy of combinations over single agents could be demonstrated using data from animal studies, thus reducing the need for human single-intervention trials. Finally, combinations that showed efficacy in animal models could be moved into phase 2 human clinical trials to evaluate their efficacy in patients.

Downstream of latency reversal, a critical question in HIV cure is whether or not CD8+ T cells will be able to clear infected cells that have been reactivated. However, few studies have evaluated the generation of HIV-specific CD8+ T cell responses in infected humanized mice. Our results show that BLT mice generate functional HIV-specific CD8+ T cells, thus providing further validation of and progress
toward the utilization of BLT mice in vaccine development. A nef-deleted mutant of SIV has been studied in macaques as a candidate live attenuated vaccine, resulting in varying levels of protection against wildtype SIV challenges after prior inoculation with SIVdeltanef [379, 380, 382, 459]. Although we did not evaluate the mice in these studies for protection against HIV challenge, we developed and implemented the needed assays to evaluate CTL responses in this model and successfully detected and measured functional HIV-specific CD8+ T cells. Remarkably, we observed CD8+ T cell-mediated control of viremia in some JRCSFNeftt-infected mice, but CD8+ T cells were not able to fully control wildtype JRCSF infection. If downmodulation of surface MHC class I by HIV Nef [400, 460] does explain this inability of CD8+ T cells to recognize infected cells in vivo, then this immune evasion will need to be further investigated and overcome in future vaccine development.

CD8+ T cells may also be unable to recognize and kill infected cells for other reasons including escape mutants in the latent reservoir [461] and CD8+ T cell dysfunction [402]. Therefore, immunomodulators may be necessary to boost the CD8+ T cell response, or targeted immunological agents may be necessary to bypass the requirement for TCR recognition of a specific peptide/MHC complex. The experiments testing the CD19xCD3 DART molecule demonstrate that CD8+ T cells are capable of being redirected in BLT mice to kill CD19+ target cells. This provides a proof-of-concept for the further evaluation of bispecific T cell-recruiting molecules for HIV-infected cells in this model. The fact that the depletion of target cells was observed in both peripheral blood and tissues is critical, as persistent HIV reservoirs are maintained both in the periphery and in tissues [73, 84, 462-464].
FUTURE DIRECTIONS

These studies show the great potential of BLT humanized mouse as a preclinical platform for multiple aspects of HIV cure research. As part of this work, we have performed experiments addressing the use of CCR5delta32 cells for transplantation in the context of HIV infection, the in vivo evaluation of latency reversal compounds, CD8-mediated control of HIV infection, and the redirecting of CTLs to kill target cell populations.

In the future, the parameters for allogeneic transplant will need to be tested and optimized in order to achieve engraftment of allogeneic CCR5delta32 stem cells in humanized mice. Maximum tolerated dose of conditioning agents such as busulfan and antithymocyte globulin will need to be assessed, and time for immune cell depletion will need to be evaluated not only in the peripheral blood but also in tissues. These parameters could also be utilized for CCR5-wildtype allogeneic transplants or donor lymphocyte infusions in order to evaluate potential graft-versus-host effects in clearing infected cells, as has been observed in graft-versus-tumor or graft-versus-leukemia effects [301, 465-467]. Persistence of HIV infection after conditioning regimens alone should also be measured.

One limitation to the study of allogeneic transplant in the BLT mouse is the difficulty of finding HLA-matched donors for the initial humanization and for the allogeneic transplant, as HLA matching is the standard for hematopoietic stem cells transplants in humans. Alternatively, BLT mice could be utilized for autologous hematopoietic stem cell transplants or adoptive transfer of autologous cells. As gene therapy technologies continue to advance, such as gene-editing and chimeric
antigen receptors, the potential for modified cells to control or cure established or suppressed infection can be evaluated in BLT mice.

For latency reversal, multiple HDAC inhibitors as well as other classes of candidate latency-reversing agents remain to be evaluated *in vivo* for HIV cure. As we have shown here, BLT mice can be used to evaluate *in vivo* activity of an HDAC inhibitor through histone acetylation analysis: biomarkers for other agents would need to be validated. Only one previous report has administered a combination of latency-reversing agents to infected, suppressed NRG-Hu mice [145], and our study is the first investigation of a single latency-reversing agent in BLT humanized mice. No combinations of latency-reversing agents have yet been reported in BLT mice.

One explanation for the lack of efficacy of panobinostat on the latent reservoir in the BLT studies or in human trials [184] is the inability of CD8\(^+\) T cells to kill reactivated cells. We provide evidence of CD8-mediated control of infection in protective-HLA mice infected with JRCSF\textit{Nef}dd. Further studies will be needed to assess more fully the polyfunctionality of CD8\(^+\) T cells against HIV-infected cells, in particular cell-killing ability. Also, a recent report suggests that broad CTL responses will be needed to clear latent HIV due to the accumulation of CTL escape mutants in the latent reservoir [461], and so mapping of the breadth of CTL response as well as sequencing of CTL escape mutants in BLT mice [386] will be informative in the characterization of CTL potential for killing infected cells in humanized mice. The role of Nef in immune evasion from CD8\(^+\) T cells should be further clarified by assessment of MHC class I downregulation in infected cells *in vivo* using flow cytometry or immunohistochemistry. Administration of a small molecule inhibitor of
Nef would be useful to assess the role of Nef in wildtype infection, and drug development in this area is in early stages [468].

CD8+ T cell dysfunction may also explain the inability of CD8+ T cells to kill HIV-infected cells after latency reactivation. Our experiments using DART molecules demonstrate that CD8+ T cells have the ability to kill when recruited to a target cell in uninfected BLT mice. The CD19xCD3 DART molecules could also be used to test in vivo CD8+ T cell killing ability in HIV-infected mice. If CD8+ T cells lose killing ability in the context of HIV infection, then we would observe no or blunted depletion of target CD19+ cells after CD19xCD3 DART molecule administration in HIV-infected BLT mice. If the CD8+ T cells retain killing ability, then CD19+ cells would still be depleted after DART molecule administration in HIV-infected BLT mice. This result would lend support to the investigation of anti-HIV DART molecules in vivo.

Several anti-HIV DART molecules have been tested in vitro and ex vivo in recent publications [232, 233]. The next step will be to test the efficacy of anti-HIV DART molecules in vivo in infected, suppressed BLT mice. Our laboratory has previously shown that an anti-HIV immunotoxin, 3B3-PE38, has the ability to reduce levels of cell-associated HIV RNA and numbers of HIV-infected cells in tissues as measured by in situ hybridization [211]; these experimental protocols can be adapted to test the same effect by anti-HIV DART molecules.

Ultimately, we will seek to combine a latency-reversing agent with a targeted cell-killing agent as the full “kick-and-kill” strategy toward HIV cure. We are currently ready to test a combination of panobinostat with 3B3-PE38 immunotoxin in the context of ART toward reduction of the latent reservoir. If panobinostat is indeed
able to reactivate HIV expression in latent cells, then the immunotoxin may be able to kill these cells and reduce the latent reservoir. Further investigations into alternative latency-reversing agents or combinations, in parallel with testing novel targeted reagents for killing infected cells, will be foundational to developing a highly effective strategy for curing HIV. BLT humanized mice will accelerate our progress toward this goal.

**FINAL SUMMARY**

To date, there is no cure for HIV. The only person to have possibly been cured is the Berlin patient. The characterization of HIV reservoirs and mechanisms for HIV latency have driven forward the development of HIV cure strategies to clear HIV infection. In this dissertation, I have described our research investigating several HIV cure strategies in BLT humanized mice, toward the long-term goal of developing an HIV cure in patients. These studies represent major progress in the *in vivo* investigation of CCR5delta32 allogeneic transplant, latency reversal, CD8+ T cell responses, and redirected lysis in a BLT mouse model of HIV infection. We have demonstrated CCR5delta32 resistance in NSG-Hu mice, and we have identified opportunities to improve conditioning regimens for future transplant experiments. We have determined that a single latency-reversing agent was insufficient to reduce HIV reservoirs *in vivo*, and our evidence suggests that CD8+ T cells are unable to recognize and kill infected cells in part due to Nef-mediated immune evasion. Finally, we showed evidence of systemic redirected lysis of target cells by CTLs after administration of a CD19xCD3 dual affinity retargeting molecule.
There is no cure for HIV, but much progress has been made in identifying steps that can be taken toward a cure. Animal models will be critical in the translation of findings in the laboratory to approaches in the clinic. Here, we have established protocols and proofs-of-concept for BLT humanized mice as a platform for future evaluation of transplant, latency reversal, vaccination, and targeted immunologic agents to clear HIV infection.
REFERENCES


43. Dorr, P., et al., *Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-


56. Organization, W.H., Guideline on when to start antiretroviral therapy and on pre-exposure prophylaxis for HIV. 2015.


