Adeno-Associated Virus (AAV) Transduction of Primary Human CD4+T Lymphocytes

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Adeno-associated virus (AAV) transduction of primary human CD4\(^+\) T lymphocytes

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Sahar Hussein Kamel
M.D., University of Tripoli, Libya

2014
Wright State University
I HEREBY RECOMMEND THAT THESIS PREPARED UNDER MY SUPERVISION BY Sahar Hussein Kamel ENTITLED Adeno-associated virus (AAV) transduction of primary human CD4+ T lymphocytes BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Development of latent HIV-infected CD4⁺ T lymphocytes is the major cause of HIV treatment failure. Adeno-associated virus (AAV) is an attractive vector for anti-HIV gene therapy due to its lack of pathogenicity, low immunogenicity, and persistent transgene expression. However, a major limitation for AAV gene transfer is the cell-specific tropism of each serotype. Only AAV serotypes 2 and 5 have been investigated in hematopoietic cells, both of these serotypes have shown low transduction efficiency. Since each of the currently described AAV serotypes demonstrate distinct tissue tropism, I hypothesized that other serotypes such as AAV1, 4, 8, or 9 may demonstrate improved transduction over AAV2 or 5. To test this, AAV infection of H9 cells (T-cell line), HeLa cells (derived from cervical cancer cells), and primary CD4⁺ T lymphocytes that were isolated from peripheral blood mononucleated cells (PBMCs) of healthy donors using a ficoll gradient, were investigated. CD4⁺ T lymphocytes were enriched to ~98% by negative selection using Easysep™ Enrichment Cocktail (Stemcell Tech.). H9 cells, HeLa cells, and CD4⁺ T lymphocytes were infected at various MOI with various recombinant AAV (rAAV) serotypes encoding the gene for GFP or luciferase. Amongst the different serotypes tested, rAAV2 had the greatest transduction efficiency in H9 cells, rAAV5 had the best transduction efficiency in HeLa cells, while none of the rAAV
serotypes appeared to infect CD4+ T lymphocytes as determined by fluorescence microscopy, flow cytometry and luciferase assay. Although none of the AAV serotypes investigated demonstrated a transduction efficiency sufficient to achieve a clinically relevant therapeutic index in primary CD4+ T lymphocytes, other serotypes or novel methods to modify tropism might yield vectors suitable for gene delivery in disease-associated leukocytes.
Table of contents

CHAPTER 1: INTRODUCTION

1.1. Proliferation and differentiation of primary human CD4^+ T cells ........................................ 2

Clusters of differentiation (CD) on primary human CD4^+ T cells .................................. 2

1.2. rAAV as a vector for gene therapy, advantages and disadvantages ........................................ 5

1.2.1. AAV2 life cycle .................................................................................................................. 6

1.2.2. AAV2 genome and capsid structure .................................................................................. 9

1.2.3. rAAV serotypes .............................................................................................................. 11

1.2.4. rAAV receptors and lectin staining .................................................................................. 11

1.3. Objective .......................................................................................................................... 15

1.4. Specific aims and hypotheses .......................................................................................... 15

CHAPTER 2: MATERIALS AND METHODS ................................................................. 18

2.1. Materials ......................................................................................................................... 18

Cell Lines ............................................................................................................................. 18

Feeder cells ............................................................................................................................ 18

Culture Media .......................................................................................................................... 20

Human CD4^+ T cell negative isolation kit and activation beads ............................................ 20

Immunofluorescent CD markers ............................................................................................ 21
CHAPTER 3: ISOLATION, CHARACTERIZATION, AND CULTURE OF PRIMARY HUMAN CD4⁺ T CELLS in vitro
Rationale ........................................................................................................................................... 40

Results .................................................................................................................................................. 42

Efficient isolation and maintenance of pure populations of primary human CD4$^+$ T cells. .................................................................................................................................................. 42

Majority of freshly isolated primary human CD4$^+$T cells are non-activated naïve cells. .................................................................................................................................................. 43

Activated primary human CD4$^+$ T cells have a phenotype of memory cells.............. 48

Conclusion .......................................................................................................................................... 50

CHAPTER 4: TRANSDUCTION EFFICIENCY OF VARIOUS rAAV SEROTYPES ON PRIMARY HUMAN CD4$^+$ T CELLS........................................................................................................................................... 51

Rationale .......................................................................................................................................... 51

Results .................................................................................................................................................. 52

rAAV serotypes 2 and 5 have a pronounced transduction efficiency on H9 cells and HeLa cells but not on primary human CD4$^+$ T cells........................................................................................................... 52

None of the other rAAV serotypes tested transduce H9 cells or primary human CD4$^+$ T cells. .................................................................................................................................................. 56

Conclusion .......................................................................................................................................... 59

CHAPTER 5: VERIFICATION THAT AAV RECEPTORS EXIST ON PRIMARY HUMAN CD4$^+$ T CELLS........................................................................................................................................... 60

Rationale .......................................................................................................................................... 60

Results .................................................................................................................................................. 64

CHAPTER 6: DISCUSSION .......................................................................................................................... 70

Conclusions ........................................................................................................................................... 73
List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Schematic of different CD markers expressed on leukocytes.</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2</td>
<td>AAV life cycle.</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3</td>
<td>The structure of wild-type AAV2 genome.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Peripheral blood mononuclear cells (PBMCs) isolation.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Negative selection of primary human CD4⁺ T lymphocytes.</td>
<td>31</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Flow cytometric analysis of cell surface markers of freshly isolated CD4⁺ T cells.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Flow cytometric analysis of various cell surface markers of freshly isolated primary human CD4⁺ T cells.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Flow cytometric analysis of CD4⁺ T cells activation CD markers.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Flow cytometric analysis of various CD4⁺ T cells activation CD markers.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Evaluation of transgene-mediated eGFP expression of rAAV2 and rAAV5 within H9 cells by flow cytometry.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 11</td>
<td>The relative transduction efficiencies of various rAAV serotypes on Hela cells as assessed by the luciferase assay.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Evaluation of transgene-mediated eGFP expression of rAAV2 and rAAV5 within primary human CD4⁺ T cells.</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 13: Evaluation of transgene-mediated eGFP expression of different rAAV serotypes within H9 cells by flow cytometry. ................................................................. 57

Figure 14: Evaluation of transgene-mediated eGFP expression of different rAAV serotypes within primary human CD4+ T cells by flow cytometry. .................. 58

Figure 15: Lectin binding profile of H9 cells. ................................................................. 62

Figure 16: Lectin binding profile of H9 cells (confocal microscopy images). ............. 63

Figure 17: Lectin binding profile of primary human CD4+ T cells. ......................... 65

Figure 18: Lectin binding profile of primary human CD4+ T cells (fluorescence microscopy images). ................................................................. 66

Figure 19: Lectin binding profile of primary human CD4+ T cells (confocal microscopy images). ................................................................. 67
List of tables

Table 1: Different rAAV serotypes with their corresponding binding ligand motifs...... 14
Table 2: Immortal cell lines ........................................................................................................ 19
Table 3: Immunoflourecent anti-human CD antibodies chosen to characterize CD4$^+$ T
cells by flow cytometry. Antibodies were purchased from e-Bioscience Inc., San
Diego, CA. ........................................................................................................................ 22
Table 4: Lectins used to determine the glycoconjugate profile on H9 and CD4$^+$ T cells,
purchased from Vector Laboratories, Inc., Burlingame, CA. .............................................. 24
Table 5: Presence (+) or absence (-) of CD markers on isolated CD4$^+$ T cells and
neutrophils as characterized by flow cytometry. ................................................................. 41
Table 6: Summary of lectin binding profile of H9 cells and CD4$^+$ T cells (relativistic
scale)..................................................................................................................................... 68
Table 7: Percentage of different CD markers expressed on CD4$^+$ T cells, isolated from all
participating donors, as characterized by flow cytometry ................................................. 80
Table 8: Data summary of rAAV transduction efficiency on primary human CD4$^+$ T
cells. ........................................................................................................................................ 81
Table 9: Data summary of lectin binding profile of primary human CD4$^+$ T cells isolated
from all participating donors. Data presented in mean fluorescence x10$^5$ (Arbitrary
unit). ........................................................................................................................................ 82
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DEDICATION

This is dedicated to

My husband, *Esmail Toumi*

My girls: *Tasnim & Nusaiba*

And

My boy, *Sufyan*

*You make all the sacrifice worth it*
CHAPTER 1: INTRODUCTION

More than 30 million people have died in the last 30 years from acquired immune deficiency syndrome (AIDS), a condition of progressive immune system failure caused by the human immune deficiency virus (HIV) (1). HIV invasion of immune system cells, including dendritic cells, macrophages and T lymphocytes (specifically CD4+ T cells), leads to the depletion of these cells. The killing of CD4+ T cells and their subsequent decline below critical levels impairs the immune system, allowing opportunistic infections that often become fatal to the host (1). The average life span of HIV infected patients, who are not subjected to any anti-HIV therapy, is estimated to be 8-10 years depending on the virus serotype (17). Currently available antiretroviral medications have succeeded in controlling HIV infections, reducing the mortality and morbidity due to AIDS and subsequently prolonging the life span of HIV-infected individuals in populations with access to these expensive medications (17). Unfortunately, the current treatments have failed to completely eradicate HIV. This failure, which is attributed mainly to the development of latent HIV-infected CD4+ T cells, is an important reason why new treatment strategies must be developed to treat HIV positive patients.

Using viral vectors to introduce a therapeutic gene is a novel approach that has been investigated as a potential treatment for many diseases. Among the clinically relevant viral vectors, adeno-associated virus (AAV) has many characteristics that make it an attractive candidate for gene therapy. AAV is a non-enveloped, ssDNA virus that
can infect many cell types with little pathogenicity, low immunogenicity, and persistent
gene expression. More importantly, AAV has also been clinically approved as the first
gene therapy vector in the developed world. Using a reliable experimental system
to characterize the tropism of various AAV serotypes to CD4\(^+\) T cells is a key step toward
the development of an anti-HIV gene therapy protocol. This thesis has focused on the
isolation, characterization of the molecular expression profile on the surface of primary
human CD4\(^+\) T cells, using different CD4\(^+\) T cell markers, and growth of primary CD4\(^+\) T
cells \textit{in vitro}. The next focus was to investigate the feasibility of using rAAV as a safe
viral vector that can efficiently transfer an anti-HIV gene therapy to primary human
CD4\(^+\) T cells.

1.1. \textbf{Proliferation and differentiation of primary human CD4\(^+\) T cells}

As part of the immune response against acute bacterial or viral infections,
antigen-specific naïve CD4\(^+\) T cells expand significantly and differentiate into effector T
helper (Th) cells, which play a critical role in antigen clearance (2, 3). As the infectious
pathogen is systematically eliminated, most of the effector cells die, leaving behind only
a small fraction to survive as memory cells in order to provide an enhanced and rapid
immunity against the same antigen. In order to design immunotherapeutic agents that
enhance the formation and function of memory CD4\(^+\) T cells, it is crucial to identify the
signals that promote the differentiation of effector (Th) cells into memory cells (5).

\textbf{Clusters of differentiation (CD) on primary human CD4\(^+\) T cells}

The Clusters of differentiation (abbreviated as CD markers) are cell surface
molecules that can act as ligands or chemokine receptors. Their activation can initiate
signaling cascades important for cell expansion, differentiation and survival. CD3, which
is a specific protein marker on T lymphocytes, makes a complex with T cell receptors (TCR) to generate activating signaling cascades important for T cells. In conjunction with CD4, a glycoprotein expressed by T helper cells, and CD8, a transmembrane protein expressed by cytotoxic T cells, these three CD markers are frequently used to distinguish T lymphocytes from other leukocytes populations. As previously mentioned, CD4⁺ T cells can be further subdivided into naïve or memory CD4⁺ T cells. As one compares the broad subsets of naïve and memory CD4⁺ T cells, it becomes evident that these two populations are heterogeneous in terms of the expression of chemokine receptors molecules on their cell surface (4).

The protein tyrosine phosphatase receptor, type C (PTPRC), also known as CD45, is a signaling molecule that is expressed by hematopoietic cells (Fig.1). CD45 can be found in several isoforms as a result of the alternative splicing of exons in its extracellular domain. CD45RA isoform is expressed on the surface of both naïve and effector CD4⁺ and CD8⁺ T lymphocytes. After their activation and differentiation into long-lived memory T cells, CD4⁺ T cells downregulate the expression of CD45RA and upregulate the expression of CD45RO (4). Furthermore, the immune activation of CD4⁺ T cells correlates with the co-expression of the activation markers CD25 and HLA-RD.

In summary, freshly isolated naïve CD4⁺ T cells are CD45RA⁺CD45RO⁻CD25⁻HLA-DR⁻, while memory CD4⁺ T cells are CD45RA⁻CD45RO⁺CD25⁺HLA-DR⁺. To assess the primary human CD4⁺ T lymphocytes isolated from PBMCs, CD3, CD4, CD45RA, CD45RO, CD25 and HLA-DR are effective markers that can be used to design panels for flow cytometry.
**Figure 1:** Schematic of different CD markers expressed on leukocytes.

1.2. rAAV as a vector for gene therapy, advantages and disadvantages.

Sustained transgene expression and an absence of vector related pathogenicity or toxicity in the infected hosts is required for the success of a virus as a safe vector for gene therapy in clinical applications (8). Using AAV as a gene therapy vector has many advantages over other commonly used recombinant viral vectors. No known disease has been recorded with AAV infection in human. In addition to a low immunogenicity against the virus capsid proteins of AAV and AAV’s ability to infect both dividing and non-dividing cells, AAV has a broad cell tropism. AAV is also a helper-dependent virus. The inability to replicate on its own adds additional safety to this vector. These factors make AAV an attractive vector for gene transfer studies (8).

Another unique feature is the ability of AAV to establish a latent infection in the host cells, in the absence of helper viruses, by stably integrating its genome into a specific locus on the human chromosome 19, called AAVS1 (13, 14, 15). While random integration into the host genome occurs on rare occasions, this predictable site-specific integration occurs in up to 20% of infected cells (13, 16), overcoming the possibility of mutagenesis that might result from random insertion, a common problem in many other viral vectors, including retroviruses.

In order to modify AAV into a vector for gene therapy, the cap and rep encoding regions from the viral genome have been removed to create space for the vector DNA. Unfortunately, deletion of cap and rep eliminated the site-specific integration capacity of the virus. Even though all of these features make AAV a stellar candidate for use as a gene therapy vector, the standard AAV gene therapy vector is not suitable for carrying
large therapeutic genes due to its limited cloning capacity (~4.5 kb) and its dependence on host cell machinery to synthesize double stranded DNA from its single stranded DNA genome (18). To circumvent the second-strand synthesis limitation, another version of AAV, containing self-complementary recombinant genomes (scAAV), has been created (18). The self-complementary genome in scAAV is able to refold into dsDNA and provide a very rapid expression of viral DNA in the host cell (19).

To date, many clinical trials that have been conducted using of AAV vectors across a plethora of chronic diseases including cystic fibrosis, hemophilia B, prostate cancer and others have showed promising results and all have emphasized the safety of all AAV vectors tested (20).

1.2.1. AAV2 life cycle

AAV is a member of the genus Dependovirus and requires help from other “helper” viruses such as the herpes simplex virus or adenovirus in order to replicate within the host cell (8, 9, 11). There are two distinct stages in the life cycle of AAV, depending on the presence or absence of the helper virus (21). After a successful host infection, helper virus co-infection ensues the start of the lytic stage of the AAV life cycle, in which the AAV undergoes productive replication and synthesis of new viral proteins and results in the production of new virions (21). AAV undergoes these following steps as it progresses through the lytic cycle:

1- Binding to the host cell membrane
2- Receptor-mediated endocytosis
3- Endosome- mediated intracellular trafficking
3- Late-endosome or lysosome escape
4- Nucleus entry
5- Uncoating
6- AAV genome ssDNA to dsDNA conversion
7- Rep gene expression
8- Cap gene expression
9- Virion assembly, and
11- Virion release from the infected host cell.

However, the absence of a helper virus results in a lysogenic stage, which involves limited virus replication, repressed viral genome expression, and the establishment of latent infection by the site-specific viral genome integration into chromosome 19 of the infected cells, as discussed before (21, 22). Once the viral genome has been integrated into the infected host genome, helper virus co-infection can rescue the integrated virus by the use of rep proteins that cut the integrated AAV genome out of chromosome 19 (22) (Fig. 2).
The AAV life cycle has two stages that are regulated by number of complex interactions that require the AAV genome and proteins, as well as adenoviral and host proteins (21). In the presence of adenovirus co-infection, AAV undergoes productive replication and synthesis of new viral proteins, and results in the production of new virions (21). The absence of a helper virus results in a lysogenic stage and the establishment of latent infection by site-specific integration into chromosome 19 (21).

**Figure 2:** AAV life cycle. Adapted from (21).
1.2.2. AAV2 genome and capsid structure

AAV is a non-enveloped, ssDNA virus that belongs to the parvovirus family (10). Wild type AAV has 4.7-kb DNA genome that contains two open reading frames (ORF) flanked by two inverted terminal repeats (ITR), 145-bp long each (21, 23). The left ORF, rep, encodes 4 proteins essential for genome replication and viral rescue (21, 22, 23). The right ORF, cap, encodes 3 structural proteins that assemble together to form the icosahedral capsid of the virus (23) (Fig.3). It is still unclear whether the ITRs of the viral genome contribute to the tissue tropism of each serotype (9). The icosahedral capsid of AAV is composed of 60 copies of 3 proteins, VP1, VP2, and VP3 that are translated from the same mRNA and arranged at a molar ratio of 1:1:18 (9, 12, 31) or 1:1:10 (21), depending on the source, for these 3 capsid proteins within the complete viral capsid. The alternative usage of the start codon results in the three proteins sharing the same C-terminal region, but having unique N-termini (9). There are projections surrounding the threefold axis of the icosahedral structure, while depressions lie at its twofold axis and around the fivefold axis (9). Differences in the structural features between the AAV serotypes may account for the differences in their ability to use various sugar motifs as viral receptors (9).
ITRs flank the rep and cap encoding genes. P5, P19, and P40 promoters produce the corresponding rep and cap transcripts as shown above.

Figure 3: The structure of wild-type AAV2 genome. Adapted from (21).
1.2.3. rAAV serotypes

A new virus serotype is identified as a virus that does not cross-react with the neutralizing antibodies specific to any of the previously discovered and characterized serotypes of the same virus (9). Although AAV2 is the most extensively investigated AAV serotype, at least another 10 AAV serotypes, named AAV1-11, have been described. Based on the previous definition, AAV6, 10 and 11 cannot be considered as true serotypes (9). AAV6 shares an identical serological profile to AAV1, which is attributed to the > 99% homology of its amino acids sequences in the capsid proteins, prominently VP3, to AAV1 (24). The serological profiles of both AAV10 and AAV11 are still not well characterized (9, 25, 26, 27).

All other AAV serotypes have significant differences in the amino acid sequences of their capsid proteins, mostly in VP3 (24). These differences are thought to determine the cell or tissue tropism of the virus as well as its efficiency to transduce a specific cell type (24). Up to 96% of the world population is seropositive for AAV2 (24). Although AAV2 shows a natural tropism to wide varieties of tissues, including the central nervous system, skeletal muscles, lung and liver tissue (9), AAV2 has a lower transduction efficiency in these tissues than AAV9, which possesses a similar tissue tropism profile (9). In contrast, AAV1 and AAV5 are better at transducing vascular endothelial cells, while AAV8 is superb in infecting the liver (9). Moreover, AAV6 and AAV7 have higher levels of transduction in skeletal muscles and AAV5 and AAV6 are superior in transducing airway epithelial cells (9, 24).

1.2.4. rAAV receptors and lectin staining
The infectious pathways of non-enveloped viruses starts with the binding of the virus to its receptors on the host cell surface (24). In AAV2, VP3 protein in the virus capsid is responsible for receptor mediated viral binding and the determination of the tissue tropism of the virus (24). VP3 often initiates the interaction of the AAV2 capsid with glycosaminoglycan receptors on the host cell surface (9). A secondary interaction of the viral capsid with co-receptors is necessary for the virus to proceed toward the intracellular trafficking pathway (9). For AAV2, heparan sulfate proteoglycans (HSPG) are important primary receptors for cell binding and transduction. Secondary interactions with the coreceptors: human fibroblast growth factor receptor 1 (FGFR1), integrins αVβ5/α5β1, and hepatocyte growth factor are further important for the attachment and intracellular trafficking of the virus (9, 28). Glycosaminoglycans are expressed on the surface of most adherent cells, which explains the broad tropism of AAV2 (24).

AAV4 and AAV5, in which VP3 displays the lowest homology to AAV2, use different receptors other than HSPG to mediate their cellular binding (24, 28). It has been reported that AAV4 uses α 2,3 O-linked sialic acid as a receptor for infection while AAV5 uses α 2,3 N-linked sialic acid (9, 24, 28, 32, 33). AAV6, whose VP3 has up to 60% homology to both AAV4 and AAV5, also uses sialic acid receptors for binding, whereas AAV3, whose VP3 is ~87% similar to AAV2 also binds to HSPG (9, 24). Unlike AAV2, AAV1 does not bind to HSPG due to its lack of heparan binding amino acids (9). However it has been reported that AAV1 uses α2,3-linked or α2,6-linked sialic acid as primary receptors for cell entry (34). Moreover, while no glycan receptor has been identified for AAV8 (35, 36), recent studies reported that while AAV9 uses N-linked galactose (35), and laminin receptor (LamR) functions as binding receptor for AAV8 and AAV9 (35, 36).
The carbohydrate binding proteins, termed lectins, have been widely used as biochemical tools to recognize the nature and diversity of carbohydrate structure on different cell surfaces. This wide use of lectins is attributed to their high specificities to sugars (29). Lectins are found in all living organisms, with plant lectins being the most intensively studied group (29). Each lectin has at least two binding sites for the carbohydrate molecules (30). Although lectins interact with carbohydrates using relatively weak bonds, these weak interactions allow easy unlinking and ensure specificity (30). Table 1 shows the different rAAV serotypes with their corresponding lectin-binding motifs.
**Table 1**: Different rAAV serotypes with their corresponding binding ligand motifs.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Ligand motifs</th>
<th>AAV binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mannose binding lectins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concanavalin A (ConA) From <em>Canavalia ensiformis</em></td>
<td>α-D-mannosyl and α-D-glucosyl residues</td>
<td>AAV9</td>
</tr>
<tr>
<td><strong>N-acetylglactosamine (Galactose) binding lectins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut agglutinin (PNA) From <em>Arachis hypogaea</em></td>
<td>Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)</td>
<td>AAV9</td>
</tr>
<tr>
<td>Dolichos biflorus agglutinin (DBA) From <em>Dolichos biflorus</em></td>
<td>Galβ1-4GalNAcβ1-R</td>
<td>AAV9</td>
</tr>
<tr>
<td>Ricinus communis Agglutinin (RCA) From <em>Ricinus communis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean Agglutinin (SBA) From <em>Soybean</em></td>
<td></td>
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<tr>
<td><strong>N-acetylglucosamine (sialic acid) binding lectins</strong></td>
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</tr>
<tr>
<td>Wheat Germ Agglutinin (WGA) From <em>Triticum vulgaris</em></td>
<td>GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)</td>
<td>AAV4, AAV5</td>
</tr>
<tr>
<td>Maackia amurensis leukoagglutinin (MAL) From <em>Maackia amurensis</em></td>
<td>Neu5Ac/Gca2,3Galβ1,4Glc(NAc)</td>
<td>AAV1, AAV4, AAV5</td>
</tr>
<tr>
<td><strong>Fucose binding lectins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulex europaeus agglutinin (UEA) From <em>Ulex europaeus</em></td>
<td>Fuca1-2Gal-R</td>
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</table>
1.3. Objective

The engineering of a safe viral vector that can efficiently transfer a therapeutic gene to primary human CD4$^+$ T cells is critical for the success of an anti-HIV gene therapy. As none of the molecular investigations of latent-HIV infection in CD4$^+$ T cells have involved the use of AAV2-based vectors, this study is a novel approach that allows for the examination of the transduction efficiencies of various rAAV serotypes on primary human CD4$^+$ T cells.

Although recent studies have discussed how various AAV serotypes use specific sugar motifs (e.g. sialic acid, mannose or heparan sulfate) on the cell surface as primary receptors, none of these studies have been performed on primary human CD4$^+$ T cells. Therefore, another goal of this study is to profile the glycoconjugate molecular basis of the cell membrane of primary human CD4$^+$ T cells. This novel glycoconjugate profile could be applied in the future to develop new methods to modify the tropism of various rAAV serotypes, yielding vectors that can efficiently deliver an anti-HIV gene therapy to HIV-infected cells.

1.4. Specific aims and hypotheses

I have focused on three distinct specific aims. The central aim is to investigate the possibility of using rAAV as a safe viral vector that can efficiently transfer an anti-HIV therapeutic gene to primary human CD4$^+$ T cells.

The first aim was to develop a method that permits detailed, replicable investigations on primary human CD4$^+$ T cells. Based on data from a previous study on HIV (6), I suggested that primary human CD4$^+$ T cells could be isolated from peripheral
blood mononuclear cells (PBMCs) and grown in vitro for a prolonged period of time to generate a population size large enough for efficient characterization and use in multiple experiments. The characterization of CD4⁺ T cells will help improve our understanding of their biology and can be used to design and test an anti-HIV gene therapy that kills HIV-infected cells.

The second aim was to examine the transduction efficiency of various rAAV serotypes on primary human CD4⁺ T cells. Including rAAV2 in this study is a novel approach, since AAV2-based vectors have not been used in any of the previous molecular investigations of latent-HIV infection in CD4⁺ T cells. Data from a previous set of experiments done in our lab demonstrated the susceptibility of H9 cells (T cell line) to infection with rAAV2 and rAAV5. Based on these previous findings, I hypothesized that at least one of the rAAV serotypes that have been characterized can efficiently transduce primary human CD4⁺ T cells. It will close a substantial gap in the literature once it is determined whether any of the previously characterized rAAV serotypes can efficiently transduce primary human CD4⁺ T cells.

The third aim was to investigate the sugar motifs on the cellular surface of primary human CD4⁺ T cells to see if these cells express any of the previously described rAAV viral receptors. A previous set of experiments conducted in the Excoffon lab has profiled the sugar motifs present on the H9 cell membrane, thereby confirming the presence of rAAV receptors on the surface of these cells. The lectin binding profile of H9 cells correlates with their susceptibility to rAAV2 and rAAV5. If any of the rAAV serotypes tested in this study failed to transduce primary human CD4⁺ T cells, one could attribute this failure to the unavailability of the primary viral receptors on the surface of
these cells. Therefore, I hypothesized that rAAV serotypes do not efficiently transduce primary human CD4\(^+\) T cells due to a lack of the appropriate AAV viral receptors on their cell surface.
CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

Cell Lines

Two immortal cell lines were used for various experiments (Table 2). The H9 cell line was a generous gift from Dr. Dawn Wooley and the HeLa cell line was purchased from ATCC, Virginia.

Feeder cells

H80 (U-251MG) cells were used as a feeder cell layer for primary human CD4+ T cells. Duke University, Durham, NC. supplied the H80 cell line (7). All cell lines were stored in liquid nitrogen in the Department of Biological Sciences at Wright State University, Dayton, OH.
**Table 2: Immortal cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>ATCC No</th>
<th>Media</th>
<th>Seeding (0.5ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H9 cells</strong></td>
<td>Cutaneous T lymphocyte</td>
<td>HTB-176™</td>
<td>RPMI-1640+10% FBS +glutamine</td>
<td>2×10⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Without red phenol</td>
<td></td>
</tr>
<tr>
<td><strong>HeLa cells</strong></td>
<td>Epithelial cells</td>
<td>CCL-2™</td>
<td>DMEM+10% FBS</td>
<td>2×10⁵</td>
</tr>
<tr>
<td><strong>H80 cells</strong></td>
<td>Malignant glioblastoma tumor</td>
<td></td>
<td>RPMI-1640+10% FBS + glutamine</td>
<td>2×10⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>With red phenol</td>
<td></td>
</tr>
</tbody>
</table>
Culture Media

The cells were cultured in appropriate sterile RPMI-1640 culture medium (MP Biomedicals, LLC, Solon, OH) supplemented with 10% fetal bovine serum immediately after removing the cells from liquid nitrogen. After the stimulation of growth, the culture medium was further substituted with a final medium-serum mix containing penicillin and streptomycin antibiotics (plus 20 IU/ml of recombinant IL-2 [R&D Systems, Inc.] for T cell culture medium). The powdered culture medium, containing 2.05 mM L-glutamine, was dissolved in distilled deionized water (ddH₂O) from a Nanopure Millipore Water System. All culture media were supplemented with 3.024 g/L sodium bicarbonate. The solution was adjusted to a pH between 7.0 and 7.1 with 1 N HCl dropwise. The culture media solutions were sterilized through a 0.2 µm-pore cellulose nitrate Nalgene vacuum filtration apparatus. For H9 cells, pre-made RPMI-1640 1X medium without L-glutamine and phenol red was used and glutamine was added, according to the manufacture instructions, right before using the media. Sterile media were supplemented with 1% penicillin/streptomycin and the appropriate heat inactivated fetal bovine serum by appropriate volume percentage. The final culture media were stored in 500 ml Pyrex bottles and kept at 4°C.

Human CD4+ T cell negative isolation kit and activation beads

EasySep™ Human CD4⁺ T Cell Enrichment Kit (catalog #19052) and EasySep™ Magnet (catalog # 18000) were purchased from STEMCELL Technologies Inc., Canada. Dynabeads® Human T-Activator CD3/CD28 (catalog #11161D) was purchased from Life Technologies AS, Oslo, Norway.
**Immunofluorescent CD markers**

The immunofluorescent CD markers used in this study for flow cytometry were purchased from eBioscience, Inc. San Diego, CA. and listed in Table 3.

**Flow cytometry**

Flow cytometry (The BD Accuri™ C6) was available through the biochemistry and molecular biology (BMB) general facilities located in Diggs laboratories at Wright State University. The flow cytometry data were analyzed using FCS Express 4 software.

**Fluorescence microscopy**

Fluorescence images were obtained from fluorescence microscopy NIKON Eclipse TE2000-S at magnification of 10X and 40X.
Table 3: Immunofluorescent anti-human CD antibodies chosen to characterize CD4+ T cells by flow cytometry. Antibodies were purchased from e-Bioscience Inc., San Diego, CA.

<table>
<thead>
<tr>
<th>Cell marker</th>
<th>Clone</th>
<th>Cat No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HumanCD4-FITC</td>
<td>OKT4 (OKT-4)</td>
<td>11-0048-41</td>
</tr>
<tr>
<td>Anti-HumanCD3-APC</td>
<td>OKT3</td>
<td>17-0037-41</td>
</tr>
<tr>
<td>Anti-HumanCD11b-PE</td>
<td>CBRM1/5</td>
<td>12-0113-41</td>
</tr>
<tr>
<td>Anti-HumanCD15-FITC</td>
<td>HI98</td>
<td>11-0159-41</td>
</tr>
<tr>
<td>Anti-HumanCD25-APC</td>
<td>BC96</td>
<td>17-0259-41</td>
</tr>
<tr>
<td>Anti-Human HLA-DR-PE</td>
<td>L243</td>
<td>12-9952-41</td>
</tr>
<tr>
<td>Anti-HumanCD45RA-APC</td>
<td>HI100</td>
<td>17-0458-41</td>
</tr>
<tr>
<td>Anti-HumanCD45RO-PE</td>
<td>UCHL1</td>
<td>12-0457-41</td>
</tr>
</tbody>
</table>
Lectins

Lectin kit fluorescein (catalog # FLK-2100) was purchased from VECTOR LABORATORIES, INC. Burlingame, CA. The list of lectins used in this study is shown in Table 4.

For cell cytopinning, cytopin (13mm double ring) slides (catalog # 22-037-242) were purchased from Fisher Scientific, Pittsburgh, PA and the double cytology funnels (catalog # 10-356) were purchased from Fisher Healthcare, Houston, TX. Vectashield mounting medium with DAPI (catalog # H-1200) was purchased from VECTOR LABORATORIES, INC. Burlingame, CA. Shandon cytopin 2 centrifuge was kindly provided by Dr. Wooley (Math and Microbiology building, Wright State University, Dayton, OH.)
**Table 4:** Lectins used to determine the glycoconjugate profile on H9 and CD4⁺ T cells, purchased from Vector Laboratories, Inc., Burlingame, CA.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Common Abbreviation</th>
<th>Cat No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricinus Communis Agglutinin</td>
<td>RCA</td>
<td>FL-1081</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td>WGA</td>
<td>FL-1021</td>
</tr>
<tr>
<td>Dolichos Biflorus Agglutinin</td>
<td>DBA</td>
<td>FL-1031</td>
</tr>
<tr>
<td>Maackia Amurensis Lectin</td>
<td>MAL</td>
<td>FL1311</td>
</tr>
<tr>
<td>Ulex Europaeus Agglutinin</td>
<td>UEA</td>
<td>FL-1061</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>Con A</td>
<td>FL-1001</td>
</tr>
<tr>
<td>Peanut Agglutinin</td>
<td>PNA</td>
<td>FL-1071</td>
</tr>
<tr>
<td>Soybean Agglutinin</td>
<td>SBA</td>
<td>FL-1011</td>
</tr>
</tbody>
</table>
Confocal microscope

Lectin staining was evaluated by laser scanning confocal microscopy (Olympus FV1000) with a 20X as well as 60X oil immersion lens. The Olympus FV1000 confocal microscope is available through the Microscopy Core Facilities in Biological Sciences Building II, Wright State University, Dayton, OH.

rAAV serotypes

rAAV1, rAAV2, rAAV4, rAAV5, rAAV8 and rAAV9 carrying either the eGFP or luciferase gene were all supplied by the University of Iowa Vector Core, Iowa City, IA.

Beta-galactosidase assay

Adenovirus serotype 5 containing either the β-galactosidase gene (Ad-β-Gal) or luciferase gene was purchased from the University of Iowa Vector Core, Iowa City, IA. The Galacto-Light Plus System (catalog T2119) was purchased from Applied Biosystems, MA. USA.

Luciferase assay

The Luciferase assay system kit (Promega Corporation, Madison, WI, catalog # E1483) was purchased through Thermo Fisher Scientific Inc. Waltham, MA. USA.

2.2 Methods

Blood Sampling

A total of five healthy subjects (22-50 years old) were included in this study. These individuals, representing several different nationalities, were recruited from different labs in the Diggs Laboratory building at Wright State University in Dayton, Ohio. The protocol was approved by the Wright State University Institutional Review
Board (IRB). Each blood donor signed an informed consent form after a thorough review of the protocol. Approximately 60 ml of blood was obtained using vacuum-driven devices through a 20-gauge butterfly needle, which was inserted into a forearm vein. This sample was then collected into six sterile, purple-top, vacutainer blood collection tubes that contained the potassium salt of EDTA (K₂EDTA) as an anticoagulant. The blood samples were then directly transferred to the Excoffon lab in Diggs and processed immediately.

**Peripheral blood mononuclear cells (PBMCs) isolation (Fig. 4)**

Peripheral blood multinuclear cells (PBMCs) were isolated by ficoll gradient. 30 ml of the isolated blood sample were transferred with a 10 ml pipette and a pipette aid to a 50 ml conical tube containing 7.5 ml of 6% dextran. The 6% dextran solution was prepared by adding 0.9 g dextran to 15 ml saline (0.9% NaCl) and warmed to 37°C for 10 min to allow the dextran to fully dissolve. After dividing the initial 60 ml of blood into (two) 30 ml samples combined with 6% dextran, as described above, both tubes were inverted twice to ensure the proper mixing of the blood with the dextran. After allowing the blood to sit at room temperature for 25-30 min, the top phase (30-35 ml volume) was removed with a 10 ml plastic pipette and added to a 50 ml conical tube (For personal safety and protection, the RBCs in the remaining bottom phase were then bleached by adding approximately 50 ml of 100% bleach for a final concentration of 70% bleach, according to the lab guidelines for handling blood). Cells in the 50 ml conical tube containing the top phase of the initial sample were spun down at 4000 rpm for 3 min. After discarding the supernatant, the pellet was gently resuspended in 1 ml saline (no bubbles), and then 34 ml of additional saline solution was added. The tube was then
separated into layers by the addition of 8 ml of ficoll with a pasture pipette that was slowly removed to allow as much ficoll as possible to be added without shaking or disturbing the developing layers. The tube was then centrifuged at 740 rfc in the Eppendorf centrifuge 5810 R at 4°C for 15 min with the brakes turned off and the acceleration set to 5. After centrifugation, a white ring, which contained monocytes and lymphocytes, was visible at the interphase. The white ring of PBMCs was recovered (in ~5 ml) and placed in a 50 ml conical tube via a p1000 pipetman with a long tip. Cells were then washed 2 times with 40 ml of Mg²⁺ and Ca²⁺ free phosphate buffered saline solution (PBS -/-) and one more time with 40 ml of PBS -/- supplemented with 2% fetal bovine serum (FBS) and 1 mM EDTA. These cells were then spun down at 150 rcf at 19°C for 10 min with the brake turned off and the acceleration set to 5. The supernatant was discarded and the cells were resuspended for counting in 1ml of T cell culture medium (RPMI-1640 with 10% FBS, 1% Penicillin and Streptomycin and 20 ul Interleukin 2 (IL-2)). The average concentration of isolated PBMCs was $5 \times 10^7$ cells/ml.
Figure 4: Peripheral blood mononuclear cells (PBMCs) isolation.

Modified from [http://www.amroboticsystems.co.uk/pbm200c.html](http://www.amroboticsystems.co.uk/pbm200c.html)
Negative selection of primary human CD4\(^+\) T lymphocytes (Fig. 5)

PBMCs were transferred to a 5 ml polystyrene tube. The EasySep\textsuperscript{TM} Human CD4\(^+\) T Cell Enrichment Cocktail was then added to the PBMCs to generate a concentration of 50 µl/ml. The unwanted cells were targeted for removal with the tetrameric Antibody Complexes in the EasySep\textsuperscript{TM} Human CD4\(^+\) T Cell Enrichment Cocktail, which recognizes CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCR\(\gamma/\delta\), glycophorin A and dextran-coated magnetic particles (STEMCELL Technologies Inc. Canada). The sample was mixed well by pipetting up and down 2-3 times and the cells were then incubated at room temperature (15-25\(^{\circ}\)C) for 10 min. The EasySep\textsuperscript{TM} D magnetic particles were vortexed for 30 sec to ensure that they were in a uniform suspension before being added to the sample at a concentration of 100 ul/ ml. The sample again was mixed well by pipetting up and down 2-3 times and the cells were once again incubated at room temperature (15-25\(^{\circ}\)C) for 5 min. After the incubation period is done, the cell suspension was brought up to a total volume of 2.5 ml by adding T cell culture medium. Sample was mixed well by pipetting up and down 2-3 times. The tube was placed into the magnet (Purple) and allowed to incubate for 5 min. Both the magnet and tube were then picked up and inverted in one continuous motion for 2-3 sec to pour off the excessive supernatant that contained the purified CD4\(^+\) T cells, into a new 5 mL polystyrene tube. Being labeled magnetically by the EasySep\textsuperscript{TM} D magnetic particles, the unwanted cells were held by the magnetic field inside the original tube. The purified CD4\(^+\) T cells (~98\% pure), unless required for experiments, were stimulated for 4 days with pre-washed Dynabeads\textsuperscript{®} (CD3/CD28 antibodies bound to magnetic beads) (life technologies) in T cell culture medium. The Dynabeads\textsuperscript{®} were first resuspended in
the vial by vortexing the vial for 30 sec. The desired volume of Dynabeads® (25ul/1×10^6 cells) was then transferred into a 5 mL polystyrene tube, and 1 ml of a buffer, consisting of PBS -/- and supplemented with 2% Fetal Bovine serum (FBS) and 1 mM EDTA, was added and mixed well by pipetting up and down 2-3 times. The tube was then placed back into the magnet (Purple) and allowed to incubate for 1-2 minutes. The supernatant was the discarded and the tube was removed from the magnet. Washed Dynabeads® were then resuspended with 2.5 ml of T cell culture medium containing the CD4^+ T Cells, purified in a previous step. The Dynabeads® bound CD4^+ T cells were grown in 2 wells of a 24 well plate within a humidified incubator at 37ºC and 5% CO_2 for 4 days. After the activation of CD4^+ T cells for 4 days, the beads were removed by transferring the cells into a 5 mL polystyrene tube and placing the tube back into the purple magnet for 1-2 min. The supernatant containing the cells was then transferred to a new tube, which could be used immediately for new experiments or to maintain a stock culture.
Figure 5: Negative selection of primary human CD4⁺ T lymphocytes.
Adapted from www.stemcell.com.
**Cell culture maintenance**

Cell culture techniques were performed under a laminar flow hood using aseptic techniques. Cell cultures were stored and grown in 25 or 75-cm² tissue culture flasks within a humidified incubator at 37°C and 5% CO₂. Fresh T cell culture medium was added every 4-5 days based upon a pH change from 7.1 to about 6.0, as indicated by a color change of the medium from pink to yellow. The culture density was maintained at 1.5 × 10⁶ - 2.0 × 10⁶ cells/ml. Cells were seeded on 30-40% confluent gamma-irradiated H80 cell layers. Every 2-3 days, fresh medium containing IL-2 replaced half of the T cell culture medium, and every 2 weeks the CD4⁺ T cells were transferred to fresh flasks of H80 feeder cells. All flasks were supplied with medium-serum mix to a final volume of 5 ml in a 25-cm² tissue culture flask and 9 ml in a 75-cm² tissue culture flask. Before each experiment, cell viability was verified using the trypan blue exclusion test. Cell viability of 80% or more was considered sufficient for conducting experiments.
Determination of cell concentration (hemocytometry)

The following technique was used during cell seeding prior to adenovirus or AAV infections. Adherent monolayers of cells were trypsinized from 1-2 flasks and combined in a 50 ml centrifuge tube while cells in suspension were collected in a 15 ml Falcon tube. The tube, containing either trypsinized adherent cells or cells in suspension, was centrifuged at 480 rpm for 5 min at 18°C. The supernatant was aspirated and the cells were resuspended in 1 ml of culture medium and mixed gently by pipetting up and down. A clean coverslip was centered on a hemocytometer between the outside railings over the two counting grids. A drop of well-mixed cell suspension was placed at each notch. The drop was added once to ensure even distribution of cells. Cells were counted with a push button counter using 400 x total magnification. Cells were counted in the four corner squares (1 mm x 1 mm) of the cytometer. These squares were 1/400 mm². The average value of the total cells counted in the 4 squares was multiplied by $1 \times 10^4$ to determine the number of cells per milliliter. The volume of the mixture was adjusted by dilution with culture medium to an appropriate concentration, depending on the demands of the particular assay.

Cryopreservation and thawing of CD4⁺ T Cells

The cells were resuspended in 95% T cell culture medium plus 5% DMSO in a cryovial then stored overnight at -80°C. On the following day, the cells were transferred to a liquid nitrogen storage tank for future use.

Thawing: Cryopreserved cells were thawed at 37°C in a water bath. When the cells were almost thawed, 1 ml of pre-warmed T cell culture medium was added dropwise at a rate of 1 ml/5 seconds. The cells were then collected from the cryovial and
added to a 15 ml Falcon tube containing 4 ml of pre-warmed T cell culture media and spun down at 840 g, 18-20°C for 5 min. To remove the cytotoxic cryopreservant DMSO, the cell pellet was washed twice with PBS -/- . Washed cells were then resuspended in 5 ml T cell culture media and cultured in a 25-cm² tissue culture flask. The cells were then incubated within a humidified incubator at 37°C and 5% CO₂ for further use. ~70-80% of CD4⁺ T cells were recovered with this protocol.

Flow cytometry

For the characterization of the molecular expression profile on the CD4⁺ T cell surface, cells were stained with antibodies against various surface markers (Table 3). Unstained CD4⁺ T cells (no antibodies added) were used as negative controls and included in each experiment. 10⁵ cells were first resuspended in a volume of 50 ul of T cell cultured medium and kept on ice. Cells were then washed with ice-cold PBS -/- in 1% BSA buffer (250ul/sample) and centrifuged at 1800 x g for 4 min at 4°C. CD4⁺ T cells were resuspended in 100 ul/sample of ice-cold PBS -/- in 1% BSA buffer and stained with 5 ul of fluorescent probe-conjugated antibodies and incubated for 45 min at 4°C. After the incubation period, CD4⁺ T cells were washed once in ice-cold PBS -/- in 1% BSA buffer and fixed by 4% paraformaldehyde (100ul/sample) and incubated for 15 min within a humidified incubator at 37°C and 5% CO₂. Cells were then washed twice with 500 ul/sample of ice-cold PBS -/- in 1% BSA buffer. The cell pellet was resuspended in 100 ul of ice-cold PBS -/- in 1% BSA buffer and analyzed by flow cytometry available in the BMB general facilities at Diggs laboratories at Wright State University. The flow cytometry data were analyzed using FCS Express Software.

Adeno-associated virus infection
Various rAAV serotypes containing either the eGFP or luciferase gene were diluted with culture medium (appropriate to the cell type included in the experiment) to the multiplicity of infection (MOI) indicated in the text. Growth medium was aspirated from adherent cells that had been seeded in 24 well plates for 24 hours, and the cells were rinsed with phosphate buffered saline with Ca$^{2+}$ and Mg$^{2+}$ (PBS+/+). 250 ul of each diluted rAAV serotype was added to each well in a 24 well plate at the MOI indicated in the text. When non-adherent cells were used for the same experiment, a cell suspension was prepared at a concentration of $2.5 \times 10^5 / 25$ ul of culture medium in each 1.5 ml tube in the case of AAV-eGFP, or $5 \times 10^5 / 25$ ul of culture medium in each 1.5 ml tube in the case of AAV-luciferase. 25 ul of diluted AAV was added to each cell suspension, in 1.5 ml microcentrifuge tubes at the MOI indicated in the text. Both adherent and non-adherent cells were then incubated for 1 hr at 37°C and 5% CO$_2$. The inoculum was then removed, cells were rinsed with culture medium, and fresh culture medium was added accordingly to each cell type. All cells were incubated in 24 well plates at 37°C and 5% CO$_2$ until further analysis.

**Adenovirus infection**

Adenovirus serotype 5 containing either the β-galactosidase gene (Ad-β-Gal) or luciferase gene (Ad-Luc) was diluted with EMEM culture medium without serum to the multiplicity of infection (MOI) indicated in the text. Growth medium was then aspirated from adherent cells that had been seeded on 24 well plate for 24 hours, and adherent cells were rinsed with PBS+/+. 250 ul of diluted adenovirus was added to each well in a 24 well plate at a MOI of approximately 100 plaque forming units/cell (PFU/cell). When non-adherent cells were used for the adenovirus transduction experiment, a cell
suspension was prepared at a concentration of $5 \times 10^5/25 \text{ ul}$ of EMEM culture medium in each 1.5 ml tube. 25 ul of diluted adenovirus was added to each cell suspension, in 1.5 ml microcentrifuge tubes at a MOI of approximately 100 plaque forming units/cell (PFU/cell), unless otherwise indicated. Both adherent and non-adherent cells were incubated for 1 hr at 37°C and 5% CO$_2$. The inoculum was then removed, cells were rinsed with EMEM, and fresh culture medium was added accordingly to each cell type. All cells were incubated in 24 well plates at 37°C and 5% CO$_2$ until further analysis.

**Beta-galactosidase assay**

The Galacto-Light Plus System (Applied Biosystems, USA) was used to analyze adenovirus-mediated beta-galactosidase activity according to manufacturer directions. Briefly, the cells were first transferred from the 24 well plate to 1.5 ml microcentrifuge tubes. Then the cells were lysed 24 hours after the initial adenovirus infection. 40 ul of Tropix lysis buffer was added to each 1.5 ml microcentrifuge tube, which was then placed on the rotator, at medium speed, for 15 min at 4°C. 20 ul of cell lysate was transferred from each tube to a new tube, followed by 120 ul of a 100X dilution of Galacton and 60 min incubation at room temperature. 200 ul of accelerator was then added just before measuring β-galactosidase luminescence in a Luminometer. Protein concentration was determined by adding 6 ul of lysate into a cuvette, followed by the addition of 1 ml Bio-Rad reagent diluted (1:10 with double distilled water (ddH$_2$O)). This mixture was then incubated at room temperature for 10 min and the optical density was determined with a spectrophotometer set to 595 nm. Each experiment was performed in 2-3 replicates and lysis buffer was used as control. The data were then analyzed by the two-tailed Student’s T-test (Prism, GraphPad).
Luciferase assay

Transfected cells were harvested 48 hours after the initial transfection in 1.5 ml microcentrifuge tubes. First, they were washed once in 1x PBS and then lysed in 30 ul of 1x cell culture lysis Buffer (Promega) at room temperature. According to the manufacturer’s directions, luciferase assays were performed on 20 ul of cell lysates by adding 100 ul of Luciferase Assay reagent (Promega) just before measuring the luciferase luminescence in the Luminometer. To calculate the number of relative light units per milligram of protein for each cell lysate, protein concentration was determined by adding 6 ul of lysate into a cuvette, followed by the addition of 1 ml Bio-Rad reagent diluted 1:10 with double distilled water (ddH2O). This mixture was then incubated at room temperature for 10 min and the optical density was determined with a spectrophotometer set to 595 nm. Each experiment was performed in 2-3 replicates and lysis buffer was used as control. The data were then analyzed by the two-tailed Student’s T-test (Prism, GraphPad).

Lectin staining

CD4\(^+\) T cells were stained with FITC- conjugated lectins (Table 4). Unstained CD4\(^+\) T cells (no fluorescent conjugated-lectin probe added) were used as negative controls and included in each experiment. \(10^5\) cells were first resuspended in a volume of 50-60 ul of T cell cultured medium/ 1.5ml tube and kept on ice. The cells were then washed with ice-cold PBS +/- in 1% BSA buffer (250ul/ sample) and spun at 1800g for 4 min at 4 °C. Cell pellets were resuspended in 100 ul/sample of ice-cold PBS +/- in 1% BSA buffer and stained with 2.5 ul of FITC- conjugated lectins and incubated for 45 min at 4 °C in a dark environment. After the incubation period, CD4\(^+\) T cells were washed three times in ice-
cold PBS -/- in 1% BSA buffer and fixed with a freshly prepared form of 4% formaldehyde (100 ul/sample) and incubated for 15 min within a humidified incubator at 37°C and 5% CO₂. The cells were then washed twice with 500 ul /sample of ice-cold PBS -/- in 1% BSA buffer. The cell pellet was resuspended in 100 ul of ice-cold PBS -/- in 1% BSA buffer and analyzed by flow cytometry in the BMB general facilities. The flow cytometry data were then analyzed using FCS Express Software. Furthermore, cells were mounted onto glass slides using Vectashield mounting media with DAPI (see cytospinning of CD4⁺ T cells) and images were taken using the fluorescence microscope in the Miller lab or the laser scanning confocal microscopy (Olympus FV1000) in the Biological Sciences building with a 60X oil immersion lens to evaluate the density of cell staining.

Cytospinning of CD4⁺ T cells

The cytospin double ringed slides were pre-labeled and inserted, bound to an empty double cytology funnel, into solid metal holders. 200 ul of lectin stained CD4⁺ T cells (in suspension) were loaded into each cuvette of the double cytology funnel. Cells were centrifuged onto cytospins (glass slides) at 500 rpm for 5 min. Then the glass slide and the double cytology funnel were held firmly together and the cytospin slide was extracted from the funnel and metal holder without damaging the freshly prepared cytospin slides. Next, the cells were fixed by adding 90-100 ul/ring of the cytospin slides of 4% paraformaldehyde for 10 min at room temperature. The fixed cells were then washed with 100 ul of PBS/ring on the cytospin slides for 10 min at room temperature. One drop of Vectashield mounting medium with DAPI was added to the cells on each ring of the cytospin slides. Each slide was then covered with a coverslip. The edges of the
coverslip were sealed to the glass slide by adding a layer of nail polish. The cytopspun-lectin stained CD4\(^+\) T cells were then inspected under a fluorescence and/or confocal microscope.
CHAPTER 3: ISOLATION, CHARACTERIZATION, AND CULTURE OF PRIMARY HUMAN CD4\(^+\) T CELLS \textit{in vitro}.

\textbf{Rationale}

In order to design and test a gene therapy vector that kills HIV-infected CD4\(^+\) T cells, we need an experimental method that permits extensive biochemical analysis of these cells and provides an excellent opportunity to improve our understanding of their biology.

I isolated cells based on a previous study on HIV, reported by Cloyd et al. (7), that suggested that primary human CD4\(^+\) T cells can be isolated from peripheral blood mononuclear cells (PBMCs) and grown \textit{in vitro} for a prolonged period of time to a population size large enough to facilitate detailed investigations. Using different CD4\(^+\) T cells markers, it is expected that one will be able to characterize the molecular expression profile on the surface of the isolated CD4\(^+\) T cells. Table 5 shows the expression of the various CD markers tested on both primary human CD4\(^+\) T cells and neutrophils.
**Table 5:** Presence (+) or absence (-) of CD markers on isolated CD4$^+$ T cells and neutrophils as characterized by flow cytometry.

<table>
<thead>
<tr>
<th>Cell marker</th>
<th>CD4$^+$ T Cells</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD25</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD45RA</td>
<td>+ On naïve T cells</td>
<td>-</td>
</tr>
<tr>
<td>CD45RO</td>
<td>+ On memory T cells</td>
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Results

Efficient isolation and maintenance of pure populations of primary human CD4⁺ T cells.

For proper identification of the purified primary human CD4⁺ T lymphocytes, cells were stained with antibodies against CD4 and CD3 T cell surface markers. The light scatter (side scatter versus forward scatter) shown in fig. 6a defined two distinct populations. A P1 gate was placed around the population of primary human CD4⁺ T cells. Data on all other dot plots were created from this P1 gated population, while unstained CD4⁺ T cells were used as negative controls (Fig. 6b). ~66% represented the purity of isolated CD4⁺ T cells in P-gated population, as shown in the dot plot in fig.6c. ~98% pure populations of primary human CD4⁺ T lymphocytes were isolated by ficoll gradient from the PBMCs of healthy donors (Fig. c and 6d). As expected, ~97% of the purified cells expressed CD3, the specific protein marker of T lymphocytes, on their surfaces (Fig. 6f and g).

The purified CD4⁺ T cells were then stimulated for 4 days with pre-washed Dynabeads® (CD3/CD28 antibodies bound to magnetic beads) in T cell culture medium containing IL-2. After 4 days of incubation at 37°C and 5% CO₂, as described by Cloyd et al. (7), the beads were removed and the cells were seeded on a feeder layer of H80 cells in the presence of IL-2. Cell expansion was confirmed by the marked increase in cell numbers when counted using a hemocytometer. Even though the division rate of CD4⁺ T cells slowed down substantially 4 weeks after their initial isolation, the percentage of dead cells remained between 15%-25% in the co-cultures, as indicated by Trypan blue dye exclusion (7). Our observations confirmed that using the H80 feeder cell
layer keeps the activated primary human CD4$^+$ T cells alive for a long enough period of time in culture to be used for multiple experiments.

**Majority of freshly isolated primary human CD4$^+$ T cells are non-activated naïve cells.**

As shown in fig.7, CD4$^+$ T cells that were freshly isolated have a mixture of ~70% CD45RA$^+$CD45RO$^-$ naïve cells (Fig.7a and b) and ~35%, CD45RA$^-$CD45RO$^+$ memory cells (Fig.7c and d). Less than 1% of the freshly isolated cells represent a population of CD45RA$^+$CD45RO$^+$ dual-positive cells that have remained in a transitional phenotype between naïve and memory CD4$^+$ T cells (6). Before stimulation with CD3/CD28 magnetically bound beads that enhance T cell differentiation into memory cells, freshly isolated primary human CD4$^+$ T cells do not express the activating CD markers, CD25 and HLA-DR, or the memory cell CD marker, CD45RO. Moreover, the majority of freshly isolated primary human CD4$^+$ T cells were non-activated naïve cells as indicated by the expression of high levels of CD45RA and low levels of CD25 (Fig.8c and d) and HLA-DR (Fig.8a and b). Very similar patterns of molecular expression profiles were observed on the surface of the freshly isolated primary human CD4$^+$ T cells obtained from all donors in this study, with the exception of the fifth donor. For the fifth donor, the majority of freshly isolated CD4$^+$ T cells were CD45R$^-$CD45RO$^+$ memory cells expressing higher level of the activation markers, CD25 and HLADR (data not shown). These exceptional observations could potentially be explained by a recent exposure of the donor’s CD4$^+$ T cells to antigens.
98.33% of the cells isolated from the blood of the human donor, using the CD4\(^+\) T cell purification kit from Stemcell Tech, were CD4 positive. (a) Flow cytometry revealed two populations upon each isolation of primary human CD4\(^+\) T cells. A P1 gate was placed around the population of cells under investigation. (b) Unstained primary human CD4\(^+\) T cells, (no antibodies added) were used as a negative control (note that cells in the bottom right hand quadrant are considered to be CD4 positive). (c) The purity of isolated CD4\(^+\) T cells, in a P1 gated population. (d and e) Primary human CD4\(^+\) T cells stained with CD4.
markers. Up to 98.33% of the purified cells were CD4 positive, based on the percentage of cells in the right hand quadrant. (f and g) The expression of T lymphocyte marker (CD3) on freshly isolated primary human CD4⁺ T cells. Based on the percentage of cells in the bottom right hand quadrant, 94.03% of the stained cells were CD3 positive.
**Figure 7**: Flow cytometric analysis of various cell surface markers of freshly isolated primary human CD4⁺ T cells. (a, b) CD45RA versus (c, d) CD45RO. The PBMCs population that expresses CD45RA⁺CD45RO⁻ represents the naive T cells while CD45R⁻CD45RO⁺ cells are memory T cells. The majority of freshly isolated primary human CD4⁺ T cells are naïve T cells (a, b) and 35.48% of them are memory cells (c, d).
**Figure 8:** Flow cytometric analysis of CD4$^{+}$ T cells activation CD markers. Before their stimulation with cross-linked anti-CD3/anti-CD28 plus IL-2 (see Material and Methods), freshly isolated primary human CD4$^{+}$ T cells did not express the activation markers as indicated by low levels of HLADR (a, b) and CD25(c, d).
Activated primary human CD4\(^+\) T cells have a phenotype of memory cells.

In order to test the phenotypic changes that might occur within the primary human CD4\(^+\) T cells population after their activation with CD3/CD28 magnetically bound beads and their subsequent expansion, the CD4\(^+\) T cells were stained once again with fluorescently labeled antibodies to different cell surface markers. These data were then analyzed using flow cytometry. As shown in (Fig. 9d), 95.97\% of the activated cells became CD45R\(^-\) CD45RO\(^+\) memory cells, while only 38.50\% of the activated cells remained CD45RA\(^-\) CD45RO\(^-\) naïve cells (Fig. 9c). Consistent with these results, we found that the activation markers, CD25 and HLA-DR that were expressed only at low levels by the non-activated naïve CD4\(^+\) T cells, were expressed at higher levels (64.8\% and 71.4\% for CD25 and HLA-DR, respectively) by the activated cells (Fig. 9a and b).
**Figure 9**: Flow cytometric analysis of various CD4$^+$ T cells activation CD markers. The majority of the primary human CD4$^+$ T cells differentiated to memory cells within 7 days of their stimulation with cross-linked anti-CD3/anti-CD28 plus IL-2. The PBMCs population that expresses CD45RA$^+$CD45RO$^-$ represents the naive T cells while CD45R$^-$CD45RO$^+$ cells are memory T cells. (a) High levels of the activation marker CD25 (64.87%) and (b) HLADR (71.47%) were observed in the activated cells. (c) The expression of CD45RA (30.50%) versus (d) CD45RO (95.97%) marks the transition from naïve cells to memory cells.
**Conclusion**

Before stimulation with CD3/CD28 magnetically bound beads that enhance their differentiation into memory cells, freshly isolated primary human CD4\(^+\) T cells are not expected to express the activating CD markers, CD25 and HLADR, or the memory cell CD marker, CD45RO. Our data supported this hypothesis, since the majority of freshly isolated primary human CD4\(^+\) T cells were non-activated naïve cells as indicated by the expression of high levels of CD45RA and low levels of CD25 and HLA-DR. Thus, most of the cells initially isolated are naïve CD4\(^+\) T cells that became memory CD4\(^+\) T cells upon their activation, by CD3/CD28 magnetic beads, in an *in vitro* culture system. Table 7 (Appendix) summarizes data of flow cytometric analysis of different CD markers expressed on primary human CD4\(^+\) T cells isolated from all participating donors.
CHAPTER 4: TRANSDUCTION EFFICIENCY OF VARIOUS rAAV SEROTYPES ON PRIMARY HUMAN CD4+ T CELLS.

Rationale

No molecular studies of latent-HIV infection in CD4+ T cells have investigated the use of AAV2-based vectors. Examining the transduction efficiency of various other rAAV serotypes on primary human CD4+ T cells is a novel approach. To prove that rAAV is a safe viral vector that can efficiently transfer an anti-HIV therapeutic gene to primary human CD4+ T cells, I hypothesized that at least one of the rAAV serotypes that have been characterized can efficiently transduce primary human CD4+ T cells that have either been freshly isolated, or have been adapted to stably replicate in vitro for an extended period of time. The H9 cell line (derived from T-cell lymphoma) has been shown in a previous study by Poornima Kotha in our lab (Fig.10), to be susceptible to AAV transduction and rAAV2 shows the greatest transduction efficiency in H9 cells, followed by rAAV5 (Fig.12). To provide a comprehensive investigation, I used this cell line as well as the intensively studied HeLa cell line (derived from cervical cancer cells) as positive controls.

CD4+ T lymphocytes were infected with various rAAV serotypes encoding the gene for GFP at various MOI. The transduction efficiency of each rAAV serotype was determined by fluorescence microscopy and flow cytometry. As an alternative approach, various rAAV serotypes encoding the gene for the enzyme luciferase were also used in
this study. The results from this set of experiments were determined by performing a luciferase assay on the infected cells. The luciferase reporter gene within each rAAV serotype is controlled by a CMV promoter. The luciferase assay is a simple and highly sensitive technique used to detect transfection efficiency and gene expression. This assay is based on the detection of photo emission, which results from the reaction between the enzyme and its substrate, detected via a luminometer. In comparison to rAAV-eGFP, with rAAV-luciferase does not need flow cytometry or fluorescence microscopy to visualize transfection efficiency.

Results

rAAV serotypes 2 and 5 have a pronounced transduction efficiency on H9 cells and HeLa cells but not on primary human CD4\(^+\) T cells.

Both H9 cells and primary human CD4\(^+\) T lymphocytes were initially infected with either rAAV2- or rAAV5-eGFP at a MOI of 10\(^6\) viral genomes/cell (vg/cells). As shown in fig.10, rAAV2 showed the highest transduction efficiency in H9 cells followed by rAAV5, 48 Hr after infection, as indicated by the percentage of the cells expressing GFP. However, neither one of the two rAAV serotypes tested in this experiment demonstrated an appreciable transduction efficiency in primary human CD4\(^+\) T cells (Fig.12). Alternatively, when rAAV2 and rAAV5 serotypes encoding the gene for luciferase at MOI of 10\(^4\) and 10\(^5\) respectively were used to infect H9 cells, HeLa cells and primary human CD4\(^+\) T lymphocytes, once again, rAAV2 and rAAV5 were able to transduce H9 cells and HeLa cells (Fig.11) but not primary human CD4\(^+\) T lymphocytes (data not shown).
Figure 10: Evaluation of transgene-mediated eGFP expression of rAAV2 and rAAV5 within H9 cells by flow cytometry.

H9 cells were infected with rAAV2 and rAAV5 at MOI $10^5$ to $10^6$ vg/cell.
- SSC-H (Side scatter) on the Y-axis describes the complexity of the cell.
- The X-axis measures the intensity of the green fluorescence.
- Cells in the right bottom quadrant represent the percentage of cells with green fluorescence.

(a) Negative control sample. (b) The percentage of eGFP expressing cells in rAAV2 infected H9 cells at MOI $10^5$ vg/cell. 75.65% of infected H9 cells were effectively transduced. (c) The percentage of eGFP expressing cells in rAAV2 infected H9 cells at MOI $10^6$ vg/cell. 78.13% of infected H9 cells were effectively transduced. (d) The percentage of eGFP expressing cells in rAAV5 infected H9 cells at MOI $10^6$ vg/cell. 29.88% of infected H9 cells were effectively transduced.
Adenovirus infected HeLa cells were used as a positive control (a) Adenovirus and rAAV were used to transduce HeLa cells at a multiplicity of infection (MOI) of $10^2$ and $10^4$ vg/cell, respectively. Luciferase assays were conducted on cell lysates 24 hours after infection. The relative light units (RLUs) were normalized to the total amount of protein in the lysate of each sample. Average values for each vector were calculated and plotted on a logarithmic scale (b). The transduction efficiency of adenovirus was an order of magnitude greater than the transduction efficiencies of the various rAAV serotypes tested. Amongst the rAAV serotypes investigated, rAAV5 showed the highest transduction efficiency in HeLa cells followed by rAAV2.

**Figure 11:** The relative transduction efficiencies of various rAAV serotypes on Hela cells as assessed by the luciferase assay.
Primary human CD4\(^+\) T cells were infected with rAAV2 and rAAV5 at MOI 10\(^4\) to 10\(^6\) vg/cell, Flow cytometry analysis was performed 48 hours after infection.

- SSC-H (Side scatter) on the Y-axis describes the complexity of the cell.
- The X-axis measures the intensity of the green fluorescence.
- Cells in the right bottom quadrant represent the percentage of fluorescent cells
- (a) Only 0.30% of cells expressed eGFP in the negative control sample
- (b) 0.29% of cells expressed eGFP in rAAV2 infected primary human CD4\(^+\) T cells at MOI 10\(^4\)
- (c) 0.15% of cells expressed eGFP in rAAV2 infected primary human CD4\(^+\) T cells at MOI 10\(^6\)
- (d) 0.51% of cells expressed eGFP in rAAV5 infected primary human CD4\(^+\) T cells at MOI 10\(^6\).

Neither rAAV2 nor rAAV5 efficiently transduce primary human CD4\(^+\) T cells.

**Figure 12:** Evaluation of transgene-mediated eGFP expression of rAAV2 and rAAV5 within primary human CD4\(^+\) T cells.
None of the other rAAV serotypes tested transduce H9 cells or primary human CD4\textsuperscript{+} T cells.

In the next step, I examined whether any of the other rAAV serotypes can efficiently transduce primary human CD4\textsuperscript{+} T cells. Both H9 cells (done in a previous set of experiments in our lab by Poornima Kotha) and primary human CD4\textsuperscript{+} T lymphocytes were infected with rAAV1, rAAV4, rAAV8 and rAAV9 encoding the gene for GFP or luciferase at various MOI (according to the viral titer of each stock available in our lab) and the transduction efficiencies were quantitated using flow cytometry for GFP-encoding rAAV serotypes or luciferase assay for luciferase-encoding rAAV serotypes. Results obtained 48 hours later showed that none of other rAAV serotypes tested was efficient at transducing H9 cells (Fig.13) or primary human CD4\textsuperscript{+} T cells (Fig.14). In contrast, these same rAAV serotypes, encoding the gene for luciferase at MOI of 10\textsuperscript{4} vg/cell, were able to efficiently transduce HeLa cells, although at an order of magnitude below the transduction efficiency of the positive control (adenovirus) (Fig.15).
Figure 13: Evaluation of transgene-mediated eGFP expression of different rAAV serotypes within H9 cells by flow cytometry.

None of the rAAV serotypes tested efficiently transduces H9 cells as indicated by a) only 1.33% of AAV1 infected H9 cells expressed eGFP at MOI $10^6$ vg/cell, (b) 1.17% of AAV8 infected H9 cells expressed eGFP at MOI $10^6$ vg/cell and (c) 0.45% of AAV9 infected H9 cells expressed eGFP at MOI $10^6$ vg/cell.
**Figure 14:** Evaluation of transgene-mediated eGFP expression of different rAAV serotypes within primary human CD4$^+$ T cells by flow cytometry. None of the rAAV serotypes tested was effective at transducing primary human CD4$^+$ T cells as indicated by (a) only 0.18% of AAV1 infected CD4$^+$ T cells expressed eGFP at MOI $10^6$ vg/cell (b) 0.49% of AAV4 infected CD4$^+$ T cells expressed eGFP at MOI $10^5$ and (c) 0.58% of AAV9 infected CD4$^+$ T cells expressed eGFP at MOI $10^6$. 
Conclusion

Amongst the different rAAV serotypes tested, rAAV2 exhibited the greatest transduction efficiency in H9 cells, followed by rAAV5. The rAAV5 serotype also elicited the highest transduction efficiency in HeLa cells. However, none of the AAV serotypes tested demonstrated a transduction efficiency large enough to achieve a clinically relevant therapeutic index in primary human CD4+ T cells. To be clinically effective, it is expected that an optimal AAV vector should transduce more than 90% of the target cells at a MOI in the range of 1-100 vg/cell. More research needs to be done to discover an AAV able to be clinically relevant for CD4+ T cell transduction. Table 8 (Appendix) summarizes the transduction efficiency of various rAAV serotypes on primary human CD4+ T cells isolated from all participating donors, as characterized by flow cytometry.
CHAPTER 5: VERIFICATION THAT AAV RECEPTORS EXIST ON PRIMARY HUMAN CD4\(^+\) T CELLS.

Rationale

Previous studies emphasize that AAV serotypes utilize specific sugar motifs (e.g. sialic acid, mannose or heparan sulfate) on the cell surface as primary receptors (9) (Table 1). However, these studies have not been performed on primary human CD4\(^+\) T cells. Because of their known high binding affinity and specificity to carbohydrates, lectins have been widely used to recognize the sugar moieties on different cell membranes. The lectin binding profile obtained from a previous set of experiments, done in our lab by Poornima Kotha on H9 cells, recognized the prominence of \(N\) – acetylgalactosamine, sialic acid, and mannose molecules on the cell surface of H9 cells, as indicated by their high binding affinities to RCA, WGA, and Con A respectively (Fig. 15 and 16). The presence of these sugar motifs was also indicated by the binding of other lectins included in this study (Table 4). Due to distinct differences in rAAV susceptibility between H9 and CD4\(^+\) T cells, I decided to assay the glycoconjugate profile of primary human CD4\(^+\) T cells to examine the existence of AAV receptors on primary human CD4\(^+\) T cell membranes. Here, I hypothesized that rAAV serotypes do not efficiently transduce primary human CD4\(^+\) T cells due to a lack of viral receptors on their cell surface. To support my hypotheses, I utilized various fluorescently labeled lectins,
listed in table (4), to specifically label different polysaccharides on primary human CD4+ T cells. This glycoconjugate profile could be applied to explore novel methods to modify viral-cell tropism or develop cell-specific rAAV serotypes that yield vectors suitable for the delivery of an anti-HIV therapeutic gene in disease-associated leukocytes.
Figure 15: Lectin binding profile of H9 cells. Cells were incubated with FITC-conjugated lectins and samples were analyzed for the mean fluorescence detected from the stained cells by flow cytometry. RCA (binds with N-acetylgalactosamine), WGA (binds with sialic acid), and Con-A (binds with mannose) are the major lectins that are interacting with sugar motifs on the H9 cell membrane as indicated by mean fluorescence of > 2x10^6 for both RCA and WGA and ~0.75 x10^6 for Con-A. (Data provided by Poornima Kotha).
**Figure 16:** Lectin binding profile of H9 cells (confocal microscopy images). Cells were incubated with FITC-conjugated lectins and samples were imaged by confocal microscopy at 60X magnification. RCA (binds to N-acetylgalactosamine), WGA (binds to sialic acid), and Con-A (binds to mannose) are the major lectins that are interacting with sugar motifs on the H9 cell membrane. (Data provided by Poornima Kotha).
Results

Primary human CD4\(^+\) T cells were stained with fluorescently labeled lectins and analyzed by flow cytometry for the mean fluorescence detected from the stained cells. To further assess the staining intensity of these cells, pictures were taken with fluorescence and/or confocal microscopes. Compared with the previously discussed glycoconjugate profile of H9 cells, the lectin staining of CD4\(^+\) T cells gave a similar profile, in that FITC-conjugated RCA, WGA, and Con-A strongly stained CD4\(^+\) T cells. This emphasized the prominence of N-acetylgalactosamine, sialic acid, and mannose sugar molecules on the cell surface of CD4\(^+\) T cells, respectively, as shown in Figs. 17-19. Interestingly, other lectins, which did not stain the H9 cell surface well, did not show any detectable staining on the CD4\(^+\) T cell surface. In other words, a marked decrease of SBN, PNA, MAL, DBA, and PNA staining in CD4\(^+\) T cells, when compared to H9 cells, was clearly shown by fluorescence and confocal microscopy (Fig. 18 and 19). Table 9 (Appendix) summarizes the lectin binding profile of primary human CD4\(^+\) T cells isolated from all participating donors.
Figure 17: Lectin binding profile of primary human CD4⁺ T cells. Cells were incubated with FITC-conjugated lectins and samples were analyzed for the mean fluorescence detected from the stained cells by flow cytometry. (a) RCA (binds with N-acetylgalactosamine), WGA (binds with sialic acid), and Con-A (binds with mannose) are the prominent lectins that are binding to the sugar motifs of the primary human CD4⁺ T cell membrane as indicated by mean fluorescence of > 0.5x10⁶ for both RCA and WGA and a mean fluorescence of ~0.2x10⁶ for Con-A. (b) Log scale of lectin binding profile of primary human CD4⁺ T cells.
Figure 18: Lectin binding profile of primary human CD4\(^+\) T cells (fluorescence microscopy images). Cells were incubated with FITC-conjugated lectins and samples were imaged by fluorescence microscopy at 10X magnification. RCA (binds with N-acetylgalactosamine), WGA (binds with sialic acid), and Con-A (binds with mannose) are the prominent lectins that bind to the sugar motifs of the primary human CD4\(^+\) T cell membrane.
**Figure 19**: Lectin binding profile of Primary human CD4\(^+\) T cells (confocal microscopy images). Cells were incubated with FITC-conjugated lectins and samples were imaged by confocal microscopy at 60X magnification. RCA (binds with N-acetylgalactosamine), WGA (binds with sialic acid), and Con-A (binds with mannose) are the prominent lectins that bind to the sugar motifs of the primary human CD4\(^+\) T cell membrane.
Table 6: Summary of lectin binding profile of H9 cells and CD4$^+$ T cells (relativistic scale)

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Lectin staining density from sparse (++) to intense (++++).

- Lectin staining with a mean fluorescence $<10^5$ is considered as a negative.
- Lectin staining with a mean fluorescence $>10^6$ is considered as an intense positive.

Conclusion

The lectin staining of primary human CD4\textsuperscript{+} T cells and H9 cells revealed a high affinity of their cell membranes to RCA, WGA, and Con-A, which bind to N-acetylgalactosamine, sialic acid, and mannose, respectively. Yet even though these sugar motifs are featured prominently on the cell surface of both H9 cells and CD4\textsuperscript{+} T cells and many AAV serotypes use polysaccharides (e.g. sialic acid, mannose, or heparan sulfate) on the cell surface of their target cells as receptors (Table 1), many of the rAAV serotypes that can efficiently transduce H9 cells cannot transduce CD4\textsuperscript{+} T cells. As such, the transduction efficiencies of the rAAV serotypes do not correlate with the results of the fluorescently labeled lectin-binding assays. Our data show that the composition of the glycoconjugates of primary human CD4\textsuperscript{+} T cells does not correlate with viral infection. Although it appears that AAV use the receptors, previously described, to bind to the host cells, this might be due to the differential arrangement of sugar molecules on the CD4\textsuperscript{+} T cell surface, which does not permit the viral cell entry. Another suggestion is that a lack of the co-receptors from CD4\textsuperscript{+} T cell surface or post-binding barriers are responsible for the differences in AAV susceptibility between H9 cells and CD4\textsuperscript{+} T cells.
CHAPTER 6: DISCUSSION

This study investigated the transduction efficiencies of various rAAV serotypes on primary human CD4+ T lymphocytes, a key step toward the development of an anti-HIV gene therapy protocol. Here, I enriched CD4+ T lymphocytes to 94.76% +/- 0.04 (+/- SD; n=4 donors) by negative selection, using Easysep™ Enrichment Cocktail (Stemcell Tech.). I then characterized the molecular expression profile on the surface of primary human CD4+ T cells, using different CD4+ T markers. I was also able to grow primary CD4+ T cells *in vitro* for a prolonged period of time to a population size large enough to facilitate detailed investigations and use in multiple experiments. My data demonstrated that the majority of freshly isolated primary human CD4+ T cells are non-activated naïve cells that do not express the CD markers associated with activation, CD25 (29.91% +/- 0.29 (SD; n= 3 donors) and HLADR (48.24% +/- 0.3 (SD; n= 2 donors), or the memory cell CD marker, CD45RO (62.50% +/- 0.12 (SD; n= 3 donors). My data also demonstrated that most of the initially isolated naïve CD4+ T cells became memory CD4+ T cells upon activation with CD3/CD28 magnetically bound beads in an *in vitro* culture system.

A previous study by Poornima Kotha in our lab has shown that the H9 cell line is susceptible to AAV transduction, with rAAV2 showing the greatest transduction efficiency in H9 cells followed by rAAV5. Therefore, I tested the hypothesis that at least one of rAAV serotypes that have been characterized can efficiently transduce primary
human CD4+ T cells that have either been freshly isolated, or have been adapted to stably replicate for an extended period of time in vitro. To provide a comprehensive investigation, I used this cell line as well as the intensively studied HeLa cell line (derived from cervical cancer cells) as positive controls. My data confirmed the susceptibility of H9 cells to rAAV2 and rAAV5. I also have shown that amongst the different rAAV serotypes tested, rAAV serotypes 2 and 5 have a pronounced transduction efficiency on HeLa cells. However, none of the AAV serotypes tested demonstrated detectable transduction efficiency in primary human CD4+ T cells. It suggests that the most likely reason for the variability in the transduction efficiencies of various rAAV serotypes on the three cell types is the difference in the viability of rAAV receptors on their cell surfaces.

The prominence of N–acetylgalactosamine, sialic acid, and mannose molecules, which are involved in the cell entry of various rAAV serotypes, has been recognized on the cell surface of H9 cells in a previous set of experiments, done in our lab by Poornima Kotha. Therefore, I lastly hypothesized that the inability of rAAV serotypes to efficiently transduce primary human CD4+ T cells is due to a lack of viral receptors on their cell surface. Surprisingly, I have shown that the lectin staining of CD4+ T cells gave a similar profile to what we obtained previously with the lectin staining of H9 cells. On CD4+ T cells, several lectins did not bind well to the cells, whereas others stained strongly. Statistical analysis of lectin staining from 5 experiments with cells from 3 different donors demonstrated that there was a significant difference in staining with WGA, RCA and Con-A (p<0.0001 by one way ANOVA). Tukey post-hoc analysis only showed a difference between control and RCA, WGA and Con-A lectin staining. When comparing
the three lectins (RCA, WGA and Con-A) that stained the CD4⁺ T cells, there was not a statistically significant difference between them (one way ANOVA, ns). Although there was a high level of N-acetylgalactosamine, sialic acid, and mannose sugar molecules on the cell surface of CD4⁺ T cells, the glycoconjugate profile of primary human CD4⁺ T cells does not seem to correlate with viral infection. Many AAV serotypes use the same polysaccharides found on the surface of CD4⁺ T cells as receptors, yet viral infection is negligible. This might be due to the differential arrangement of sugar molecules on the CD4⁺ T cell surface, which makes them inaccessible to the virus and does not permit the viral cell entry. Further investigations for detailed molecular architecture of primary receptors could be carried out by Western blot analysis and immunocytochemistry for the specific viral receptor proteins. Further study to determine the virus-cell binding by using a radioactive AAV or various AAV serotypes carrying another fluorescent gene rather than GFP, are also important to circumvent this hurdle. Furthermore, a secondary interaction of the viral capsid with co-receptors is necessary for the virus to proceed toward the intracellular trafficking pathway (9) and a lack of these co-receptors from CD4⁺ T cell surface could be the reason behind the inability of various AAV serotypes to infect these cells. Thus, an experiment to verify the existence of the virus secondary receptors on the surface of primary human CD4⁺ T cells should be carried out in the future. Western blot analysis or immunocytochemistry with antibodies specific to AAV secondary receptors could be used to verify the existence of these co-receptors on primary human CD4⁺ T cells. Another explanation could be the presence of post binding barriers that might affect any critical step involved in the virus lifecycle including: receptor-mediated endocytosis, endosome-mediated intracellular trafficking, late-
endosome or lysosome escape, nucleus entry, uncoating, AAV genome ssDNA to dsDNA conversion, Rep gene expression, Cap gene expression, virion assembly and virion release from the infected host cell. Intensive future biochemical studies for each of these steps are needed to determine the exact mechanisms. All above suggested studies should include the use of another cell line with proven susceptibility to the various AAV serotypes investigated, as a positive control.

Conclusions

Collectively, my findings suggest the possibility that the differential arrangement of sugar molecules on the cell surface or post binding barriers could be responsible for the differences in AAV susceptibility between H9 cells and CD4$^+$ T cells.

Future studies

The engineering of a safe viral vector that can efficiently transfer a therapeutic gene to primary human CD4$^+$ T cells is critical for the success of an anti-HIV gene therapy. This study demonstrated low to no transduction efficiency for all rAAV serotypes tested on primary human CD4$^+$ T cells. As such, none of the AAV serotypes that were investigated demonstrated the minimal transduction efficiency required to achieve a clinically relevant therapeutic index. Future experiments should focus on building a deeper understanding of the biology of the various AAV serotypes, including the molecular architecture of AAV primary and secondary receptors and intensive future biochemical studies for each critical step involved in the virus lifecycle, or novel methods to modify the tropism of existing AAV serotypes to yield vectors suitable for gene
delivery in disease-associated primary human CD4\(^+\) T lymphocytes.
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APPENDIX

Table 7: Percentage of different CD markers expressed on CD4\(^+\) T cells, isolated from all participating donors, as characterized by flow cytometry

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<tr>
<td></td>
<td></td>
<td>2 weeks old cells</td>
<td></td>
<td></td>
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<td>1 week old cells</td>
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</tr>
<tr>
<td>CD4: 95.8%</td>
<td>CD4: 80</td>
<td>CD4: 90.5%</td>
<td>CD4: 83</td>
<td>CD4: 97.2%</td>
<td>CD4: 98</td>
<td>CD4: 88.0%</td>
<td>CD4:</td>
<td>CD4: 97.9%</td>
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</tr>
<tr>
<td>CD45RA: 30.8%</td>
<td>CD45RA:</td>
<td>CD45RA: 11.9%</td>
<td>CD45RA:</td>
<td>CD45RA: 46.5%</td>
<td>CD45RA:</td>
<td>CD45RA: 37.9%</td>
<td>CD45RA:</td>
<td>CD25: 7.1%</td>
<td>N/A</td>
</tr>
<tr>
<td>CD45RO: 39.1%</td>
<td>CD45RA:</td>
<td></td>
<td>CD45RO:</td>
<td></td>
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<td></td>
<td>CD45RO:</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>CD25: 4.3%</td>
<td>CD45RA:</td>
<td></td>
<td>CD3:</td>
<td></td>
<td>CD45RO:</td>
<td></td>
<td>CD3:</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>CD45RA:</td>
<td></td>
<td>11.9%</td>
<td></td>
<td>CD25:</td>
<td></td>
<td>3.04%</td>
<td></td>
<td>N/A</td>
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<td></td>
<td>CD45RO:</td>
<td></td>
<td>83.31%</td>
<td></td>
<td>HLA-DR:</td>
<td></td>
<td>62.7%</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>CD25:</td>
<td></td>
<td>27.7%</td>
<td></td>
<td>HLA-DR:</td>
<td></td>
<td>25.4%</td>
<td></td>
<td>N/A</td>
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<tr>
<td></td>
<td>HLA-DR:</td>
<td></td>
<td>28.7%</td>
<td></td>
<td>HLA-DR:</td>
<td></td>
<td>26.9%</td>
<td></td>
<td>N/A</td>
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</tbody>
</table>

Table 7: Percentage of different CD markers expressed on CD4\(^+\) T cells, isolated from all participating donors, as characterized by flow cytometry.
Table 8: Data summary of rAAV transduction efficiency on primary human CD4$^+$ T cells.

<table>
<thead>
<tr>
<th>rAAV-eGFP serotypes</th>
<th>Donor 1 Fresh cells</th>
<th>Donor 2 Fresh cells</th>
<th>1 week old cells</th>
<th>9 days old cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV1</td>
<td>MOI 10^6: 0.18%</td>
<td></td>
<td></td>
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<tr>
<td>rAAV2</td>
<td>MOI 10^6: 0.49%</td>
<td>MOI 10^6: 0.24%</td>
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<td></td>
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<tr>
<td>rAAV4</td>
<td>MOI 10^5: 0.49%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rAAV5</td>
<td>MOI 10^5: 0.51%</td>
<td></td>
<td>MOI 10^5: 0.45%</td>
<td></td>
</tr>
<tr>
<td>rAAV8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rAAV9</td>
<td>MOI 10^6: 0.58%</td>
<td></td>
<td></td>
<td>MOI 10^6: 0.36%</td>
</tr>
</tbody>
</table>
Table 9: Data summary of lectin binding profile of primary human CD4\(^+\) T cells isolated from all participating donors. Data presented in mean fluorescence \(x10^5\) (Arbitrary unit).

<table>
<thead>
<tr>
<th>Lectin staining Mean fluorescence (x10^5) (Arbitrary unit)</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week old frozen</td>
<td>2 week old cells</td>
<td>Fresh cells</td>
</tr>
<tr>
<td>Control</td>
<td>0.04</td>
<td>0.019</td>
<td>0.006</td>
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<tr>
<td>Con-A</td>
<td>7.24</td>
<td>5.27</td>
<td>3.71</td>
</tr>
<tr>
<td>DBA</td>
<td>0.16</td>
<td>0.03</td>
<td>0.17</td>
</tr>
<tr>
<td>PNA</td>
<td>0.35</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>RCA</td>
<td>11.2</td>
<td>6.31</td>
<td>4.83</td>
</tr>
<tr>
<td>SBA</td>
<td>0.72</td>
<td>0.15</td>
<td>0.1</td>
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<tr>
<td>MAL</td>
<td>0.82</td>
<td>0.42</td>
<td>0.17</td>
</tr>
<tr>
<td>WGA</td>
<td>9.31</td>
<td>8.71</td>
<td>4.55</td>
</tr>
<tr>
<td>UEA</td>
<td>0.14</td>
<td>0.12</td>
<td>0.27</td>
</tr>
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</table>