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Novel Approaches for the Eradication Of HIV Latently Infected Cells

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NOVEL APPROACHES FOR THE ERADICATION OF HIV LATENTLY INFECTED CELLS

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sally Al Ali ENTI TLED Novel Approaches For The Eradication Of HIV Latently Infected Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THEREQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

AlAli Sally M.S. Department of Microbiology and Immunology, Wright State University, 2013. TLED Novel Approaches For The Eradication Of HIV Latently Infected Cells.

The development of a suitable experimental cell model to study HIV latency in primary cells could have a massive effect on the current approaches to eradicate virus in latently infected cells. The main proposal of this paper is to develop an *in vitro* HIV cell model that represents HIV latency *in vivo,* then to create a more effective viral vector in order to target HIV reservoirs. For this goal, a directed evolution method is suggested to be used in order to mutate the AAV *cap* gene to generate a recombinant AAV vector that is capable of infecting primary resting CD4+ T cells previously infected with HIV-1 and in a latent stage.

Table of Contents

I. INTRODUCTION

Human immunodeficiency virus (HIV) is a lentivirus in the *Retroviridae* family. HIV causes acquired immunodeficiency syndrome (AIDS) in humans. Although the development of highly active antiretroviral therapy (HAART) has increased life expectancy, HIV reservoirs are still an obstacle to the eradication of the virus.

HIV latency is a big challenge scientists face to completely eliminate HIV from patients' bodies. In order to eradicate HIV latently infected cells, there is a big need to understand the mechanism of HIV latency. One strategy is to prevent latency. Another strategy for complete HIV eradication is to identify biomarkers for latently infected cells, in order to target them *in vivo*. Targeting HIV reservoirs can be done either by anti-retrovirus drugs, or gene therapy. Anti-retrovirus drugs could be a possible way for full HIV eradication if it is specifically designed for eliminating HIV reservoirs. Gene therapy is another targeting strategy; it is a promising approach for targeting latent reservoirs.

The main challenge in understanding HIV latency is to have a paradigm that is similar to *in vivo* latency. Studying HIV latency *in vitro* is an obstacle due to the lack of a cell model that represents HIV latent reservoirs. The lack of cell model representing HIV latency is behind the reason that latency establishment is possible to start in multiple mechanisms. . A cell model representing HIV latency would allow the investigation of

these mechanisms. Moreover, HIV reservoirs are likely to be persistent in several cell types (Bosque and Planelles, 2011).

Numerous efforts have been undertaken to create a novel cell model to study HIV latency. Some of the early efforts to study latency have investigated chronically infected cell lines such as JΔK (Antoni *et al.*, 1994) and ACH2 (Jordan *et al.*, 2003). JΔK cells originated from the Jurkat T cell line. These cells are infected with HIV-1 virus, which has deletion on its long terminal repeat (LTR) at the NFκB binding site. (Antoni *et al.*,1994) these cells are different from primary cells because it is proliferating constantly. Therefore, these cells never enter a quiescent stage. In the ACH2 cell model, tumor necrosis factor – alpha (TNF-α) can reactivate the HIV latent virus (Jordan *et al.*, 2003). However, Tyagi, *et al.* (2010) reported no latent HIV-1 reactivation by TNF-α in a primary CD4+ latently infected cell model.

Brooks, *et al.* (2001) generated an animal model by implanting a human fetal thymus and liver tissues into severe-combined immunodeficient mice (Thy/Liv SCID-hu). This animal model is likely to be a promising alternative to *in vitro* research. The model created by Brooks and colleagues allows the study of latency in naïve CD4+ T cells, which contributes to 1.9% of HIV-1 reservoirs, while T central memory cells (T_{cM}) contribute with 51.7% and T transitional memory (T_{TM}) gives about 34.3% (Chomont et al., 2010).

There have been several models developed that utilize primary T cells in order to study latency. Primary T cells are permitted to enter latency after activation and infection with HIV vectors. Several strategies have been used for this purpose such as, incubating latently infected T cells with IL-7 (Marini *et al*. 2008), or taking advantage of CD4+ T cells which can have an intermediate level of differentiation between two T cell subsets, naïve T cells and effector T cells (Bosque and Planelles, 2011). This intermediate or un committed phenotype will be referred to as non polarized CD4+ T cells in this paper.

Studies of HIV latency in previous cell models have given us deeper knowledge about the possible mechanisms of latency. Understanding the mechanism of latency could provide insights into promising approaches to prevent latency. However, there is a greater possibility that latency may not be preventable from occurring. Therefore, targeting HIV-1 reservoirs may be a more efficient way to achieve full eradication of HIV from the body.

Identifying biomarkers for HIV reservoirs is an important step to target latently infected cells. Several studies have reported that the majority of HIV reservoirs are T_{cM} and T_{TM} subsets (Chomont *et al*., 2010), which both share the expression of $CD45RO^{+}$ (Bosque and Planelles, 2011). Tyagi *et al.* (2010) identified HIV reservoirs, in the novel HIV latently infected cell model created by his team, as $CD45RA^{-}$, $CD45RO^{+,}$ $CD27^{low}$, $CCR7^{low}$, $CD25^{-}$, and $CD38^{+}$. This finding is consistent with previous studies, which suggest that T_{cM} subsets represent HIV latently infected cells (Okada *et al*., 2008).

Several previous studies have used HIV-based vectors or replication deficient HIV virus to infect CD4+ cells. In this paper, I propose to use wild type HIV to infect primary restingCD4+ T cells in order to determine whether the latently infected cells will express the same biomarkers reported in previous studies. The second objective in this study will be to infect the HIV latently infected cells with an adeno-associated virus (AAV), which will be created using a directed evolution technique, in order to originate a novel therapeutic approach using gene therapy. This approach would be a more promising and convenient method if combined with HAART regimen for full HIV eradication.

II. BACKGROUND

The development of the high active antiretroviral therapy (HAART) has a huge impact on controlling HIV infection, by acting on different phases of HIV life cycles. This treatment has improved HIV patients' life expectancy, by maintaining immune system functions. It also protects patients from the opportunistic infections that frequently cause death cases among acquired immunodeficiency syndrome (AIDS) patients. Despite HAART improving the quality of life in HIV patients; therapy approaches have not been able to eradicate HIV reservoirs. These reservoirs can persist in patient's body for approximately 44 months; these resting memory CD4+ T cells have low decay rates. Thus, it takes about 60 years for full HIV eradication (Siciliano *et al*,. 2003).

HIV latently infected cells or HIV reservoirs are established early, during HIV acute infection after initial infection. It is believed that memory T cells are the main reservoir for HIV (Chomont *et al.*, 2010). Moreover, Chomont *et al*. (2010) reported T_{cM} isolated from HIV-1 aviremic patients forms the majority of HIV-1 latently infected cells. The same team also reported the presence of T_{TM} subsets in the pool of HIV-1 reservoirs (Chomont *et al*., 2010).

HIV latently infected cells are in resting stage *in vivo*. Resting stage is identified by the lack of cell activation markers, such as CD25, CD69, and HLADR (Bacchus *et al.*, 2013). HIV reservoirs are found in G_0, which means decreased levels of cellular DNA and RNA synthesis (Richman., 1980). The necessity of cells entering G_0 to become HIV

latent cells has not been investigated. Another question with no answer is whether transition from G_0 to G1 phase is a crucial step for HIV reactivation.

There are three possible mechanisms for HIV latency *in vivo*. The first mechanism suggests the return of an activated CD4+ T cell to the resting state after the integration of HIV provirus. But for an activated, infected CD4+ T cell to survive, it must escape activation-induced cell death because it eradicates the majority of activated cells. Moreover, the cell must survive the HIV cytopathic effect (Shen *et al*., 2000).

Another possible theory for the establishment of HIV latency is that T cells allow reverse transcription and integration. However, this reverse transcription is not sufficient for viral replication. Infection through this time will allow the cell to avoid the HIV cytopathic effect (Shen *et al.,* 2000).

A third suggested mechanism for the establishment of HIV latency reported the ability of resting CD4+ T cells to allow HIV integration and gene expression when these cells are treated with cytokines such as IL-2, IL-7, IL-15 (Unutmaz *et al.*, 1999).

These three hypotheses have not been investigated due to the difficulty in creating an HIV latently infected cell model. It is hard to create a cell model that represents all cell types that associate with HIV latency *in vitro* because multiple cell types are involved in HIV latency *in vivo*. A second reason for the difficulty is that we still don't know which cytokine mechanism or pathway is significant in HIV reactivation *in vivo* (Yang *et al*. 2009). Last two decades have provided several studies aimed focused on understanding the latency mechanism. Some of these studies investigate latency in chronically HIV-

infected cell lines (Antoni *et al*., 1993; Jordan et al., 2003), animal models (Brooks *et al.*, 2001), And primary cell culture (Tyagi *et al,*. 2010; Bosque and Planelles, 2011; Saleh *et al*., 2010).

Jordan *et al*. (2003) created a latent HIV cell model using the ACH2 cell line. This model is based on chronically infected T cells generated from the parental T cell line, A3.01, that includes latent pro virus (Jordan *et al.*, 2003). In this cell line Jordan and colleagues reported that reactivation of HIV-1 from latency could occur after adding TNF-α to the medium. However, the ACH2 cell line cannot represent true HIV latency because this finding is different than Tyagi et al. who found no evidence of HIV-1 reactivation in HIV-1 latently infected cells in a primary isolated T cells upon TNF-α treatment (Tyagi *et al.*, 2010).

Another cell model based on a chronically infected cell line is the JΔK cell model. In this model, cells are derived from the Jurkat cell line and chronically infected with an HIV serotype that has a deletion in the long terminal repeat (LTR) at the NFκB binding site (Antoni *et al.*, 1993). In this model HIV-1 reactivation occurs in a fashion independent of NFκB by incubating cells with PMA, hexamethylene bisacetamide or sodium butyrate (Antoni, *et al.*, 1993). More investigation is needed to know the effect of the previous materials on any cell model that represents HIV latency in a more similar way.

Antoni and Tyagi cell models gave us a deeper knowledge of a group of cytokines that are associated with HIV-1 reactivation. However, the previous cell models are not

completely parallel to the resting CD4+ T cells in G_0 phase in HIV infected individuals (Chun *et al*., 1997; Finzi *et al*., 1999)

Another different approach to create an HIV latently infected cell model was originally created by Brooks *et al.* (2001). Using an animal model, Brooks and coworkers developed an animal model using severe-combined immunodeficient mice that have an implanted human fetal thymus (Brooks *et al*., 2001). Brooks and colleges generated latency at a high rate during thymopoiesis. The vast majority of latent cells in this *in vivo* model are naïve CD4+ T cells (Brooks *et al*., 2001). Naïve CD4+ T cells represents 1.9 % of HIV latently infected cells while T_{cM} and T_{TM} represent 51.7 and 34.3% respectively (Chomont *et al.,* 2009; OstrowskI. *et al.,* 1999). Therefore, using this animal cell model is promising for studying HIV latency in naïve CD4+ T cells. However, naïve CD4+ T cells represent the minority in the population of HIV reservoirs.

Another possible strategy to create HIV latency cell model is the *ex vivo* culturing of peripheral blood mononuclear cells (PBMC). By growing CD4+ T cells *ex vivo*, we are generating a more parallel paradigm to HIV latency *in vivo*. There are two main challenges in using primary cells to create an *ex vivo* cell model for HIV latency. The first challenge is the short half-life of the primary cells. These cells cannot survive more than 20 days *in vitro* (Saleh *et al*., 2007; Peace *et al*., 2012). The second challenge is the limited cell numbers obtained from these models (Peace *et al*., 2012; Bosque *et al*., 2009). Several trials were established to obtain a cell model that represents HIV

reservoirs *in vivo* using different strategies such as, non-polarized T cells (Bosque *et al*., 2009), Bcl-2 cDNA transduced CD4+ T cells (Yang *et al*., 2009), and activated T cells co-cultured with a feeder cell line (Tyagi *et al*., 2010).

Bosque and coworkers (2009) took advantage of non-polarized cells since they can be activated to Th1 and Th2 T cells or stay inactivated as $T_{cM.}$ The cells were infected with an *env* deficient HIV (DHIV), then cultured with medium that included IL-2 (Bosque *et al,*. 2009). Although this cell model generated a large number of latently infected cells with different subtypes, cell viability was 20% at the end of the resting stage (Bosque *et al.,* 2009). An important advantage of this model is the convenience of drug screening that could target various steps of HIV latency. However, this model is not perfect for studying HIV that targets chemokine receptor (R5 tropic virus) since these cells express lower CCR5 levels due to the activation step.

Yang *et al*. (2009) transduced primary cells with lentiviral vector that expressed the B cell lymphoma-2 gene (BCL-2), which encodes an anti-apoptotic protein located downstream of IL-7 signaling (Yang, *et al.,* 2009). Therefore, Inhibition of apoptosis allowed cells to survive longer independent of cytokine stimulation. This model generated enough cells, which is optimal for drug screening. However, the majority of model cells developed by Yang *et al*. (2009) generated effector memory T cells. These cells are the second minority after naïve T cells in HIV reservoirs pool (Chomont, *et al*. 2009). Moreover, mutated HIV vector could potentially affect the results because it does not represent HIV wild type accurately.

Tyagi and colleagues (2010) infected activated primary CD4+ T cells isolated from healthy donors. After activation and infection with mutated HIV vector, cells were co-cultured with an H80 feeder cell line (Sahu *et al,*. 2006; Tyagi *et al.,* 2010). This cell model overcame the problem of the short half-life of latent cell models *ex vivo* (Sahu *et al*., 2006; Tyagi *et al*., 2010). Also, this cell model showed that epigenetic silencing and restricted P-TEFb levels contribute to HIV latency (Tyagi, *et al*., 2010). However, co-culturing activated CD4+ T cells with the H80 feeder cell line results in a comparatively homogenous population of T_{cM} (Tyagi *et al*., 2010). This means that the Tyagi and colleagues (2010) cell model does not allow the study of other T cell subsets that contribute to HIV latent cells pool such as the T_{TM} subset (Chomont, *et al*., 2009). Furthermore, cells in nature are not completely resting as it appears in Tyagi and coworkers' cell model, $CD25^{high}$ and $Ki67^{high}$ which means that these cells are actively proliferating (Tyagi *et al*., 2010). Finally, the use of mutated HIV may affect the behavior of the latent virus.

The latent cell models discussed previously used activated T cells (Bosque *et al*., 2009; Yang *et al.,* 2009; Sahu *et al.,* 2006: Tyagi *et al.,* 2010), using activated T cells in HIV latent cell models can produce a large number of cells, which is suitable for drug screening (Bosque *et al*., 2009; Yang *et al.,* 2009). However, activated T cells in a latent model result in a homogenous population of one T cell subset, such as a Tc_M population, resulted after activated T cells were co-cultured for a long period of time with H80 feeder

cells (Sahu *et al*., 2006; Tyagi *et al*., 2010). Thus, using activated T cells, as an HIV latent cell model does not parallel HIV latency *in vivo.* There is a big need to study HIV latency in a cell model that represents HIV reservoirs *in vivo.* The more similar the model is to *in vivo* HIV latency the greater benefit we get in identifying reservoirs and the biomarkers on these cells. Identifying biomarkers of HIV reservoirs is a promising strategy in targeting these cells to full HIV eradication.

The using of resting T cells in creating an HIV latent cell model could represent *in vivo* HIV latency in a more precise way. Direct infection of primary resting CD4+ T cells prevents any stimulation or activation that could change cellular characteristics, thus we can obtain CD4+ T cells potentially as they exist in the body. It is predicted that using resting cells will give us more accurate results, and it will allow us to study latency in all T cell types. Peace and colleagues (2012) developed an HIV latent cell model using resting T cells. In this cell model resting cells were spinoculated with HIV WT to increase infection. This experiment was done to measure the integration, transcription, and translation of HIV proteins; it shows that resting CD4+ cells have the ability to produce HIV Gag protein without enhancing the spread of infection (Peace *et al*., 2012). This study reflects the latency features of HIV *in vivo*. Also, it can give us more accurate results for T cells biomarkers as they are in the body. However, T cells in the Peace and coworkers cell model do not survive for longer than 20 days *in vitro* (Peace *et al.*, 2012). This is considered a disadvantage in studying HIV latent cells, particularly when studying

HIV latency for drug screening or gene therapy.

Identifying biomarkers for HIV reservoirs is necessary to develop strategies to target these cells efficiently. Several studies identified biomarkers for HIV latent cells in primary CD4+ T cell (Tyagi *et al*., 2010; Bosque *et al*., 2009). These two studies revealed similar features of latently infected CD4+ T cells, which are $CD45RA^{-}$, $CD45RO^{+}$, $CCR7^{+}$, $CD27^{+}$. These cell markers show that HIV latent cells have the central memory T cells phenotype (Tyagi *et al*., 2010; Bosque *et al*., 2009). Additionally, Tyagi and colleagues investigated the cell proliferative state by detecting the nuclear proliferation markers Ki67 and BrdU in HIV latently infected cells in the cell model (Tyagi *et al.,* 2010). The results show that the majority but not all latent cells are quiescent. Tyagi and coworkers suggest that the Ki67 positive cells are viable for a longer time when treated with IL-2 (Tyagi *et al.,* 2010). However, both Tyagi and Bosque previous studies were done in cell models that were not quite parallel to in *vivo* HIV latency since Tyagi's model resulted in a homogenous T cells subset of T central memory cells (Tyagi *et al*., 2010), and the Bosque cell model had a majority of non-polar T cells that express $CD45RA^{+}$ (Bosque *et al.,* 2009). On the other hand, Bacchus and colleagues identified biomarkers for HIV reservoir *in vivo*, which were $CD25^{-}$, $CD69^{-}$, and $HLADR^{-}$ (Bacchus *et al*. 2013). Additionally, this study revealed four resting CD4+ T cells subsets that contribute to the different percentages of subsets found in the *in vivo* HIV latency pool. These four resting CD4+ T cells subsets are: naive (CD45RA+CCR7+CD27+) 2%,

central-memory (CD45RA-CCR7+CD27+) 17%, transitional memory (CD45RA-CCR7-CD27+) 58%, and effector memory cells (CD45RA-CCR7-CD27-) 9.7% (Bacchus *et al.*, 2013). Comparing HIV latent cells isolated as described in the method section below to these studies described above using the same biomarkers will allow us to compare the similarity of our cell model to the *in vivo* HIV latent cell populations.

Pace *et al.* (2012) developed a cell model that revealed an important feature of HIV reservoirs, which is the Gag protein persistence in these cells. Resting T cells produce a smaller quantity of HIV proteins and fewer types of HIV proteins (Pace *et al.*, 2012). Results found by Pace and coworkers are consistent with the *in vivo* results that the Gag protein is more predominant in resting cells than *env* and *vif* proteins (Zhang *et al*., 1999). Thus, targeting HIV reservoirs producing Gag protein will be more beneficial than targeting latent HIV cells expressing *env* (Berger, and Pastan, 2010)

In this paper I propose to use primary resting CD4+ T cells without activation, then to infect these cells with HIV WT in order to have the advantage of a parallel cell model to HIV latency *in vivo*. Thus, identifying cell markers before and after infection will be more efficient to determine the changing that occurs in T cells subsets and phenotypes before and after infection. Another purpose of this system is to target HIV latent cells with adeno-associated virus (AAV). Targeting cells needs HIV latent cells to survive for long period. Thus, cells will be co-cultured with H80 feeder cell line to ensure cell survival in our cell model.

Gene therapy is a potential method to cure several diseases. It has shown encouraging results in treating diseases such as cystic fibrosis (Wagner JA, *et al.* 1998; Excoffon *et al.,* 2009), improvement of vision in Leber congenital amaurosis patients (Maguire *et al.* 2008), and severe combined immunodeficiency SCID-X1 disease (Cavazzana *et al.,* 2000). With using gene therapy, scientists can deliver a gene in order to correct a defective gene in patient's body, or to improve cell capability to fight infectious agents. Thus, it can be said that gene therapy is a developed form of drug delivery, and it could be applied for a vaccination strategy.

AAV is a member of of the *Parvoviridae* family and is a *Dependovirus.* The AAV wild type genome is 4.7 Kb, it is a linear, single stranded DNA virus with 2 open reading frames ORFs. The first open reading frame is *rep*, this area encodes transcripts for 4 proteins that are required for AAV genome replication. The second ORF is *cap* and encodes 3 proteins (VP1-3) that build the AAV capsid (Muzyczka , Berns , 2001). AAV is a potential viral vector candidate that may target HIV reservoirs for several reasons; first, AAV capsid is highly evolved; this capsid could be engineered in a way that it can change AAV tropism since it is encoded by a single gene (Summerford , Samulski ,1998; Walters , *et al.*, 2001; Gao *et al.* 2004). Mutating the AAV *cap* is a possible strategy to target cells other than those that AAV WT usually infects. The second reason that makes AAV a better viral vector is the high level of safety when using this virus in

humans. AAV has never been documented to cause human disease (Moss *et al.*, 2007). Also, the ability of AAV Type 2 to infect lymphocytes was investigated in 3 B cell subsets and 4 T cell types; the results show that AAV is able to infect all tested lymphocyte subsets (Mendelson *et al.*, 1992). Moreover, AAV has been reported to successfully infect alveolar cells, which are mature, non-dividing cells (Mendelson *et al.*, 1992). Finally, the development of synthetic biology techniques such as, directed evolution would be promising if it is used with AAV to create a mutated cap gene, which can change AAV tropism and target more cell types in a more efficient and specific fashion.

Inouye and coworkers reported a strong inhibition of HIV-1 in primary T cells and alveolar macrophages when they delivered combined anti-Rev single-chain immunoglobulin (SFv) or Rev response element (RRE) decoy genes delivered by AAV vector (Inouye, *et al.*,1997). It is also a promising strategy to create a vaccine that prevents HIV infection. Furthermore, it shows that immune cells (T cells and alveolar macrophages) are capable of being infected with more than one virus, AAV and Ad5 and HIV (Inouye, *et al.*, 1997). However, the efficient delivery of this strategy has not been yet investigated.

Mendelson *et al*.(1992) reported an increase of about 60-70% in HIV-1 reverse transcription when lymphocytes are co-infected with either adenovirus or adeno-associated virus alone (Mendelson *et al.*, 1992). The increase of reverse

transcription activity was decreased by about 20% when cells were infected with AAV plus Ad2 virus together (Mendelson *et al.,* 1992). The study highlights an important advantage of AAV, which is the ability of these viruses to interact with HIV-1 after infecting lymphocytes. Thus, T cells can survive after being infected with 3 viruses (Mendelson *et al.,* 1992). However, the yield of infectious AAV was low in most cases (Mendelson *et al*., 1992). The low rate of infectious AAV could be due to the low viral tropism to lymphocytes. Therefore, developing AAV *cap* gene for better infection of lymphocytes using directed evolution would be a possible way to target HIV reservoirs in our HIV latent cell model.

The directed evolution strategy has a potential effect in changing the functional properties of enzymes, proteins, and genes. Directed evolution occurs when we applied selective pressure to a library of objects in order to get the most desired properties of these objects.

Excoffon *et al*. (2009) demonstrated the capability of AAV to develop remarkably greater infectivity within the airway epithelium when a library of AAV *cap* mutants were placed under selective pressure. When the AAV vector was customized with the mutated AAV *cap* gene after directed evolution, it was better able to infect human airway epithelia (Excoffon *et al.,* 2009). Thus, using directed evolution to mutate AAV *cap* gene in order to infect HIV latent cells is likely a promising approach in targeting HIV reservoirs.

The first goal in this paper is to generate an efficient HIV latency model that is parallel to the *in vivo* HIV latency pool. Our HIV latent cell model will allow us to identify HIV latent cells' biomarkers before and after being infected with HIV WT. To guarantee that cells will survive for longer time, cells will be co-cultured with the H80 feeder cell line (Sahu *et al.,* 2006; Tyagi *et al.,* 2010). Additionally, our novel cell model will give us the chance to investigate the reasons behind the predominant levels of HIV *Gag* protein in latently infected cells reported by Pace and colleagues (Pace *et al.,* 2012). The second goal of this study is to engineer a viral vector that is capable of infecting HIV reservoirs and express a GFP gene or an anti-HIV gene within these latent cells. AAV is a potential candidate for targeting HIV reservoirs due to its high safety profile in humans. Targeting HIV latent cells with AAV vector that is capable to get high gene expression could be a significant step towards the eradication of HIV reservoirs in patients' body.

III. AN OVERVIEW OF THE METHOD

T cell isolation:

T cells will be isolated from PBMC. To purify resting T cells, we can stain the cells with R-Phycoerythrin (PE) Conjugated Antibodies against CD25, CD69, and HLA-DR, and then use Anti-PE magnetic beads (Pace *et al.*, 2012). Pace and colleagues method in isolating resting CD4+ T cells has a purity greater than 98% (Pace *et al.*, 2012).

Purified cells can go through more investigation to identify resting CD4+ T cells biomarkers, and compare the obtained subtypes with the *in vivo* studies by Bacchus *et al.* (2013) The four resting CD4+ T cells subsets in this study are: naive (CD45RA+CCR7+CD27+) 2%, central-memory (CD45RA-CCR7+CD27+) 17%, transitional memory (CD45RA-CCR7-CD27+) 58%, and effector memory cells (CD45RA-CCR7-CD27-) 9.7% (Bacchus *et al*., 2013)

Incubating resting CD4+ T cells:

Resting CD4+ T cells will left in RPMI medium, containing 10% fetal calf serum (FCS) and antibiotics, with no stimulation (Pace et al., 2012).

Other resting CD4+ T cell groups will be stimulated with either IL-7 or anti-CD28/CD3. Stimulated CD4+ T cells will used to evaluate the differences

between cell markers in resting CD4+ T cell with/without stimulation, and then compare our results with the *in vivo* results by Bacchus *et al.* (2013).

Stimulated and non-stimulated resting CD4+ groups will be tested for the following biomarkers: CD45RA, CD45RO, CCR7, CD27, CD25, CD69. Identifying CD4+ T cells biomarkers will be helpful to determine the difference in T cell subsets in each CD4+ T cells groups (stimulated/ non-stimulated).

Infecting CD4+ T cells:

Stimulated and non-stimulated CD4+ T cells will be infected with HIV WT. Cells will be stained with trypan blue stain to ensure cell viability.

One of the big concerns in this stage is that HIV WT could induce cell death. Therefore, using Drug resistant HIV variants is likely to be a good alternative strategy if HIV WT caused cell death. Another pitfall in our HIV latent cell paradigm is the spread of HIV in cell culture. Therefore, cell media will include antiretroviral drug to select for cells with HIV integration but not replication.

Identifying Biomarkers for CD4+ T cells post infection:

Same cell biomarkers in Bacchus et al. (2013) study will be identified which are: naive (CD45RA+CCR7+CD27+), central-memory (CD45RA-CCR7+CD27+) , transitional memory (CD45RA-CCR7-CD27+) , and effector memory cells (CD45RA-

CCR7-CD27-) (Bacchus et al. 2013). in order to determine CD4+ T cells subsets after entering latency. Moreover, Ki67 and BrdU staining will be performed to check the proliferation stage of the cells.

For further investigations, chromatin immunoprecipitation (ChIP) assay will be performed as described in Tyagi *et al*. (2007). ChIP assay will be performed in order to determine the levels of chromatin in resting primary CD4+ models.

Long-term incubation:

Cells will be incubated for long period using the H80 feeder cell line as previously described in the Tyagi and coworkers paradigm (Tyagi *et al.,* 2010).

Pitfall: HIV WT could infect H80 feeder cells. Thus, using H80 feeder cells supernatants may be a possible way to maintain HIV latent cells if our model cells survive, and to avoid an HIV cytopathic effect on H80 feeder cells if it occurs.

AAV viral vector construction:

AAV viral vector will be constructed using the directed evolution method as previously described by Excoffon et. al (2009). Briefly, AAV2 and AAV5 *cap* genes will be amplified via PCR. DNA shuffling will be performed as previously described (Zaho *et al.,* 1998; Stemmer, 1994). Next, the chimeric *cap* genes will be cloned in pSub2 for recombinant AAV (rAAV) construction (Maheshri *et al.,* 2006). Error-prone PCR will be

done for subsequent evolution sequences (Maheshri *et al.,* 2006).

The method for viral production will be as previously described by Excoffon and colleagues (Excoffon *et al,*. 2009).

rAAV *in vitro* selection and characterization :

To select the best AAV vector that is able to infect HIV latent cells, AAV will be diluted in EMEM and added to HIV latent cells. After 16 hours of incubation, unbound viruses will be washed off with PBS (Excoffon *et al.,* 2009). Three days later, AAV vectors will be amplified by adding Ad5 as a helper virus, as previously described (Maheshri *et al.,* 2006). After 3 days, AAV DNA will be recovered by PCR amplification and cloned into pSub2 to produce more rcAAV, and selected for pXX2 NotI for rAAV production (Koerber *et al.,* 2008).

To determine the relative efficiency of AAV transduction in HIV latent cells, the latter will be infected with rAAV vectors that carry cDNA encoding green fluorescent protein (GFP). The percentage of GFP cells will be quantified using flow cytometry 48 hours post infection (Excoffon *et al.,* 2009). Cells will be stained with Trypan blue to check viability.

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