Creating an ecological trap for HIV using combinatorial gene therapy

[redacted]

Project Summary

SCIENTIFIC MERIT

Recent clinical trials have proven the safety and some efficacy in knocking out the CCR5 co-receptor to block HIV infection[1,2]. This CCR5- cell type is only resistant to the CCR5 strain of HIV. However many people living with HIV have a strain that uses the CXCR4 co-receptor. To obtain a functional cure for HIV we need to do more than just block the infection by a single strain of HIV[3,4]. Instead we need to generate innately resistant cells by combining restriction factors that target multiple stages of the HIV life cycle[5]. We must also address latently infected cells through viral genome removal[6] and increase the cytotoxic T-lymphocyte (CTL) immune response[7] to increase the clearance of infected cells. We have previously shown the effectiveness of stacked restriction factors at inhibiting HIV infection from both strains, however this was combined with the CCR5 knock-out[8]. Thinking in terms of an ecological trap model of viral growth rates[9,10], we think that the CCR5- cell type actually hinders the process of viral clearance by passively avoiding infection. Instead an ecological trap model, utilizes cells that look like hosts but are unproductive for viral replication, a viral trap. Further in a structured environment, such as gut-associated lymphoid tissue, HIV uses the CCR5 receptor to recruit host cells to sites of infection[11–15]. A viral trap cell would be more effective at forcing the virus to extinction. We think a HIV resistant hematopoietic stem cell (HSC) thearpy that maintains CCR5 function would require fewer transfused cells and be more effective at clearing the virus.

Specific aims

1. **Generate Hematopoietic Stem Cells Resistant to HIV while maintaining CCR5 function** through homologous recombination of a stacked restriction factor cassette containing: RevM10, ApobeC3G 128k, hrhTrim5a, Tre recombinase, and the SL9 epitope specific T-Cell Receptor into the AAVS1 “safe harbor” locus. *The hypothesis being tested is that CCR5 knock out is not required to obtain HIV resistance in modified hematopoietic stem cells.*
2. **Use modified HSCs to model an ecological trap of HIV infection** during acute and continuous challenge with HIV. *The hypothesis being tested is that CCR5+ modified t-cells are resistant to HIV and will show an increased ability to clear viral infection compared to Wildtype or CCR5- modified t-cells.*
3. **Develop a microfluidic device for cell sorting and electroporation using sterolithography**. *The hypothesis being tested is that a microfluidic device will improve cell viability and transduction efficiency while increasing cell membrane permeation at low voltage.*

BROADER IMPACT

The human immunodeficiency virus (HIV) has killed over 35 million people since the epidemic began, and nearly the same number of people are currently living with HIV. The use of combinatorial anti-retroviral therapy (cART) using pharmaceutical drugs has greatly improved the lifespan and quality of life for these individuals. However access, cost and adherence remain insurmountable barriers to treating all people living with HIV. Autologous stem cell therapy using gene therapy is a possible method of treatment. Using an ecological trap in such treatments may significantly decrease the quantity of cells required for effective treatment.

The methods used for the production of these therapies may also prove to be too costly and greatly limit the applicably of gene therapy for HIV. Our methods address these issues by improving the efficiency of creating modified HSCs and could greatly expand the reach of gene therapies for HIV and other diseases.

Project Description

Our long term goal is to generate CCR5+ HIV resistant HSCs and to characterize the dynamics of an ecological trap on HIV infection. This first requires developing an HIV resistant HSC line which maintains CCR5 function and therefore supports viral fusion into an ecological trap containing intracellular restriction factors. Utilizing an array of Genomic, Proteomic, and Transcriptomic measurements we will use bioinformatic approaches to integrate these measures and model these cells in structured and unstructured environments when challenged with HIV.

Our focus is on improving the current understanding of HIV infection in an environment containing both wildtype host cells and HIV resistant cells. Utilizing an ecological trap model of viral infection we will quantify levels of modified cells required to obtain HIV resistance and viral clearance from the host. Improving our understanding of HIV infection in heterogeneous host environments and thereby helping the development of effective gene therapy treatment strategies.

We also aim to address the need for a standardized protocol for combinatorial gene therapy in HSCs. We think an easily reproducible microfluidic device for cell sorting and transduction of vectors is needed. Using the open-source hardware model of development we will produce a 3D microfludic device using high resolution stereolithography[16]. Recently this process has been shown to be economical but also more effective than current strategies that are limited by cost, complexity, and difficult to translate into clinical use[17]. Microfluidics however have shown great success in translating to point-of-care diagnostics[18].

Previous studies have shown the effective inhibition of HIV infection by stacked restriction factors when combined with the knock-out of the CCR5 co-receptor[8]. An ecological trap model has demonstrated the dynamics of bacteriophage infection in the presence of trap cells over-expressing viral receptors[9]. We therefore think that maintaining CCR5 function and the use of intracellular restriction factors will create a trap cell capable of altering HIV growth rates while maintaining normal cell function.

S*pecific aims*

1. **Generate CCR5+ Hematopoietic Stem Cells Resistant to HIV** through homologous directed repair mediated recombination of a stacked restriction factor cassette containing: RevM10, ApobeC3G 128k, hrhTrim5a, Tre recombinase, and the SL9 gag-specific T-Cell Receptor into the AAVS1 “safe harbor” locus. *The hypothesis being tested is that CCR5 knock out is not required to obtain HIV resistance in modified hematopoietic cells.*

2. **Use modified HSCs to model an ecological trap of HIV infection** during acute and continuous challenge of CCR5(+) HIV resistant HSC and downstream cell types. *The hypothesis being tested is that CCR5+ t-cells that are resistant to HIV will show an increased ability to clear viral infection compared to Wildtype or CCR5(-) t-cells in accordance with an ecological trap model.*

3. **Develop a microfluidic device for cell sorting and electroporation using sterolithography**. This approach is aimed at improving current methods use in the modification of hematopoietic cells. *The hypothesis being tested is that a microfluidic device will improve cell viability and transduction efficiency while increasing cell membrane permeation at low voltage.*

STATEMENT OF THE RESEARCH PROBLEM

HIV Resistant Hemtaopoetic Stem Cells for the elimination of HIV:

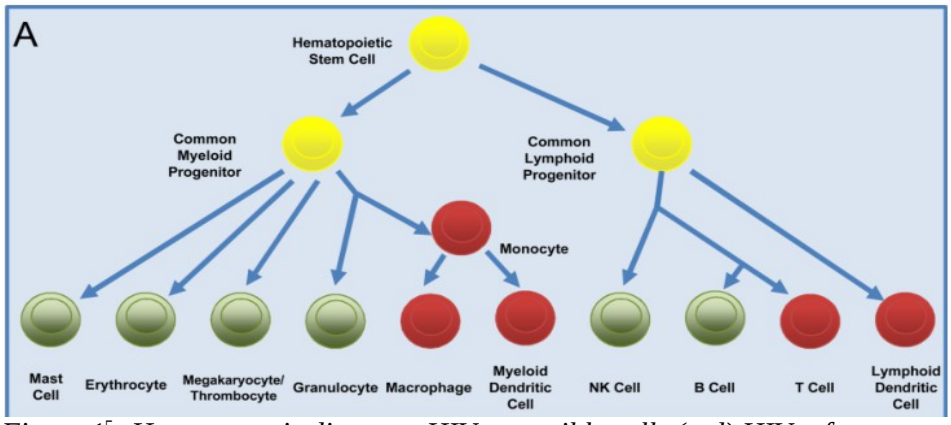


Figure 1[5]: Hematopoesis diagram, HIV suceptible cells (red) HIV refactory cells (green) HIV infection controversial (Yellow)

Since the announcement of the Berlin Patient, a patient now considered cured of HIV, many research projects have studied the role of the CCR5Δ32 deletion in HSC and downstream cells as a possible cure for HIV[2,4,8,19,3]. Yet removal of the CCR5 co-receptor only confers sub-optimal resistance to one strain of HIV[5]. To address this problem the pairing of CCR5 deletion with the knock-in of restriction factors hrhTrim5a, APOBE3k, and RevM10 proved to be an effective multi-strain inhibitor of HIV[8]. This blocks the fusion of CCR5 tropic strains of HIV, and inhibits CXCR4 strains that still infect the modified cell type. It was originally thought that CCR5 deletion would confer a selective advantage upon modified cells and, during anti-retroviral therapy interruption, out-compete HIV susceptible cells. However this has proven untenable because of the low numbers of modified cells remaining in the body post HSC transfusion and the incomplete removal of HIV host cells from the environment. This limits the use of CCR5 knock-out as a cure for HIV.

The incomplete removal of HIV can be explained by an ecological trap model[9,10]. The model first presumes a heterogeneity in habitat for species, and that there are signals for such habitats that influence the affinity a species has for the habitat. In this case the CD4 receptor and the CCR5 and CXCR4 co-receptors are considered cue used by HIV to determine competent host cells for replication. The Theory further explains three types of habitat; Host, Neutral, and Sink. While a host habitat such as CD4 CCR5+cxcr4+ cells are the desired habitat for producing competent viruses a non-host is a non-compatible habitat for virus production. While non-hosts can either be neutral meaning they don't attract or send the appropriate signals to the virus, Sinks instead send the right signals but trap the virus in a habitat that is not conducive to viral replication such as one containing intracellular restriction factors. Essentially the CCR5 knock-out only creates a neutral habitat for HIV, simply deflecting the virus toward fusion with host cell-types. Unfortunately a neutral cell-type is required to compose at least 50% of all available cell-types to force the virus the toward extinction[9,10]. Since the blockage of CCR5 is not required for effective HIV treatment the knock-out of CCR5 shouldn't be required for creating a HSC line resistant to HIV. Further, leaving CCR5 function should create a sink phenotype that is not only an effective sink but is also actively recruited to sites of HIV infection such as Gut-associated lymphoid tissue (GALT)[11,13,12,14] . Sink phenotypes are likely to require to compose a lower proportion of cells to effectively push the virus to extinction, such as 30%[10]. This however is only part of the overall disease ecology model for HIV.

HIV also relies on latent viral genomes that are incorporated in active and resting cd4 cells to hide from the immune system[20,21]. This is a critical issue in the development of a cure for HIV. Current treatment strategies only limit the replication of virus and do not remove latently infected

cells or integrated viral genomes. Recently the development of Tre-recombinase (TRE) has shown one method of viral genome excision[6]. TRE is a selectively mutated cre recombinase that specifically recognizes the LTR region of inserted HIV genomes and then excisses the viral genome from the host genome. It was shown to be highly effective in the removal of viral genomes and also lead to distinct decreases in viral load levels and increases in cd4/cd8 ratios in patients both important determinants of effective therapies. However as with most treatment protocols for HIV a single approach is not possible in the effective treatment of HIV. For this reason we will include a tat promoter dependent version of TRE to address latency but also to further safeguard our sink cells from viral incursion.

The competition of sink cells against host cells in a viral infection is only part of the overall disease ecology model. This is because the source/host cells, especially with HIV, are not maintaining the same absolute number or ratio as the virus cell cycle slowly kills host cells. This is good as it increases the ratio of sink cells to host cells further driving the virus toward elimination. However a distinct pathology of HIV infection is the inability of CD8 cells to completely remove HIV infected cells[22,23]. It is unlikely the sink cells alone will be able to eliminate all HIV infected cells in the body. For this reason and to further drive the virus toward elimination it will be important to also improve the function of CD8 cells. To do this the incorporation of an HIV specific T-cell Receptor in the HSC line will greatly improve the clearance of infected cells.

PRELIMINARY RESULTS

We have previously shown the effectiveness of combinatorial gene therapy for creating HIV resistant HSC using in vitro cell culture and in vivo mouse models(Table 1)[8]. However these methods relied upon the bi-alleleic knock-out of the CCR5 co-receptor. Recent clinical trials have proven the safety of such a strategy however it showed treatment failure in one patient due to only mono-allele knock out. Also the expected selective pressure for CCR5- cell lines proved to be insufficient in eliminating the virus. For these reasons we have begun to focus on creating modified cells that address these issues and those recently raised by Younan et al[4]. To better understand the in vitro selective pressure of modified cells we have decided to model the infection of cells using the source-sink ecology model for viral extinction introduced by Dennehy et el and further improved upon by Long et al [9,10]. To do this we have decided to create a

	R5 HIV			X4 HIV		
Cells	Cumulative RTCN	Fold protection	95% CI	Cumulative RTCN	Fold protection	95% CI
Wild type	5,095	1	1–1	1,904	1	1–2
puroR	551	9	8–10	1,651	1	1–2
hTRIM	3,846	1	1–1	3,011	1	1–1
hrhTRIM	79	64	37–251	726	3	1–20
APO	51	100	96–105	10	198	146–308
Rev	95	54	48–61	863	2	1–642
APO/hrhTRIM	177	29	26–33	2,014	1	1–2
Rev/hrhTRIM	607	8	8–9	1,716	1	1–2
Rev/APO	26	193	185–202	15	131	115–153
hrh triple	4	1,212	595–1,212	1.1	1,782	328–1,782

Abbreviations: h, human; hrh, human-rhesus hybrid; rh, rhesus; RTCN, ratio to cell negative.
[a]Infection, quantified as cumulative RTCN values, and fold protection were calculated following 25 days of challenge by R5- or X4-tropic HIV. 95% CI, confidence interval is given.

Table 1[8]:Protection of CCR5- modified cells after long-term exposure

Ecological trap model

An ecological trap is an environment that is equally or more attractive to a species, while also being an unproductive environment trapping the species and driving it toward extinction. Dennehy et al modeled an ecological viral trap using bacteriophages and engineered trap cells. In this model the trap cells had increased receptors for the phage leading to a more attractive host environment, thereby increasing the rate at which the phage associated with trap cells. A comparison of cell composition in a serial passage experiment showed that the virus persisted in a pure host environment, and a heterogenous environment containing 50% neutral non-host cells. However an environment with 50% trap cells was able to drive the virus to extinction, even at increased original viral levels. Indirect positive effects on susceptible host cells could also be a positive outcome of the use of trap sink cells. Protecting natural host cells from infection by driving the virus to extinction before viral proliferation by decreasing overall viral density.

Applying this model to HIV we can consider the recent CCR5 knockout treatment strategy to create a neutral non-host cell, essentially perceptual traps that hide an otherwise productive environment from CCR5 strains of HIV. This however relies on the assumption that the host cells will eventually be decimated by the infection leaving only neutral non-hosts. However in a spatially structured environment density dependence looses efficacy especially when neutral hosts are no longer actively recruited to sites of infection.

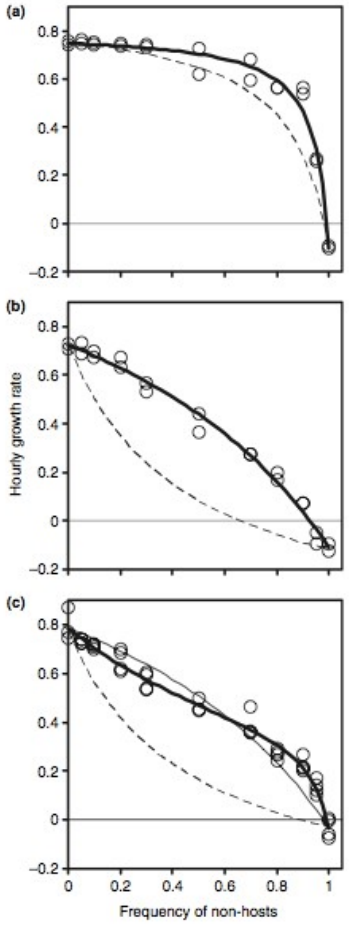


Figure 2[9]: Bacteriophage hourly growth rates A) neutral non-hosts in unstructured environment B) trap cells in unstructured environment C) trap cells in structured environment. Solid lines - best fit Dashed lines- null model

This ecological trap model measures the growth rate of HIV dependent on the density and composition of host and non-host cells in the environment. A CCR5- cell is measured under non-hosts cells because their neutral efficacy restricts the active pool of HIV infection to CCR5+ host cells, maintaining an immune system but not forcing the virus to extinction. Under a neutral system such as this the non-hosts cells would have to compose well over 50% of an unstructured environment to force a virus to extinction.

A sink cell would instead maintain the CCR5 and cxcr4 receptors as environmental cues which lead to a committed fusion of the viron with the cell. The sink cell would then be resistant to HIV essentially tricking the virus into a nonproductive environment. Unlike the previous experiment HIVs fusion with the cell is a committed and irreversible event, the previous system modeled a phage that could disassociate from the sink cell.

With HIV there is an additional importance to maintaining CCR5 function, the cytokines released by HIV infected cells actively recruit host cells for infection. By maintaining CCR5 function modified cells will be recruited to active sites of infection, altering the decreased efficacy of sink cells in a structured environment which was seen in the bacteriophage experiment. This structured environment recruitment has been extensively discussed within gut associated lymphoid tissues. The efficacy of sink cells can be measured by the level of HIV viron rna in the system and cell type environment composition such as CD4/CD8 ratio.

In a structured environment virion release can lead to infection or clearance of the viron. Introducing sink cells would decrease the number of virions in the environment by promoting death through immigration. An equally attractive host in the form of sink cells are still required to compose a large proportion of the

environment. There are however additional factors that can be utilized to alter the balance of the model toward HIV extinction. The use of an active immune response increases the death rate of the model and will further lower the growth rate of the virus by clearing infected cells prior to virion release. Elite controllers have been shown to maintain increased CD8+ cell function in GALT and thereby control the infection through improved elimination of infected cells. Through the use of engineered CTL TCRs improved CD8 response has been shown to mimic the immune response of elite controllers. Utilizing both sink cd4 cells and improved cd8 cells will push the virus toward extinction while requiring a lower number of cells in the environment. Making a treatment utilizing this model would require fewer modified cells then a treatment relying simply on neutral CCR5- cells.

One concern with the use of engineered sink cells is that HIV could mutate to ignore the environment however by simply maintaining normal cd4 cell function HIV would have no cue to discern between host and non-host cells. Only resistance mutations of the virus to three distinct restriction factors, a latency targeted recombinase, and the increased CD8 cell function would lead to viral escape. As with combinatorial anti-viral therapy the target of multiple parts of the HIV life cycle and previous results above show the efficacy of combinatorial gene therapy and that this situation is unlikely to occur.

Utilizing cell culture as an unstructured environment we can model the effect of environmental composition on viral growth rates. However with the lack of an active immune system in cell culture we can only model the effect of sink cells on the ecological trap model. To model the effects of a structured environment including immune function and sink cells requires the use of mice engrafted with modified hematopoietic stem cells.

WORKPLAN

Data Management

All genomics data will be aligned to the human reference genome to detect SNPs. Transcriptomics data will be aligned to the modified cell genome. Ecological model will be modeled in Matlab. All statistical analysis will be conducted in R using bio-conductor.

AIM 1: <u>Generate CCR5+ Hematopoietic Stem Cells Resistant to HIV</u>

Primary Objectives

1. Create a stacked restriction factor cassette containing RevM10, ApobeC3G, hrhTrim5a, Tre-recombinase, and an engineered TCR.
2. Use Crispr/Cas9 nickases to insert the cassette at the AAVS1 locus of HSC
3. Characterize modified HSC for HIV resistance in cell culture.

Perspective: The hypothesis being tested is that the targeted insertion of a stacked restriction factor cassette will confer robust resistance to both CCR5 and CXCR4 strains of HIV.

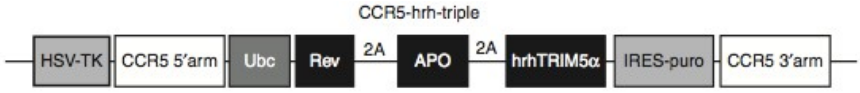


Figure 3[8]: previously used hrhTriple cassette targeting CCR5 insertion

Defensive components target defense against initial HIV infection.

HrhTrim5a is a human-rhesus hybrid form of the Trim5a restriction factor that limits any

antigenicity experienced when using a rhesusTrim5a[24–26]. It acts as a post fusion pre-integration restriction factor, preventing the virus from shedding the viral capsid and infecting the cell.

RevM10 is a dominant negative mutant of a viral protein. This mutant inhibits the export of viral RNA from the nucleus. It has been extensively researched and was one of the first trans-genes used in HIV gene therapy research[4,8,5,27,28]. It has been successful in limiting the progression of HIV infection but is not effective enough on it's own to stop HIV infection.

ApobeC3G is a single amino acid mutant cytidine deaminase that is resistant to degradation by the viral infectivity factor (Vif) of HIV[8,29]. This mutation allows the ApobeC3G to effectively inhibit replication of the virus.

Offensive components target post infection control of HIV infection.

Tre recombinase is a site specific recombinase targeting a 34bp sequence of the LTR region of the integrated HIV-1 viral genome[6]. When expressed TRE effectively and specifically excises the viral genome that has been incorporated into the Human genome. This is especially important to address latency of viral genomes in infected cells. Limiting the expression of Tre to HIV infected cells is achieved by placing it under the control of a TAT mediated promoter. TAT is a viral protein that is highly expressed during infection and drives the replication of the virus. Linking TRE to TAT creates a specificity in expression to only cells that have active infection and thereby integrated viral genomes.

SL9 specific T-cell Receptor is an engineered TCR that is specific to the HIV Gag SLYNTVATL epitope[30,7,31]. In vivo models using HSCs have shown the effective development of CD8+ cytotoxic T lymphocytes (CTL) that are HIV specific and effectively suppress HIV infection. CTL dysfunction is a major component of the pathogenesis of HIV[12,22,23]. HIV specific CTLs are an important component of the innate immune response critical to the clearance of HIV infected cells.

CRISPR/CAS9n

Crispr/Cas9 is an efficient system for directed genome editing and gene insertion[32]. However Crispr/Cas9 nucleases as with zinc-finger nucleases and Talens can cause off-target indels. The Cas9 D10A mutant is instead a nickase capable of causing only a single stranded break in the genomic DNA[32]. Paired nickases increase the specificity and decrease the off-target effects while maintaining efficiency[32–34]. The CRISPR system has also shown a faster response rate, half that of TALENs[32]. We will use the Mali et al plasmids available through addgene.org (41816...18) for the D10A mutant and two guide RNAs T1 and T2 specific for the AAVS1 locus. We will then combine the two guide RNAs and the restriction factor cassette onto a single U6 promoted plasmid.

AAVS1

The adeno-associated virus integration site 1 is considered a safe harbor for the insertion of genes into the human genome[35]. There are no known phenotypic changes due to the disruption of this locus in human cells[35]. The open chromatin structure also ensures consistent transcription of inserted genes and little chance of down-regulation. For these reasons we chose it as the best site for integration of our restriction factor cassette.

Transduction

There are many options for the transduction of HSCs however current methods rely heavily on viral based vectors either for the insertion of genes or the expression of zinc-finger nucleases or TALENs. This poses severe risks for the handling of such vectors and decreased efficiency with resting cells like some HSCs. There is also great difficulty in producing lentilviral or adenoviral particles and concern about immunogenicity of such vectors[5]. Electroporation has been used for decades however is commonly restrictive by the low quantity of cells transduced at a time as well as low cell viability due to the high voltages used[36]. Recent advancements in microfluidic electroporation however shows great efficiency for the introduction of plasmids at lower voltage, with increase cell viability, and in a highly scalable flow-through system, perfect for hematopoeitic cells. For these reasons our third aim is dedicated to the development of a 3D multilayer microfluidic system for cell sorting and electroporation. These methods will be discussed in detail later. However should we be unable to achieve high efficiency compared to other methods we will use the following as our previously demonstrated methods[8].

Cell-Sorting

We will use the EasySep Human CD34 positive selection kit from Stemcell technologies to select HSCs either from whole blood or mobilized PBMC. The EasySep system utilizes immunomagnetic nanobeads that conjugate to a CD34 antibody that magnetically retain CD34 cells through positive selection.

Transfection

In culture transfection of the Crispr plasmid and the gRNA restriction factor cassette using the previously described transfection procedure[8]. Briefly, HSCs and plasmids will be placed in a culture with nucleofection buffer (KH_2PO_4,$NaHCO_3$,$MgCl_2$, H_2O, ATP, Glucose, Ph 7.4) with the plasmids. Post transfection positive selection is performed using puromycin.

Cell Culture

Modified HSCs will be cultured in expansion medium and placed under puromycin positive selection to obtain a pure culture of modified cells. The modified HSCs will then be differentiated in culture to obtain CD4+ t-cells for infection assays.

Measurements

Genomics

Deep sequencing of whole genome of unmodified and modified cells for off-target analysis

Targeted deep sequencing of aavs1 integration site for indel and efficiency measure.

Transcriptomics

RNA-SEQ: restriction factor, hiv infection associated gene expression,
qPCR: Restriction factor transcription level, plasmid transduction efficiency,
qRT-PCR: HIV viral load quantification

Metabolomics

GC-MS – Targeting CCL5, RANTES, IFN-A and other HIV infection related cytokines.

AIM 2: Use HIV resistant HSC cells to model an ecological trap of HIV infection
Primary objectives

1. Use HIV resistant cells to model an ecological trap in an unstructed cell culture.
2. Use HIV resistant HSCs to model an ecological trap in a humanized mouse structured environment.
3. Determine differences in HIV resistant cell recruitment to mouse Gut Associated lymphoid Tissue.

Perspective: The hypothesis being tested is that modified CCR5+ HSC and their lineage will act as trap cells in an environment when challenged with HIV infection.

Unstructured Environment Cell Culture model

Ecological traps have shown higher efficiency in spatially unstructured environments such as suspended cell cultures where trap cells are density dependent in function[10]. Utilizing cell culture will allow us to model the trap cell efficacy in an unstructured environment such as the well mixed peripheral blood environment. We will perform a number of replicates including 50% wild type cells 50% CCR5- modified cells or 50% CCR5+ modified cells, and a final set using a mixed hetrogenous environment containing all three cell types. The CCR5+ modified cells will also undergo a dose-dependent assessment where they constitute 10-40% of the environment to further model the HIV growth rate in an ecological trap environment.

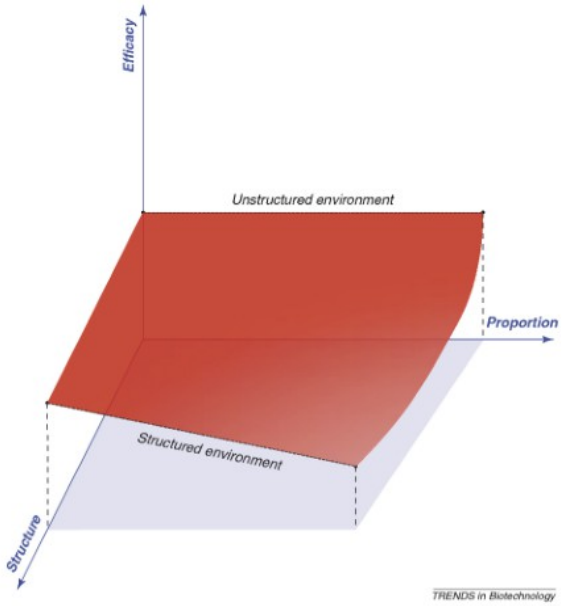


Figure 4[10]: Decreased efficacy of trap cells in structured environments

Structured Environment Humanized Mice model

The use of Rag1−/−γc−/− humanize mice has become the standard small animal model for studying HIV infection due to it's ability to support multi-lineage hematopoiesis from engrafted human hematopoietic stem cells[37]. The use of Rag1−/−γc−/− humanized mice to model a structured environment will be fundamental to understanding the effects of maintaining CCR5 function during HIV infection[37]. Humanized mice allow for the infection of HIV through vaginal and intraperotoneal routes[37]. CCR5 is not only used as an entry portal for HIV but also a homing beacon stimulated by cytokines during HIV infection to attract susceptible host cells to sites of HIV infection[15].Maintaining this chemotactic ability while making cells resistant to HIV should allow trap cells to migrate actively to sites of HIV infection thereby altering the previously shown decrease in efficiency of trap cells in a structured environment.

Each mouse expriment will contain three replicates for statistical significance.. As previously explained we will test CCR5+ modified cells, CCR5- modified cells and wild type cells during HIV infection. We will also model the dose dependent manner of trap cells in viral growth rate. Finally we will model the role existing infection plays on using trap cells during chronic and latent infections. We will first engraft the mice with unmodified HSCs and then post HIV infection we will transfuse a dose-dependent quantity of trap cells.

Measurements

Proteomics

To better understand the role recruitment plays on trap cell efficiency in a structured environment Maldi-MS imaging will be used to visualize cell-type localization in mouse Gut associated lymphoid tissue (GALT)[38]. Using a ferulic acid matrix and a high density mass spec detector we will be able to resolve the presence of CCR5, HIV coat protein GP120, and histological differences due to HIV infection[38].

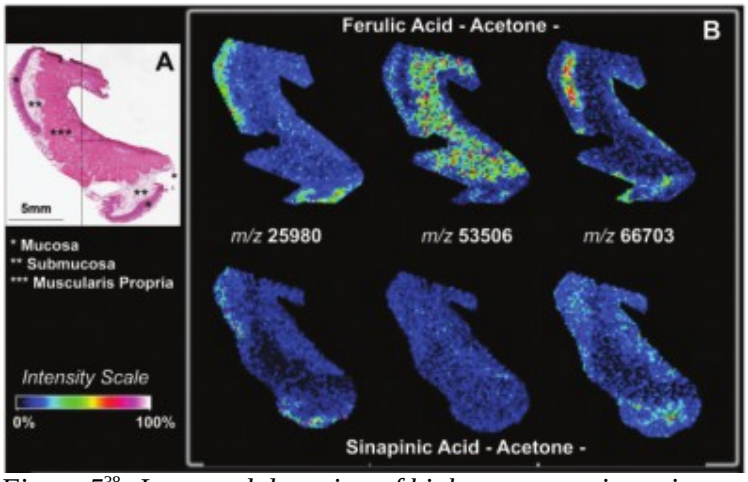


Figure 5[38]: Improved detection of high mass proteins using Ferulic Acid Acetone in mouse gut tissue

Transcriptomics

RNA-SEQ will be used on extracted GALT tissue to quantify restriction gene expression, and HIV infection related gene expression.

qRT-PCR will be used to quantify HIV viral load and validate RNA-SEQ quantifications of specific GALT tissue genes such as CCR5 and restriction factor expression.

Expected Results

We expect to see a distinct alteration in the HIV infection ratio in vitro and in vivo. The viral growth rate will be distinctly lower in trap cell containing environments. Finally using our model cell density requirements can be elucidated so that we can optimize treatment strategies used in gene therapy.

Future directions

-Increased CCR5 cell expression to make trap cells more attractive to the Virus.
-Modify CD4, CD8 and HSCs to improve cell density.

AIM3: Develop a microfluidic device for cell sorting and electroporation

Primary objectives

1. Develop a multilayer device for whole blood sorting of HSC
2. Develop a cell size specific multi-channel electroporation array
3. Combine objective 1 and 2 into an integrated device.

Perspective: The development of a microfluidic device capable of separating and modifying HSCs and other white blood cells such as CD4 and CD8 cells is needed for gene therapy research and clinical applications. Our hypothesis is that using proven high resolution stereo-lithographic techniques we will be able to design and create a device that allows improved cell viability and efficiency on both tasks.

Currently photo-resistive masks and Polydimethylsiloxane are used for the creation of most microfluidic systems in research labs. However this process is limited to single layers without complex 3D structures. The stacking of PDMS slabs has proven useful technically complex for developing multi-layered devices[18,39,40]. The use of stereolithography and biocompatible resins can

overcome these hurdles as has been recently shown[17].

CELL SORTING

Currently the most common way to isolate HSCs is to use cd34+ immunomagnetic nanobeads to positively select and separate HSCs. However cd34 only selects for a subclass of HSCs and progenator cells[41,42]. Instead using cell mediated rolling on potential HSCs will provide a wider selection of HSCs with more potential for long-term renewel and better engraftment potential[43,44]. P-selectin has proven to be effective at mediating this interaction shown in Fig 7[41].

As shown in figure 7 an PDMS chip has been coated in p-selectin along the riged surface. These ridges guide cells that interact with the p-selectin toward a gutter, while non-HSCs do not interact with the p-selectin coated surface and roll through. This process allows for a wider selection of HSCs than an immunomagnetic bead process dependent on CD34 expression.

Electroporation

The use of microfluidic electroporation has been extensively researched and shows increased cell viability, increased efficiency and easily scalable to large volumes[36,46,47]. Figure 8 depicts a simple microfluidic device made using photo-resist and PDMS. The channels width actually dictates the voltage a cell experiences during the electroporation process. This allows for transient higher voltages for the formation of pores in the plasma membrane of the cell, while the wider longer parts of the channel help to further trans-locate the plasmids being transduced[36]. By using small defined channels the duration and exact voltage of a cell can be quantified and poration can occur at sub-kilovolt range significantly less voltage than current systems. This decreased voltage increased cell viability post transduction. The use of spiral vortexs such as in figure 9D have proven to greatly increase the efficiency of transduction but permealizing the entire cell membrane rather then just it's poles[36].

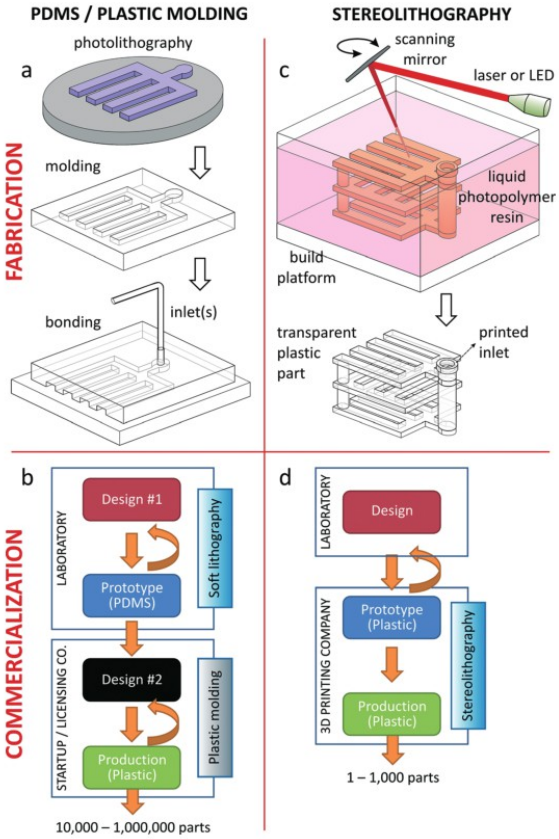


Figure 6[17]: Comparison of Photolithography techniques versus

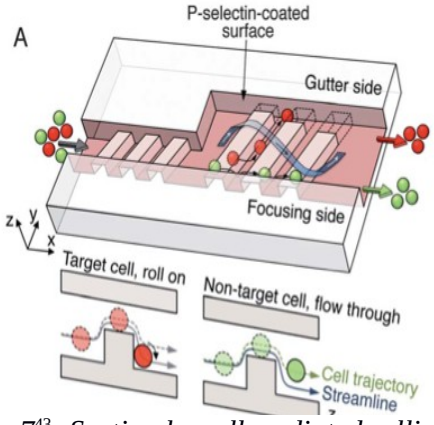
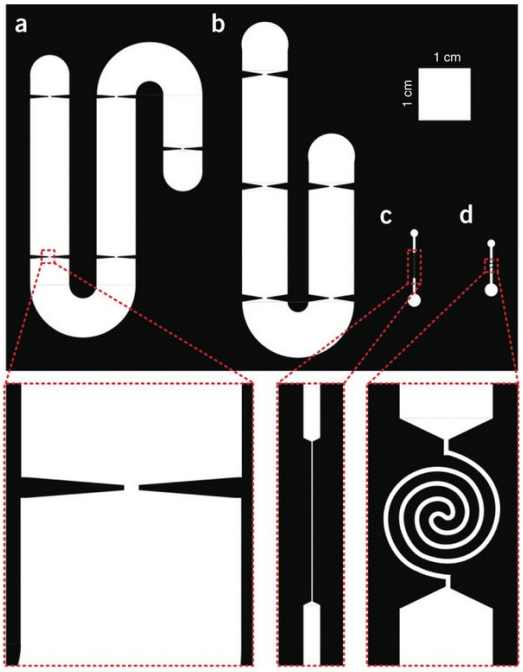


Figure 7[43]: Sorting by cell mediated rolling



	Wide section		Narrow section		H (μm)
	W_1 (μm)	L_1 (μm)	W_2 (μm)	L_2 (μm)	
a	7,500	4,000 + 4 × 30,000 + 4,000	500	5 × 200	480
b	8,730	4,000 + 4 × 20,000 + 4,000	873	5 × 200	412
c,d	500	2 × 3,000	35	1 × 4,768	75

Figure 9[36]: Microfluidic Electroporation channels

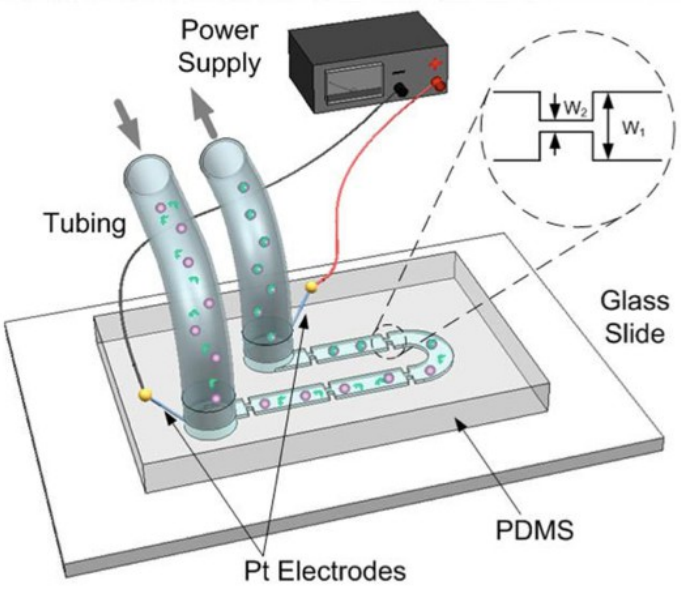


Figure 8[46]: Simple microfluidic electroporation device design

Design

We will use the open-source Kicad and Blender 3D modeling systems for design of the devices. We will make all models publicly availably through Github to facilitate an open-source hardware model of design. Researchers will be able to download, improve and print their own devices for specific uses.

Future Directions

-Sort and electroporate additional cell types

-Include microfluidic cell culture[45]

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